Introduction to Clinical Chemistry

Derek A. Woodrow, FIMLS, MBIM
Senior Chief Medical Laboratory Scientific Officer,
Chemical Pathology Department, St Thomas's
Hospital Medical School, London

Butterworths

London Boston Durban Singapore Sydney Toronto
Wellington

First published, 1987

British Library Cataloguing in Publication Data

Woodrow, Derek A.
Introduction to clinical chemistry.
1. Chemistry, Clinical
I. Title
616.07'56 RB40

ISBN 0-407-00254-5

Library of Congress Cataloging-in-Publication Data

Woodrow, Derek A.
Introduction to clinical chemistry.

Bibliography: p.
Includes index.
1. Chemistry, Clinical. I. Title. [DNLM:
1. Chemistry, Clinical. QY 90 W893i]
RB40.W66 1987 616.07'56 87-6372
ISBN 0-407-00254-5

Photoset by Latimer Trend & Company Ltd, Plymouth
Printed and bound by Robert Hartnoll Ltd, Bodmin, Cornwall

Preface

Medicine has been practised from the very earliest times, and there is evidence that the priests of ancient Egypt and Greece had both considerable knowledge and success in this art. During the Middle Ages the standard of medical care seems to have declined, and many of the old techniques were either forgotten or discarded. The evolution of the scientific basis of medicine appears to have started with Fransiscus Sylvius, who was Professor of Medicine at Leyden University in the 17th century. He introduced laboratory teaching into his syllabus and attempted to correlate physiological with chemical changes. It was not, however, until the 19th century that the chemical investigation of biological fluids was seriously attempted. Probably the first real advance came with the introduction of a method for the determination of urea by Liebig. In the same generation Robert Bunsen discovered the principle of spectral analysis, and Thomas Graham the phenomenon of dialysis. The stage was now set for further scientific advancement, and methods of analysis began to appear from men whose names are remembered to this day because of their association with the apparatus and methods which they developed. Otto Folin, Kjeldahl, Haldane and Van Slyke are still very familiar names in analytical and clinical chemistry.

One of the major problems in the early days was that the methods of analysis required very large quantities of biological fluids, which severely restricted the number of determinations that could be performed without exanguinating the patient. Furthermore, the techniques were often technically difficult and time-consuming, and many were non-specific and insensitive. Increasing sophistication and refinement of equipment have led to the development of fast, sensitive and precise methods for an ever increasing number of chemical constituents, and improved separating techniques now provide us with a means of detecting and estimating minute quantities of substances which only a short time ago was not possible.

The intention of this book is to provide the elementary student of clinical chemistry with some physiological background to the reasons for a number of investigations, to explain the principles

involved in the more common determinations, and to describe the types of analytical techniques which may be used.

As my intention is to cover the basics of clinical chemistry, I have taken the advice of the Red Queen to Alice – to begin at the beginning – and have therefore not selected the most modern and complex equipment to illustrate principles, but in some instances have deliberately selected the simplest equipment that will illustrate the principle involved. Reference will be found, however, to the refinements available in more modern equipment. It is not the purpose of this work to give details of methods of analysis, but to concentrate on giving the reader an overall basic understanding of chemical pathology.

I would like to express my thanks to the staff of the Chemical Pathology Department of St Thomas's Hospital, London, for much helpful advice and criticism, to the Technicon Instruments Company, the Medical Division of Vickers Ltd, Union Carbide and DuPont de Nemours Ltd for permission to include details of their instruments.

Finally, I would like to thank the publishers, Butterworths Ltd, for their infinite patience and help.

DEREK A. WOODROW

Chapter 1

Basic principles of metabolic biochemistry

Carbohydrate metabolism

Carbohydrates are a group of organic substances containing carbon, hydrogen and oxygen which form the principal source of body energy. They occur naturally in the form of linked sugar units called saccharides, e.g. as monosaccharides (glucose, fructose), disaccharides (sucrose, lactose, maltose) or polysaccharides (glycogen – a polymer of glucose units). The term carbohydrate (carbon and water) derives from the originally held view that the hydrogen and oxygen elements of these substances were always present in a ratio of 2:1. It is, however, more correct to regard the simple sugars as polyhydroxyaldehydes or ketones – linked alcoholic groups with a terminal aldehyde (—CHO) or oxo (═O) group.

