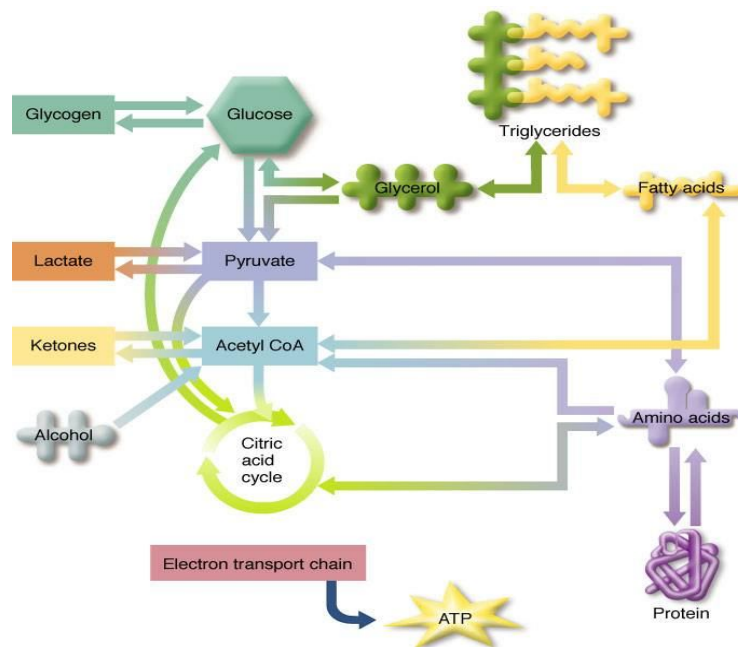


LABORATORY MANUALS

Fundamentals of Biomedical Science I



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PREFACE

As a part of the ongoing evolution/revolution in medical education, the basic sciences and clinical sciences are to be integrated throughout the curriculum. Although there has been an orchestrated and organized integration of the clinical sciences, such has not been the case generally for the basic sciences. Student in most modern medical schools, especially in the basic science courses, are required to memorize a large number of “facts”. Often the sets of facts they are required to memorize are chosen by the instructor based on what he or she knows and is familiar with. The sets of facts may or may not be relevant to medical practice. Furthermore, the student, a passive recipient in this approach, may or may not learn the facts in such a way that they are useful in future practice, regardless of whether the student is able to demonstrate on examination that his or her memory is good.

Bridging the basic science years with the clinical years using problem-based learning will enhance the skills of creating a differential diagnosis and appropriately using diagnostic tests. Problem-based learning is the learning that results from the process of working toward the understanding or resolution of a problem. Learning through problem-solving is much more effective for creating in a student’s mind a body of knowledge *useable* in the future than is traditional memory-based learning. The physician skills most important for patients are problem-solving skills, not memory skills.

In line with this idea we introduce laboratory practical work based on clinical case extended with small group discussion. The purpose of this idea is to identify intellectual principle and concept that can advance the integration of teaching and learning across the basic sciences and between the basic sciences and clinical medicine in medical education. This kind of structure of laboratory activity will not only enhance the psychomotor and knowledge competency of the students, but will also improve their behavior as well, as this program can endorse the students to build team work and have higher motivation to study medicine.

This book is design as the manual for the students, which will guide them in doing their laboratory practical work and assist them to understand better the theory they learn during the lecture. In its chapter it consists of general objective of the laboratory activity, patient's case presentation, topics to be discussed, which are related to topics they learn during the class lecture, laboratory methods, short review of the clinical case and some assignments.

Without support, encouragement and participation of many people, this book would never been accomplished. Our personal and very deep appreciation and acknowledgement goes to all of the contributors for accepting the challenge of preparing the contents, for sharing their ideas and recommendations to construct this manual, for accepting so readily suggestions to modify their contributions and for their understanding and cooperating throughout the period of the preparation. To each we extend our sincerest thanks for a job well done.

In any project, somebody must accept the responsibility for the final product. The decisions concerning the selection of topics and format, reviewing the drafts, and responsibility for the final checking of the manual were entirely ours. We welcome comments, criticisms, and suggestions from the students, faculty, colleagues, and professionals who read this manual. It is our hope that this work will be of a value to those searching on the exciting experience of learning biochemistry where the basic of medical science begins and is expanding so rapidly.

Editors

GENERAL INSTRUCTION

Before starting any experiment the student should read the instructions carefully, paying attention to all details, and should be quite certain about what he/she is trying to do. Be prepared for the discussion, read a lot about the discussion topic. During the discussion, do not hesitate to ask or to express opinion. Try to think and speak logically. Try to listen to your friend's opinion. The result of the experiment and discussion should be written and handed over to the tutor on the next meeting.