Ingested polysaccharides are hydrolysed by enzyme activity in the small intestine to form mainly monosaccharides. Both monosaccharides and disaccharides are absorbed into the blood stream, but since disaccharides cannot be transported across the cell membrane they are excreted in the urine. However, this occurs only when there is a disaccharidase deficiency which prevents their conversion to monosaccharides in the intestine. Monosaccharides are small enough to diffuse into the cells, but their rate of unassisted diffusion is very slow and insufficient to provide the amount of energy required for cellular metabolism. Insulin, secreted by the β-cells of the islets of Langerhans in the pancreas in response to high blood glucose concentrations, increases the rate of glucose diffusion into the cells by altering the permeability of the cell membrane and allowing glucose, bound to a carrier, to be transported into the cell. Here the carrier is released and the glucose further metabolized, producing energy-rich phosphate bonds of adenosine triphosphate (ATP) in the process. The principal stages in the metabolism of carbohydrates are shown in *Figure 1.1*. Each stage is controlled by a specific enzyme, deficiency of which gives rise to one of the *inborn errors of carbohydrate metabolism*. Glucose which is not immediately required is converted to glycogen and stored in the liver and muscle

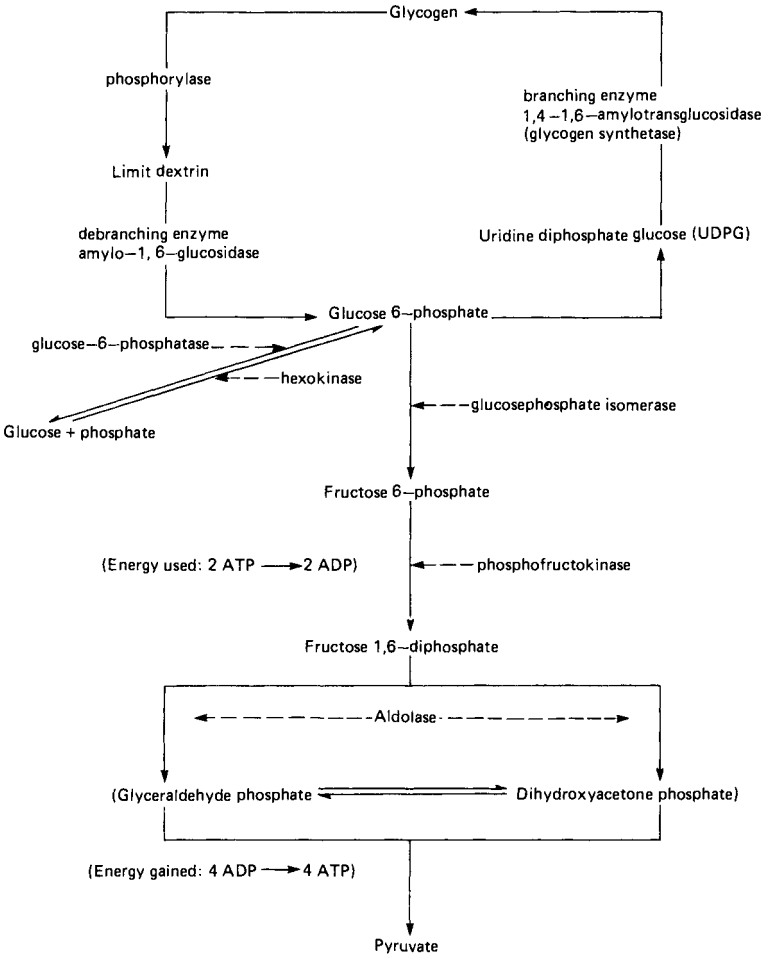


Figure 1.1 Carbohydrate metabolism. In the above metabolic pathway there has been a net gain of two moles of energy-rich ATP from each mole of glucose (glucose + 2ADP + $2PO_4^{3-}$ → 2 pyruvate + 2ATP) (This is part of the Embden-Meyerhof-Parnas pathway.)

tissue. Deficiency of the specific enzymes necessary for the formation and breakdown of glycogen gives rise to glycogen storage diseases, while diabetes mellitus is caused by a deficiency of insulin.

When sufficient glucose is available, the liver, stimulated by the action of cortisol and glucagon, is able to convert stores of amino acids and glycerol to glucose (a process known as gluconeogenesis).

This process occurs to a marked degree in starvation (where there is an insufficient supply of carbohydrates) and in diabetes mellitus (where there is insufficient transport of glucose due to insulin deficiency).

Glucose filtered by the glomerulus into the renal tubules is specifically reabsorbed into the blood until the blood glucose concentration reaches approximately 8 mmol/ℓ, at which point (the renal threshold) no further reabsorption takes place, the remainder being excreted in the urine. The presence of glucose in the urine (glycosuria) is therefore an indication of one of the following:

1. A raised blood glucose level (as in diabetes mellitus)
2. A lowered renal threshold for glucose (as in pregnancy)
3. Renal damage permitting glucose and other substances to be excreted in excess.

Disorders of carbohydrate metabolism

Diabetes mellitus

The characteristic laboratory findings in this condition are hyperglycaemia and glycosuria. In severe and uncontrolled diabetes there may also be keto-acidosis caused by increased conversion of acetyl-coenzyme A to acetone, acetoacetic acid and β-hydroxybutyric acid in the liver. Acetone and acetoacetic acid may be detected in the urine, and the resultant excessive liberation of hydrogen ions produces a metabolic acidosis, with low plasma sodium and bicarbonate concentrations.

Laboratory investigation includes:

1. Blood glucose determination (fasting, postprandial and glucose tolerance tests)
2. Tests for urinary acetone and acetoacetic acid
3. Plasma urea and electrolyte determinations

Galactosaemia

This condition is caused by a deficiency of the red cell enzyme galactose-1-phosphate uridyltransferase, which is responsible for the conversion of galactose 1-phosphate to glucose 1-phosphate. The deficiency causes an accumulation of galactose 1-phosphate in the blood, and galactose is therefore excreted in the urine. Excessive galactose acts as an enzyme poison with an effect similar to that of heavy metals, and will cause severe liver and renal tubular damage if the deficiency is not recognized and treated. Later in life, an alternative pathway using the enzyme UDPgalactose pyrophosphor-

ylase can be used, but in infants this enzyme is present only in small amounts which are insufficient to cope with the necessary conversion. If the deficiency is diagnosed early and the patient given a galactose-free diet, normal development can occur. The metabolic pathway is shown in *Figure 1.2*.

Laboratory investigation includes:

1. Chromatographic detection of urinary galactose
2. Determination of red cell galactose-1-phosphate uridyltransferase.

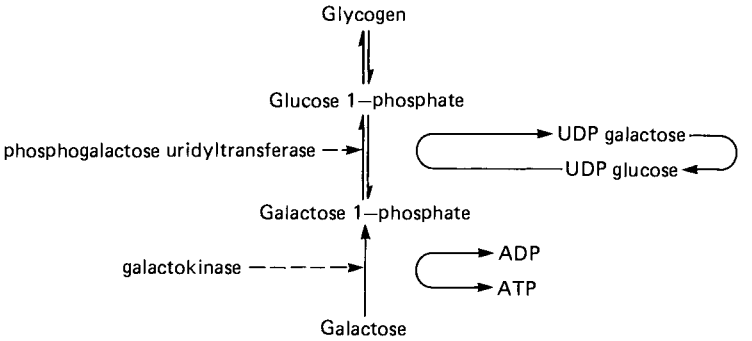


Figure 1.2 The metabolism of galactose

Disaccharide intolerance

Maltose, lactose and sucrose are hydrolysed in the small intestine to monosaccharides (*Figure 1.3*). Deficiency of the appropriate brush border enzymes (maltase, lactase or sucrase) causes malnutrition accompanied by chronic diarrhoea with pale, watery stools. The symptoms are alleviated by removal of the specific offending disaccharide from the diet.

Chromatography to detect and quantitate the specific sugars after an oral load may be used to confirm the diagnosis.