LABORATORY RULES

1. Do not replace any solution in a reagent bottle; take only the minimum amount required.
2. Replace the stopper immediately a solution has been taken from a bottle and take care not to mix stopper.
3. Do not remove bottles of special reagents from side shelves to your own benches.
4. Never mouth-pipette a corrosive fluid, always use pipette filler.
5. Cleanliness is essential in all biochemical work. Make sure that your glassware is clean and dry.
6. When you have finished work, leave your bench clean and dry, just as you expect to find it. See that all waste material is put in the wastebasket provided and not into the drains. Strong acids or bases should not be poured down the drain, unless accompanied by a large volume of water.
7. Be careful with the use of centrifuge or spectrophotometer equipment; do not use them before you know exactly how to operate them.
8. Inspect your the content of your bench every time before and after you use them. Any damage or loss must be reported immediately.
9. The person responsible for it must exchange damage or loss of the equipment in a short time.

CHAPTER I. BIOMOLECULES

Living organisms are enormously complex. Higher organisms correspondingly have a greater complexity. Human beings (*Homo sapiens*), for example, may contain 100,000 different types of molecules, although only a minor fraction of them have been characterized. Living organisms have an underlying regularity that derives from their being constructed in a hierarchical manner. **Anatomical** and **histological** studies have shown that multicellular organisms are organizations of **organs**, which are made of **tissues** consisting of **cells**, composed of subcellular **organelles**. At this point in our hierarchical descent, we enter the **biochemical** realm since organelles consist of **supramolecular** assemblies, such as **membranes** or **fibers**, which are organized clusters of **macromolecules** (**polymeric molecules** with molecular masses from thousands of dalton on up).

Living things in general, contain different types of macromolecules: **protein** (Greek: *proteios*, of first importance), **nucleic acids**, and **polysaccharides** (Greek: *sakcharon*, sugar). All of these substances have a modular construction; they consist of linked monomeric units that occupy the lowest level of our structural hierarchy. Thus proteins are polymers of amino acids, nucleic acids are polymers of nucleotides, and polysaccharides are polymers of sugars. **Lipids** (Greek; *lipos*, fat) the fourth major class of biological molecules, are too small to be classified as macro molecules but also have a modular construction. Proteins are all synthesized from the same 20 species of amino acids, nucleic acids are made from 8 types of nucleotides (4 each in **DNA** and **RNA**), and there are ~ 8 commonly occurring types of sugars in polysaccharides. The great variation in properties observed among macromolecules of each type largely arises from the enormous number of ways its monomeric units can be arranged and, in many cases, derivatized. Besides the existing macromolecules, living cells also contain **minerals** in form of cations and anions, such as Na^+ , Ca^{+2} , Cl^- , SO_4^{-2} and PO_4^{-3} .

The aim of studying biochemistry is to understand the biological structure and function of living organisms in chemical terms. One of the most excellent approaches to understand biological phenomena was to purify an individual chemical component, the biomolecules, such as protein, and to characterize its chemical structure or catalytic activity.

In this chapter of the laboratory exercises, students will learn more about molecules and macromolecules that build the living cells. A clinical case of patient with chronic renal failure post streptococcal glomerulonephritis, which presents several sign and symptoms due to biomolecular changes of the body, will be presented as an entry to discuss the functional related structure of biomolecules. The students will conduct plasma protein electrophoresis, the assays of plasma sodium, blood urea nitrogen and creatinine using spectrophotoscopic principle, and determine several minerals in the urine. Since dietary management is one of the modality of treatment in this patient, the students have also to take biomolecular aspect of dietary resources into their consideration during the discussion.

CLINICAL PROBLEM

This is a routine visit of Mrs. Gloria Nefrita Koronis to the Renal Dialysis Unit, Hasan Sadikin Hospital. She is 35 years old diagnosed to have Chronic Renal Failure. As usual, she comes as a very anxious, **pale** woman. She stated notifying increased weakness and fatigue with gradually increasing shortness of breath over the last 6 months. She lost her appetite gradually without significantly decrease in bodyweight. She is turned down for life insurance a couple of years ago because of albumin in her urine.

Physical Examination

Pulse 110 bpm; BP 180/110 mm.Hg; Respirations 30/ min and deep.

Eye conjungtivae are pale;

Chest : rales in the bases of both lungs.

Extremities : **2 + pedal edema**

Chest X-ray

Mild pulmonary edema; **generalized osteomalacia** with resorption of the distal portions of the clavicles, consistent with secondary hyperparathyroidism.

Clinical Course

She is placed on dialysis. She does well on dialysis treatment and is placed in the waiting list for a renal transplant. A diet with protein restriction (0.6 gr./Kg) and mild dietary sodium restriction was instituted.