Glycogen storage diseases

With the exception of the Mauriac syndrome, this group of diseases is characterized by the absence or deficiency of enzymes necessary for the synthesis or breakdown of glycogen. The nine types of storage disease each relate to a specific enzyme in the metabolic pathway. The most frequently occurring (type 1) is von Gierke's disease, which is caused by the absence or deficiency of glucose-6-

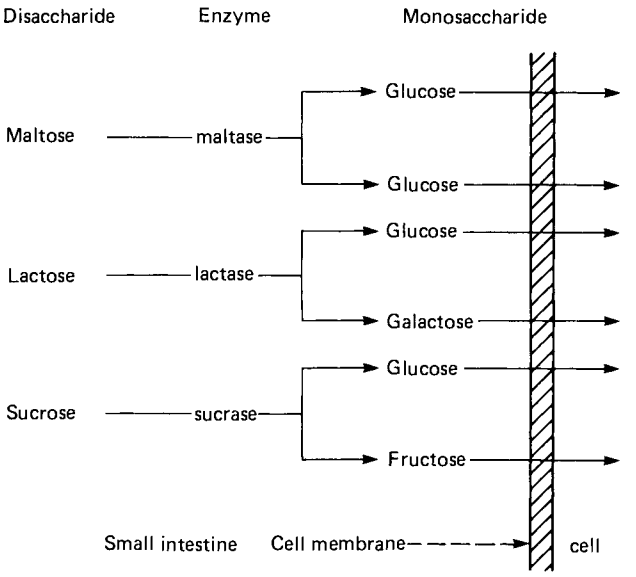


Figure 1.3 The intestinal metabolism of disaccharides to monosaccharides, which can be absorbed by the cells of the small intestine

phosphatase, the enzyme responsible for the conversion of glucose 6-phosphate to glucose.

$$\text{Glucose 6-phosphate} \xrightarrow{\text{glucose-6-phosphatase}} \text{glucose}$$

The clinical features of this condition include hypoglycaemia and hepatomegaly due to glycogen accumulation in the liver. The laboratory diagnosis depends on the demonstration of a specific enzymatic error in the glucose–galactose metabolic pathway.

Lipid metabolism

Lipids form a large group of substances which, although of differing structures, have the common property of being relatively insoluble in water but soluble in organic solvents. In man there are four main types: fatty acids, triglycerides, phospholipids and cholesterol and its esters.

Fatty acids

These are aliphatic (straight-chain) substances which have at least eight carbon atoms and a terminal carboxylic acid (COOH) group. The general formula may be given as R.COOH, where R is the non-carboxylic acid radical of the molecule. For monocarboxylic acids this may be taken as C_nH_{2n+1}. In human metabolism the most important fatty acids are palmitic and stearic acids, which are both saturated (have no double bonds) and conform to the general formula given above, i.e.

Palmitic acid ($n=15$) $C_{15}H_{31}COOH$

Stearic acid ($n=17$) $C_{17}H_{35}COOH$

Unsaturated fats (which have double bonds) are also found. Linoleic acid ($C_{17}H_{31}COOH$) is an essential unsaturated fatty acid present in vegetable oils. Fatty acids not bound to other substances are termed *free fatty acids* (FFA) or *non-esterified fatty acids* (NEFA). More commonly, however, they are combined with glycerol to form glycerides.

Triglycerides

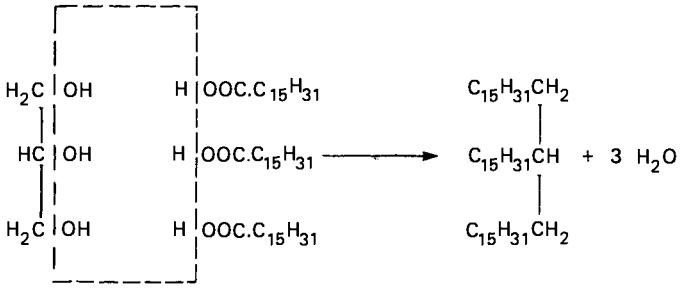
Esterification of fatty acids with glycerol produces triglycerides, as shown for glycerol tripalmitate in *Figure 1.4*. On alkaline hydrolysis (saponification) of a triglyceride, the component fatty acid is released in the form of its alkaline salt (soap) and glycerol. Triglycerides are the main form in which lipid is stored in the body.

Phospholipids

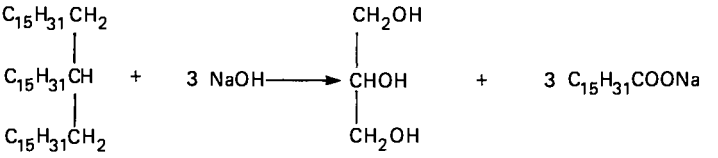
These are synthesized in the body from lipid, phosphate and nitrogen, and are important in the regulation of cell permeability. The principal phospholipids in man are lecithin (a diester of glycerol, two fatty acids and choline) and sphingomyelin, which contains sphingosine instead of glycerol (*Figure 1.5*).