Laboratory Data of Gloria Nefrita Koronis

	Present	Normal
Hb (Haemoglobin)	6.1	12 – 16 g/dL
Creatinine	14.1	0.6 – 1.2 mg/dL
BUN	183	7 – 18 mg/dL
Calcium	6.5	8.4 – 10.0 mg / dL
Phosphate	12,6	3.0 – 4.5 mg / dL
Sodium	140	135 – 147 mEq/L
Potassium	6.2	3.5 – 5.0 mEq/L
Chloride	105	96 – 105 mEq/L
Bicarbonate	10	22 – 26 mEq/L
Albumin	1.2	3.5 – 5.5 gm/dL
Urine		
Protein	4 +	0

Overview of Chronic Renal Failure and Post Streptococcal Glomerulonephritis

Chronic renal failure (CRF) is a progressive disease characterized by an increasing inability of the kidney to maintain normal low levels of the products of protein metabolism (such as urea), normal blood pressure and hematocrit, and sodium, water, potassium, and acid-base balance. Renal function is clinically monitored by measurement of serum creatinine and blood urea nitrogen (BUN) and by urinalysis. Once serum

creatinine in an adult reaches about 3 mg/dL and no factors in the pathogenesis of the renal disease are reversible, the renal disease is highly likely to progress to end-stage renal disease (ESRD) over a very variable period (from a few years to as many as 20 to 25). Unless contraindications are present such as terminal irreversible disease in another organ system(s) or the patient does not wish it, almost all patients in industrialized nations then receive renal replacement therapy (RRT). The symptoms of chronic renal failure depend on the severity and rapidity of the underlying renal disorder. When chronic renal failure develops slowly, most individuals renal failure is far-advanced (GFR < 10-15 mL/min).

Topic for discussion

- Hierarchy and classification of biomolecules
- Structure related function of important biomolecules
- General principle of analysis of biomolecules (electrophoresis and spectroscopy)
- Biomolecules alterations in chronic renal failure and impact on its function (edema)

Biomolecules in living organism: An overview

The human body is composed of a few elements that combine to form a great variety of molecules. Four elements compose over 95 % of the human body. These elements and their percentages are : hydrogen (H) 10 %, oxygen (O) 65 %, carbon (C) 18 %, and nitrogen (N) 3 %, they are the major constituents of most biomolecular.

Most chemical reactions occurring in the body take place within cells. The elementary composition of the human body has been determined. Carbon (C), oxygen (O), hydrogen (H), and nitrogen are the major constituents of most biomolecules. Phosphate (PO_4^-) is a component of the nucleic acids and other molecules and is also widely distributed in its ionized form in the human body. Calcium plays a key role in innumerable biologic processes and is the focus of much current research. Elements such as K^+ , Na^+ , Cl^- , and Mg^+ are most encountered on an almost daily basis in medical practice in dealing with

patients with electrolyte imbalances, whereas Fe^{2+} is found in iron-deficiency anemia, and I- in thyroid diseases.

The plasma proteins constitute 7 to 9 percent of the plasma and are substances characteristic for the plasma. They are large enough that they pass through blood capillary membranes with difficulty and tend to remain within the bloodstream. Here they are primarily responsible for osmotic return of filtered water to the capillaries from the interstitial fluid. Other functions served by proteins include : Contributing to the viscosity of the plasma; Creating a suspension stability in the blood that aids in maintaining dispersion of materials; Serving as reserve of amino acids (not usually used for metabolism but available); and Serving as buffers. By relatively techniques such as adding certain concentrations of salts to the plasma, three main fraction of proteins may be separated. They are designed as albumins, globulins, and fibrinogen.

Albumins are the most plentiful (55 to 64 %) of the proteins, and are present to the extent of 4 to 5 gm per ml of blood. They are also the smallest (molecular weights 69,000 to 70,000) of the proteins. They contribute most of the osmotic pressure of the plasma that “pulls” water into capillaries, and serve to bind many substances for transport through the plasma [barbiturates, thyroxin (partly on albumins)].

Globulins constitute about 2 % of the plasma and are present to the extent of 2 to 3 gm per 100 ml of blood. Their molecular weight range 150,000 to 900,000. Three fractions may be separated in the globulins by electrophoresis, in which the plasma is subjected to an electric current rates and the proteins migrate at different rates according to their size and electrical charge.

Alpha globulins have molecular weights of 150,000 to 160,000. They serve the general functions of the proteins and also bind many substances for transport including lipids, thyroxin (TBG - thyroxyn binding globulin), copper (as ceruloplasmin), and cortisol.

Beta globulins have molecular weights of 160,000 – 200,000. They also serve the general functions of the plasma protein and bind certain substances such as iron and cholesterol.

Several of the clotting factors, including prothrombin, are members of the alpha and beta groups.

Gamma globulins have molecular weights of 150,000 to 900,000 and this group contains the immunoglobulins (Ig) or antibodies. By further procedures, such as ultracentrifugation, five types of Ig have been isolated from human. They are designated as : IgA(1,2), IgM, IgG(1,2,3,4), IgD, and IgE. Each arises as a result of particular type of antigenic challenge, and some are found in a variety of secretions.

Fibrinogen is a soluble plasma protein with a molecular weight of about 200,000 and it occurs in the plasma to the extent of 0.5 to 0.3 gm per 100 ml. It is converted to insoluble fibrin as the blood coagulates.

Albumins and globulins normally maintain about a 2 : 1 ratio; this ratio is designated as the A/G ratio. Levels of proteins decrease during starvation, liver damage, and renal disease. A primary sign of lowered plasma protein levels (hypo proteinemia) is the development of edema, as filtered plasma water is not returned to the bloodstream and remains in the interstitial compartment.

Water and electrolytes of the plasma are derived almost entirely by absorption from the alimentary tract. Amino acids produced by digestion of proteins are also absorbed from the guts, as are simple sugars resulting from digestion of carbohydrates. Such substances are also delivered to the bloodstream by body cells. In starvation, protein intake is decreased and fewer amino acids are available for synthesis into plasma proteins. This accounts for the decrease of plasma proteins during starvation. Albumins, alpha and beta globulins, and fibrinogen are produced by liver. Gamma globulins are contributed by plasma cells, and by the disintegration of white cells in the bloodstream. Waste materials such as CO₂ are contributed by all body cells as they carry on cellular metabolism. Urea is derived from liver cells as they metabolized amino acids.

Electrophoresis

Electrophoresis is a method widely used in the analysis of biological macromolecules (serum of proteins, membrane of proteins, hemoglobin, DNA/RNA, etc), adapted for serial investigations, e.g. in the clinical laboratory. Many biological molecules carry an electrical charge, the magnitude of which depends on the particular molecule and also the pH and composition of the suspending medium. These charged molecules migrate in solution to the electrodes of opposite polarity when an electric field is applied, and this principle is used in electrophoresis to separate molecules of differing charges.

The electrophoresis mobility depends mainly on the ionizable groups present on the surface of the particle, and the sign and magnitude of the charge carried by the ionizing groups varies according to the ionic strength and pH of the medium in a characteristic manner. Separation of molecules can therefore be affected by selecting the appropriate medium.

Some of electrophoresis methods are:

1. Moving boundary electrophoresis.
2. Zone electrophoresis.

The effects of convection can be reduced to a minimum if electrophoresis is carried out on supporting medium impregnated with buffer solution. Complete separation of a maximum can be effected into distinct zones, and this technique has replaced the classical method of free boundary electrophoresis. Some commonly used supporting media are now considered.

Many materials can be used as a medium for running the electrophoresis such as :

1. Paper
2. Cellulose acetate. This material shows minimal adsorption and clear separation of a mixture into discrete zones. Because of this, compounds are easily eluted with good recovery. Very small quantities of material are needed and the separation can be

completed in only an hour or so compared with overnight for paper electrophoresis. Cellulose acetate is, however, more expensive than paper.

3. Agar-gel
4. Starch-gel
5. Starch grain
6. Polyacrylamide – gel

Separation of plasma proteins by electrophoresis on cellulose acetate.

Principle

There are numerous applications of electrophoresis in clinical and biochemical fields, and one test frequently carried out is the analysis of serum/plasma for changes in proteins during disease. A rapid and convenient method for this is cellulose acetate electrophoresis illustrated in the following experiment.

Cellulose acetate membranes have been used advantageously for the electrophoresis separation and study of numerous protein mixtures. They are thin (130 μm) and micro porous which, along with the fact that most of their constituent hydroxyl groups contain derivative groups, makes them practically non-adsorptive. Since adsorption is minimal, tailing is eliminated and solutes can be separated as sharply defined bands. The membranes are opaque and brittle when dry but become quite strong and pliable when wet.

In addition to the elimination of tailing, cellulose acetate also offers the following advantages over paper electrophoresis.

1. Separation is much more rapid
2. Very small quantities can be separated
3. The membranes can be made transparent, which decreases the back-ground error in quantitative scanning.
4. Proteins can be separated more readily.

5. Immune diffusion techniques can be applied.
6. Solutes can be easily eluted the membrane is soluble in certain solvents
7. Many proteins stain much better.
8. Low background radioactivity enables effective isotope counting.