Cholesterol

Cholesterol is the most abundant steroid in the body. It is synthesized mainly in the liver and also released following the breakdown of low density lipoproteins (LDL) by the cellular lysosomes. It plays a significant role in the synthesis of all steroid hormones and in lipid metabolism. It is a major component of cell walls and is necessary for membrane function. Excess cholesterol is removed by combination with high density lipoprotein (HDL) and transported to the liver, where it is converted to neutral steroids and incorporated into

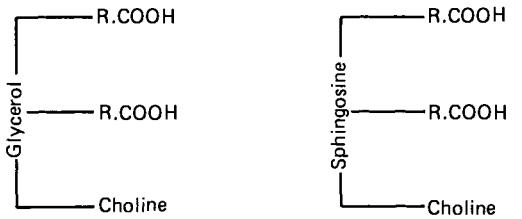


Glycerol + Palmitic acid ⟶ Glycerol tripalmitate (triglyceride)



Glycerol tripalmitate + Sodium hydroxide ⟶ Glycerol + Sodium palmitate (soap)

Figure 1.4 The formation and saponification of triglycerides



(*a*) (*b*)

Figure 1.5 Phospholipids (*a*) lecithin; (*b*) sphingomyelin

bile acids, which are secreted into the bile duct. The structure and basic metabolism of cholesterol are shown in *Figure 1.6*.

The accumulation of cholesterol-containing material (atheroma) is a major contributor to one of the commonest causes of death in

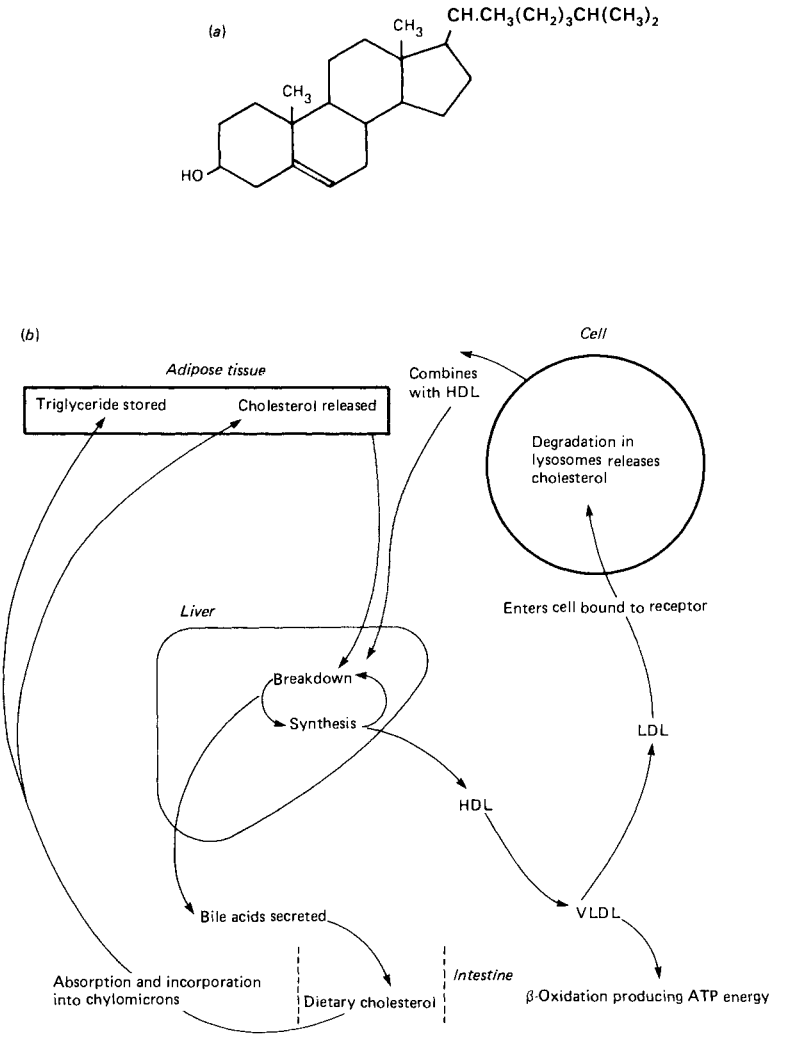


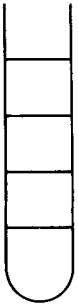
Figure 1.6 (*a*) The structure and (*b*) metabolism of cholesterol (*see text for details*)

the Western world. Narrowing of the arteries by atheromatous deposits obstructs blood flow, thus depriving muscle tissue of oxygen and causing ischaemia, which in turn produces infarction (zone of dead tissue). Drugs used to reduce the blood cholesterol concentration include cholestyramine, which binds with cholesterol

to reduce intestinal absorption, and clofibrate, which inhibits cholesterol synthesis.

Lipoproteins

In order to make transportation possible, lipids are converted to soluble lipoproteins by binding with specific *apoproteins*, the protein surrounding the lipid fraction making it water-soluble. Ultracentrifugation of plasma lipoproteins reveals four major fractions: chylomicrons; very low density lipoproteins (VLDL); low density lipoproteins (LDL); and high density lipoproteins (HDL) (*Figure 1.7*).



Fraction	Size (Å)	Density (g/cm^3)
Chylomicrons	5000 (500 nm)	0.94
Very low density lipoproteins (VLDL)	500 (50 nm)	1.00
Low density lipoproteins (LDL)	200 (20 nm)	1.06
High density lipoproteins (HDL)	100 (10 nm)	1.09

Figure 1.7 Ultracentrifugation of plasma lipoproteins. Diagram showing the size and density of the different fractions

Chylomicrons

These are comparatively large particles, with an average diameter of approximately 5000 Å (500 nm) and a density of about 0.94 g/cm^3. The dominant protein is apoprotein C. Chylomicrons consist mostly of triglycerides and have a very low protein content. Their approximate mean composition is shown in *Table 1.1*.

On ultracentrifugation chylomicrons form the upper lipoprotein fraction. On electrophoresis they remain at the origin.

Very low density lipoproteins

VLDL have a maximum diameter of 500 Å (50 nm) and a density of 0.95–1.005 g/cm^3. The dominant protein is apoprotein C. Compared to chylomicrons they contain more protein, phospholipid and cholesterol, and less triglyceride, although this is still the main lipid component. Their approximate mean composition is shown in *Table 1.1*.

On ultracentrifugation VLDL form the fraction immediately

Table 1.1 Approximate composition (mean values) of the four lipoprotein fractions

	Chylomicrons	*VLDL*	*LDL*	*HDL*
Protein (%)	2	10	22	54
Phospholipid (%)	7	16	22	24
Cholesterol (%)	3	21	43	17
Triglyceride (%)	88	52	12	4
Fatty acid (%)	0	1	1	1

beneath the chylomicrons, and on electrophoresis they separate with the α_2-globulins.

Low density lipoproteins

LDL have a diameter of 150–250 Å (15–250 nm) and a density of 1.006–1.063 g/cm^3. The dominant protein is apoprotein B. In comparison to both VLDL and chylomicrons, the triglyceride fraction is markedly reduced, cholesterol being the main lipid component. Both protein and phospholipids are increased. The approximate mean composition of LDL is shown in *Table 1.1.*

On ultracentrifugation LDL form the fraction immediately beneath the VLDL, and on electrophoresis they separate with the β-globulins.

High density lipoproteins

HDL are the heaviest and smallest of the lipoproteins, with an average diameter of 100 Å (10 nm) and a density of 1.063–1.121 g/cm^3. The dominant protein is apoprotein A. The protein content is considerably higher than in the other lipoproteins, and the triglyceride fraction is much lower. The approximate mean composition is shown in *Table 1.1.*

On ultracentrifugation HDL form the lower lipid fraction, and on electrophoresis they separate with the α_1-globulins.

The role of HDL in coronary heart disease is protective rather than causative, as it combines with excess cholesterol and transports it to the liver, where it is further metabolized and removed from the circulation. It has been demonstrated that patients with myocardial infarction tend to have low HDL levels, whereas Greenland Eskimoes (who do not suffer from coronary heart disease) have higher HDL levels than their mainland counterparts.