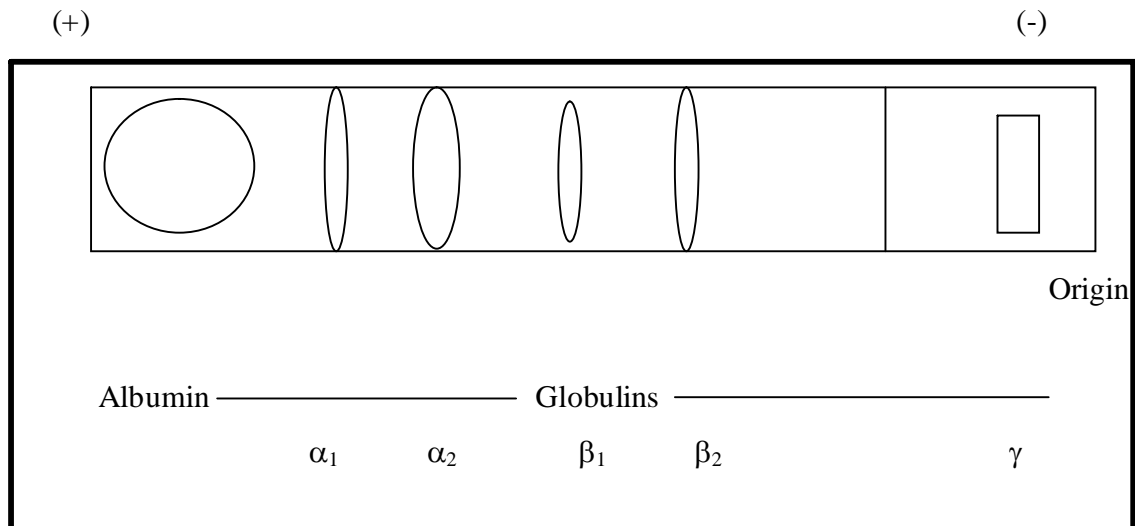
Materials

1. Horizontal electrophoresis apparatus
2. Power pack
3. Barbiturate buffer (0.07 mol/L, pH 8.6)
4. Ponceau S protein stain (2g/L in 30 g/L TCA)
5. Acetic acid (5 % v/v)
6. Serum/plasma
7. Cellulose acetate strips (Oxoid Ltd)
8. Whatman 3 MM paper
9. Methanol : water (3:2)
10. Citrate buffer (0.1 mol/L, pH 6.8)

Method

Moisten cellulose acetate strip (2.5 x 14.5 cm) by placing it on the surface of the buffer in flat dish and allow the buffer to soak up from below. Immerse the strip completely by gently rocking the dish, then remove it with forceps. Lightly blot the strip, place in the apparatus, and connect it to the buffer compartments with filter paper wicks. Switch on the current and adjust to 0,4 am per centimeter width of strip. Apply a streak of serum from a micro liter pipette or melting point tube about one-third of the length of the strip from cathode. This is best carried out by guiding the application with a ruler placed across the tank.

Carry out electrophoresis for 1.5 – 2 h. Then remove the strip, and stain with Ponceau S for 10 min. Prior heating is not needed here since the TCA fixes the proteins for staining. Remove excess dye from the strip by washing repeatedly in 5 % v/v acetic acid. Compare your electrophoresis pattern with that shown in the following Figure.



**The separation of human serum / plasma proteins
by electrophoresis on cellulose Acetate at pH 8,6**

Determinations of calcium plasma concentration

This element presents abundance in the body than any other. An adult, weigh about 70 kg, contains about 1200 gr. of calcium. As the chief constituent of bone, calcium is present as salt resembling minerals of the apatite group; 99 % of the total amount of calcium in the body is found in the bones. The element may exist as a double salt of the carbonate and phosphate, $\text{CaCO}_3 \cdot n\text{Ca}_3(\text{PO}_4)_2$, where the range of n is 2-3.

There also evidence that the principal inorganic salt in bone may be a hydroxyapatite or a hydrated tricalcium phosphate. Smaller amounts of calcium are found in the teeth (36 % in enamel), skin, and blood. In blood, calcium is found almost exclusively in the plasma, where it occurs to the extent of about 9 to 11.5 mg/dl, or 4.5 – 5.5 milli-equivalents per

liter. The determination of calcium concentration in vitro in serum, plasma or urine can be use by the method Moorhead and Brigs, from ST- Reagents product.

These ST- Reagents contain:

Cat No :	Reagents A	Reagents B	Standard
037 - 0149	1 x 100 ml	1 x 100 ml	1 x 3 ml

Principle:

The Cresolphthalein Complexon reaction occurs with calcium and magnesium in alkalis. The violet color produced to be due to coordination complex between them can be read at 575 nm in spectronic- 20 against the reagents blank.

Materials :

- Micropipette 20 ul
- Pipette 1.0 ml
- Test tube
- Specimen (plasma by heparin)
- Reagents A
- Reagents B
- Photometer / Spectrophotometer: $\lambda = 575 \text{ nm}$ (540 – 600).

Procedure:

1. Mix reagents A and reagents B (1 : 1), stabile along 1 day at temperature $2 - 8^{\circ} \text{C}$. The absorbency blank reagents must be (0 – 0.3) if reading to aquadest in wavelength 575 nm.
2. Set up 3 test tube and deliver the reagents (in ul) according to the protocol shown in the following table:

Test Tube	Blank	Standard	Test
Reagents A&B (1:1)	1000 ul	1000 ul	1000 ul
Aquadest	20 ul	-----	-----
Standard	-----	20 ul	-----
Sample	-----	-----	20 ul

3. Mix the materials perfectly all of the 3 test tube, and read the absorbency in spectrophotometer $\lambda = 575 \text{ nm}$ respectively.
4. Calculations :

Absorbency Test	
Calcium (mg/dl) =	$\frac{\text{-----}}{\text{Absorbency standard}} \times \text{Concentration Standard}$

Note:

Normal calcium in serum : 8.4 – 10.4 mg/dl (9.4 mg/dl)

Identification of albumin in urine

Principle of the experiment:

White sediment will be shown if urine contained albumin is heated. In base condition, phosphate and carbonate will also be sediment. To distinguish albumin sedimentation from phosphate or carbonate sedimentation, acetic acid is added. Albumin will still sediment, while phosphate or carbonate will be solved in acid condition.

Objective:

To identify protein in urine

Materials:

- Urine from patients with Glomerulonephritis
- Acetic acid 0.1 M.

Method:

Centrifuge first if the urine is cloudy. Use the supernatant for the experiment. Add into two clean tubes 3 ml urine each. Heated one tube and let the other as control. White sediment will be observed from the heated tube. Add two drops acetic acid 0.1 M, and the sediment will be persisted.

Chapter II. Biomolecules and nutrition

Introduction

We have all witnessed through the medium of television and the newspapers harrowing pictures of starving children dying in various areas in the country due to the prolonged multi dimension crisis. Our immediate reactions are of compassion and we feel compelled by our common humanity to contribute what we can to charitable agencies engaged in famine relief. Almost certainly we will also feel anger and helplessness in the face of the apparently insuperable problems of drought, wars, foreign debt, local corruption and adverse trading arrangements with Western countries which are at the root of such calamities.

Food aid, channeled through relief agencies, is of course an immensely important immediate response. However, it has to be recognized that the affected individuals are likely to have multiple nutritional deficiencies, not only of protein and calories, but also of vitamins, minerals and other dietary components. Moreover, the nutritional needs of different groups within the population, such as young children, pregnant women, nursing mother, manual workers and elderly, are quite different. Local conditions also vary enormously; parasitic infections are usually rife and resistance to even minor infectious diseases may be very low. Food storage conditions are usually far from ideal, resulting in spoiling of locally-produced and imported foodstuff alike. Consequently, relief schemes must be devised and coordinated by experts well acquainted with local conditions. Famine and its consequences are not intractable. Relatively simple measures would make an immense difference if only there was the political and humanitarian will to do it!

The problem.

Protein-rich food supplement and vitamin A soft capsules were hurriedly dispatched to two West Java famine zones, the south-west region of Banten and the south region of Subang. Relief workers in Subang reported that about 80% of the children in their region were saved by the food supplements. In contrast, the Banten workers found that less than

15% survived and even claimed that the protein appeared to have hastened the children's deaths.

The children in Subang were clearly emaciated whereas the Banten children often had a deceptively plump appearance with swollen abdomens and edematous limbs. Ten percents children in Banten indicated vitamin A deficiencies (night blindness, xeroftalmia, bitot spot) and in Subang only 1%, whereas vitamin A supplementation were government program which routinely supplemented at POSYANDU each month.

Many people in Subang have domestic fish-pond and as traditional tempe or tofu producer.

Table 2.1 Serum albumin concentration groups of children from each region

	Serum Albumin (g/dL)
Banten Children	1.6-2.5
Subang Children	2.3-2.9
Well-nourished controls	3.5-5.0

Topics of discussion:

- Resource and utility of some biomolecules
- Biological function of important biomolecules
- Biomolecules as nutrition
- Interrelated function of biomolecules

Questions guide

What is the basic biochemistry behind the Subang children's emaciated appearance?

Why do the Banten children have oedema?

Why was feeding large amounts of protein-rich food paradoxically detrimental to the Banten children?

How would you recommend the Banten relief workers modify their feeding procedures in the future?

Why are the children in Subang have lower percentage of vitamin A deficiencies?

Qualitative Tests for Ketone Bodies in Urine (Gerhardt's and Rothera's Tests):

Introduction:

The most important consequence of the increased metabolism of fat in starvation or diabetic patients is the accumulation in blood of the following substances:

- acetoacetic acid ($\text{CH}_3\text{CO}\cdot\text{CH}_2\text{COOH}$).
- β -hydroxybutyric acid ($\text{CH}_3\text{CHOH}\cdot\text{CH}_2\text{COOH}$).
- acetone ($\text{CH}_3\text{CO}\cdot\text{CH}_3$).