Metabolism

Most dietary fat is ingested in the form of neutral fats (triglycerides)

which are emulsified by bile salts and hydrolysed to glycerol and fatty acids by pancreatic lipase. These are absorbed by the intestinal mucosal cells and recombined with protein and phospholipid to form chylomicrons, which are transported by the lymphatic system and enter the blood stream via the thoracic duct. In adipose tissue, lipoprotein lipase hydrolyses chylomicrons to release free fatty acids and glycerol. In the presence of insulin, fatty acids combine with glycerophosphate to form triglycerides, which are then stored in the adipose tissue until required.

Triglycerides from the fat store are hydrolysed by lipoprotein lipase to fatty acids which enter the blood stream bound to albumin. β-Oxidation of fatty acids, which sequentially splits off two carbon atoms, releases energy in the form of acetyl-coenzyme A and ATP, and ketone bodies (β-hydroxybutyric acid, acetone and acetoacetic acid) are formed as waste products.

Unoxidized fatty acids are returned to the liver and re-esterified to form VLDL and HDL. On entering the cell membrane VLDL is reduced to LDL, which then enters the cell and is further metabolized by the lysosomes, releasing cholesterol which is returned to the liver combined with HDL.

Disorders of lipid metabolism

Hyperlipoproteinaemias

These are diseases associated with a high concentration of cholesterol or triglyceride, or both. Most are inherited (e.g. hyper-β-lipoproteinaemia) while about 15 per cent are secondary to an underlying disorder such as hypothyroidism, renal disease, diabetes, gout and biliary obstruction.

Various classifications have been proposed, depending on the characteristic findings of different investigations.

1. Electrophoretic pattern (Fredrickson)
2. Apoprotein pattern (Alaupovic)
3. Particle size (Stone and Thorpe)
4. Density (Strisower)

The most generally accepted (and that used in this book) is the modified WHO (Fredrickson) classification.

Type I This is a rare disorder, accounting for about 1 per cent of hyperlipoproteinaemias, and is caused by a deficiency of lipoprotein lipase. This results in inefficient removal of chylomicrons from the

blood following a meal and an intolerance to all types of dietary fat. It is always detected in childhood. The serum or plasma is very creamy in appearance and the triglyceride level is characteristically very high. On electrophoresis the increased chylomicrons give an abnormal dense band at the origin.

Type IIa This type, which accouts for about 25 per cent of all cases, may be either primary (familial hypercholesterolaemia) or secondary to another condition. It is more common in women than in men. The serum or plasma is clear, with normal triglyceride but high cholesterol concentrations. Electrophoresis shows an increase in β-lipoproteins.

Type IIb This type also accounts for about 25 per cent of cases, but is more common in men than in women. The rate of removal of LDL is decreased, resulting in excess cholesterol accumulation and thus an increased risk of coronary atherosclerosis. Both cholesterol and triglyceride concentrations are raised. Electrophoresis shows an increase in pre-β-lipoproteins.

Type III This rare type (3 per cent of cases) is mainly inherited. It results from the accumulation of the cholesterol-rich remnant of lipoprotein lipase activity. Both cholesterol and triglycerides are raised, usually in equal proportions. The plasma has a milky appearance. Electrophoresis shows an abnormal broad band in the β-lipoprotein region, and for this reason type III is often referred to as 'broad β' or 'floating β' disease.

Type IV This is a very common type, accounting for about 50 per cent of the hyperlipidaemias. Although it can be inherited there are many secondary causes, and it is often found associated with diabetes mellitus. It is due to LDL depletion. The most characteristic finding is the increase in pre-β-lipoproteins (VLDL) on electrophoresis. The plasma is often turbid, with a raised triglyceride concentration. The cholesterol concentration may be normal or slightly raised.

Type V This rare disorder (3–4 per cent of all cases) is a combination of types I and IV in which both cholesterol and triglyceride concentrations are raised. Electrophoresis shows the presence of chylomicrons at the origin and an increase in pre-β-lipoproteins.

Hypolipoproteinaemias

As its name implies, a-β-lipoproteinaemia is characterized by the absence of this lipoprotein. Cholesterol, triglycerides and phospholi-

pids are all markedly reduced in the plasma. In hypo-β-lipoproteinaemia only 10–15 per cent of the normal lipoprotein is found, and cholesterol, triglycerides and phospholipids, although reduced, are less so than in the previous condition. α-Lipoprotein deficiency (Tangier disease) is characterized by an increase in plasma triglycerides and a decrease in the plasma cholesterol concentration. In Tangier disease, cholesterol esters are deposited in the reticuloendothelial tissues.