It is now held that the liver is the main site of formation of the two acids given above. Unless adequate glucose is available, the liver is compelled to use an increased amount of fat, so that these acids are formed in quantities too great for the tissues to metabolise them. Then they begin to accumulate in the blood and to be excreted in the urine. It has been shown that diabetics are able to metabolise them normally. It is the increased production which is significant. In addition to the ketosis of diabetes, starvation ketosis arises in the same way. Testing the urine for these substances is an important part of the routine examination of diabetic or starved patients.

Gerhardt's Ferric Chloride Test:

Principle:

The definite reaction is not yet unknown, probably the oxidation of acetoacetic acid by FeCl_3 produces a red brown colour substance.

Procedure:

Add ferric chloride solution (100 g/l), drop by drop, to a few ml of urine in a test tube. A red brown colour is given by acetoacetic acid. If considerable amounts of phosphate are present a precipitate of ferric phosphate is produced. It may be necessary to filter this off.

Add more ferric chloride after filtering and again observe. Similar colour is given by salicylic acid and salicylates as well. This can be differentiated from acetoacetic acid by their different behaviour towards heat. On thoroughly boiling the urine, acetoacetic acid loses carbon dioxide, and is converted into acetone, which no longer gives the test. If the heating is done after adding the ferric chloride, the colour already formed disappears. Salicylates are unaffected by boiling the urine and the colour persists.

This test is only given by acetoacetic acid, not by the other two keton bodies. However, it has now been superseded by a version of the Rothera test, usually in the convenient stick form.

Rothera's Nitroprusside Test:

Principle:

Sodium nitroprusside (sodium nitroferricyanide) in an acid condition will split to $\text{Na}_4\text{Fe}(\text{CN})_6\text{-NaNO}_2$ and $\text{Fe}(\text{OH})_3$, a strong oxidizing agent. Acetoacetic acid and acetone will be oxidized and produce a red purple colour mix of substances. To stabilize these substances, a buffer solution $(\text{NH}_4)_2\text{SO}_4$ is needed.

Procedure:

Saturate about 5 ml urine with solid ammonium sulphate and add a little sodium nitroprusside, either about 0.5 ml 20 g/l solution or a small quantity of powdered solid. Mix and add about 0.5 ml concentrated ammonia. A purple color is given by acetoacetic acid and acetone which is maximal in 15 min. If the ammonia is layered on, a purple ring is obtained at the junction of the liquids. This test is not given by salicylates.

Rothera's test is much more sensitive than Gerhardt's, being positive at about one part in 40 000 compared with one in a few thousands for the Gerhardt test.

Blood Urea Nitrogen (BUN) Determination

Urea is the end product of nitrogen metabolism in humans and is normally excreted by the kidneys. In serum, normal values range from 8 to 20 mg urea/dL of blood. In this

analysis, urease catalyzes the hydrolysis of urea to NH_3 and CO_2 . The ammonia is converted to indophenol blue in the presence of sodium nitroferricyanide-phenol and hypochlorite reagents. The color of the unknown is read against a blank at $A_{625\text{nm}}$ and calculations are made by comparison with absorbency values of a standard of known concentration. Follow the procedure shown in the following Table.

Procedure for Determination of Blood Urea Nitrogen (BUN)^a

Additions (μL)	Tube no.						
	1	2	3	4	5	6	7
	Blank						
Standard (0.2 mg/ml)	-	10	10	10	-	-	-
Serum	-	-	-	-	10	10	10
H_2O	20	10	→				
Urease	20	→					
	Incubate at 37°C for 30 min.						
Phenol reagent (mL)	0.5	→					
Hypochlorite reagent (mL)	0.5	→					
	Mix and incubate at room temperature for 40 min. Add 4 ml of H_2O , mix, and read in a Spectrophotometer against the blank						
$\% T_{625\text{ nm}}$							
$A_{625\text{ nm}}$							

^aUse 13 x 100-nm test tubes for this assay

Commentary - Malnutrition

Nutritional status assessment is essential, especially in children, for identifying either the undernourished or over nourished state and estimating the optimum energy intake to promote growth and well-being. In children with moderate-to-severe disabilities or many of the chronic diseases, nutritional assessment is complicated by interaction of the primary diseases process (i.e., muscle atrophy, contractures, chronic malabsorption), drug-nutrient interactions, and acute and/or chronic malnutrition.

Malnutrition, defined as an unintentional weight loss of more than 10% associated with a serum albumin level below 3.2 g/dL, is not infrequent in hospitalized patients in the modern era of medicine. Regardless of the etiology, malnutrition is associated with a suboptimal surgical outcome, increased rate of infection, longer hospital stay, impaired wound healing, frequent hospital readmission for elderly, more frequent postoperative complications, and increased risk death. Malnutrition results from alterations in the intake, digestion and/or absorption, metabolism, excretion, and/or metabolic requirements of dietary energy, protein, and other nutrients. Understanding the pathophysiology of the patient's disease is essential to the likelihood of finding a component of malnutrition. Furthermore, the severity of clinical illness may be an independent variable of nutritional compromise. Bedside nutritional assessment requires integration of the medical history, the physical examination, selected laboratory data, estimation of nutritional requirements, and approaches to nutritional intervention and its outcome. Accurate bedside assessment allows the physician to determine which patients may benefit from nutritional intervention, and careful patient selection is essential to the cost-benefit of nutritional support.

As a result of the widespread famine, the Subang children are suffering from a severe dietary deficiency of protein and energy foodstuffs known as marasmus (from Greek, meaning 'to waste'). Occurrence of marasmus is not related to climate and used to occur in industrialised areas of Europe

In the absence of adequate food intake, the body mobilises its fat stores. However fatty acid cannot be used to maintain blood glucose, which is vital for a variety of organs, particularly the brain, and erythrocytes. Liver glycogen stores are rapidly exhausted, and so muscle protein must be catabolised to provide amino acid carbon sources for gluconeogenesis.

Although the Banten children are also severely malnourished, their oedeme conceals the underlying muscle wasting and poor growth. Hence, when these children are given high quality food, the first improvement usually seen is a reduction in their oedeme, which then

reveals the effects of the malnutrition. Among the principal protein produced by the liver is serum albumin, a major osmotic buffer of extracellular fluid. The marked deficiency of serum albumin in the Subang children leads to water retention in the tissues. Another important biosynthetic role for the liver is to provide apolipoproteins for the formation of serum lipoprotein complexes. These are responsible for the transport of lipids, especially triglycerides, around the body. When lipoprotein synthesis is compromised, lipids accumulate in the liver.

Ingestion of large amounts of protein results in an excess of amino acids over the immediate requirements for protein synthesis. The surplus amino acids cannot be stored; instead they are catabolised. The unwanted amino groups give rise to ammonia which must be detoxified by conversion to urea. This takes place largely in the liver via the urea cycle. Presumably the Banten children's damaged livers have only a limited ability to handle excess amino acids. Thus, feeding large amounts of protein supplements, far from saving the Banten children as intended, inadvertently overloaded their weakened livers and contributed to their eventual demise. Smaller amounts of the supplements should be given at more frequent intervals to spread the load on the failing liver.

The Banten children are suffering from a form of malnutrition known as kwashiorkor, first described medically by Cecily Williams on the Gold Coast (now Ghana) in 1933. Kwashiorkor is largely restricted to the (sub)tropical regions of west and central Africa. The word comes from the Ga language of Ghana and loosely translated means 'the sickness of the child who is displaced from the breast'. This is because the condition is frequently associated with the weaning of a child onto an inadequate diet, following the birth of a younger sibling. Kwashiorkor is often said to result from predominantly starchy diets which may be adequate in calories but are grossly deficient in protein. However, protein-energy malnutrition is not the complete story and does not explain kwashiorkor's limited geographical occurrence. While the exact aetiology is still controversial, it is clear that kwashiorkor is complicated by other factors such as liver damage due to contaminated food, as in the case described here, and various parasitic infections. The hot

and humid climate provides ideal conditions for the growth of moulds in foodstuffs, particularly when they are stored under rather primitive conditions in rural areas.

In both marasmus and kwashiorkor the malnutrition leads to a generalised debility and an inability to resist infections, so that death usually results from pneumonia or the consequences of chronic diarrhoea.

Further Questions

1. Malnutrition is not simply a matter of dietary insufficiency of protein and/or calories. Vitamins and minerals will also be limited. Vitamin A deficiency is frequently a complicating factor. What is vitamin A, what are its roles and what are the symptoms of its deficiency? From what you can discover about vitamin A transport in the body, can you explain why vitamin A deficiency is particularly noticeable in kwashiorkor?
2. Which hormones are involved in controlling energy metabolism in starvation?
3. Why are fatty acids not gluconeogenic?
4. In a hitherto well-nourished individual who starves, why does muscle metabolism take place at a much higher rate in the first few days than later?
5. Chronic diarrhoea can be fatal. Why is this and how would you treat it?

Case :

Chronic renal failure

Laboratory activities:

1. Electrophoresis of plasma protein
2. Identification of albumin in urine
3. Blood calcium assay

Topic for discussion

- a. Hierarchy and classification of biomolecules
- b. Structural related function of important biomolecules
- c. General principle of analysis method of biomolecules
- d. Biomolecules alterations in chronic renal failure and impact on its function

Case :

Famine in West Java

Laboratory activities:

1. Blood Urea Nitrogen assay
2. Rothera reaction
3. Gerhardt reaction

Topic for discussion

- a. Resource and utility of some biomolecules
- b. Biological function of important biomolecules
- c. Biomolecules as nutrition
- d. Interrelated function of biomolecules