Laboratory investigation of lipid disorders is usually confined to the measurement of plasma cholesterol and triglyceride concentrations. However, preparative ultracentrifugation studies and electrophoretic studies may have to be carried out if additional diagnostic information is required. These are, however, only available in specialized laboratories.

Nitrogen metabolism

Nitrogen is an extremely important element, and is the fourth most common in human biochemistry. It is an essential constituent of proteins, nucleic acids and creatine.

Man depends on the nitrogen 'fixed' by the bacterium *Rhizobium* in the roots of leguminous plants for his survival since the body is unable to convert nitrogen into organic compounds. Nitrogen is ingested in the diet in the form of proteins and amino acids. The proteins are hydrolysed by proteolytic enzymes to peptides and amino acids, which are then absorbed. Amino acids which are not used are not stored in the body, but excreted. The basic pattern of nitrogen metabolism is shown in *Figure 1.8*.

The average intake of nitrogen in adults is approximately 15 g/24 hours. Normally this is balanced by the daily excretion, and the body is said to be in 'nitrogen balance'. During growth, when intake exceeds excretion, the balance is positive, but in starvation the reverse is true and the balance is negative.

The total non-protein nitrogen fraction of plasma consists mainly of urea, but also contains uric acid, creatinine, creatine and ammonia.

Urea

Urea is the end product of the metabolism of amino acids in the liver (*Figure 1.9*). The ornithine also produced from arginine in this process is recycled. The excretion of urea depends on the glomerular filtration rate, retention occurring when this is reduced to about 30

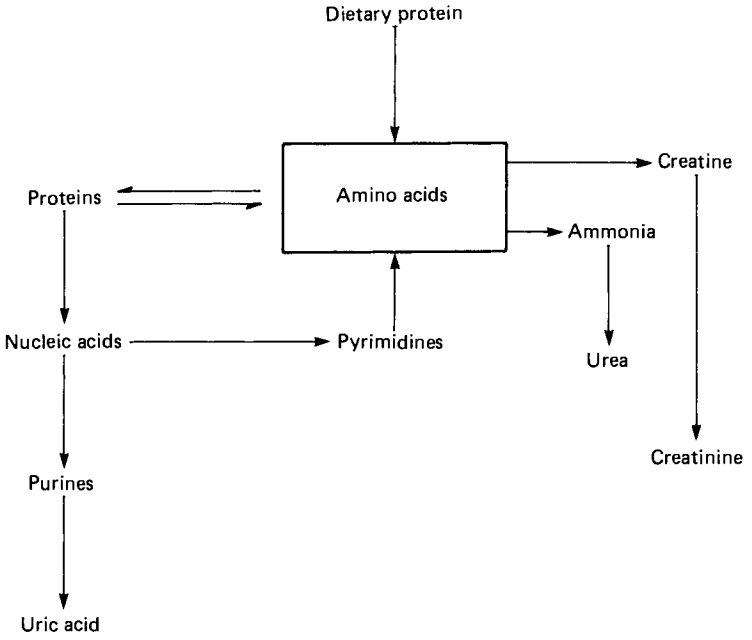


Figure 1.8 The basic metabolism of nitrogen

per cent of the normal rate. Approximately 40 per cent of the urea reaching the renal tubules is reabsorbed into the blood.

Ammonia

This is formed in the liver as a result of the deamination of amino acids, and is converted to urea. It is also released by the distal renal tubules during the conversion of glutamine to glutamic acid and α-oxoglutaric acid.

$$\text{Glutamine} \xrightarrow{\text{glutaminase}} \text{glutamic acid} + \text{ammonia}$$

$$\text{Glutamic acid} \xrightarrow{\text{glutamic dehydrogenase}} \alpha\text{-oxoglutaric acid} + \text{ammonia}$$

It plays an important role in the buffering action of the kidneys by accepting hydrogen ions to form ammonium ions:

$$NH_3 + H^+ \rightarrow NH_4^+$$

As the pH in the renal tubules decreases, the activity of the enzyme glutaminase increases, thus producing more ammonia to

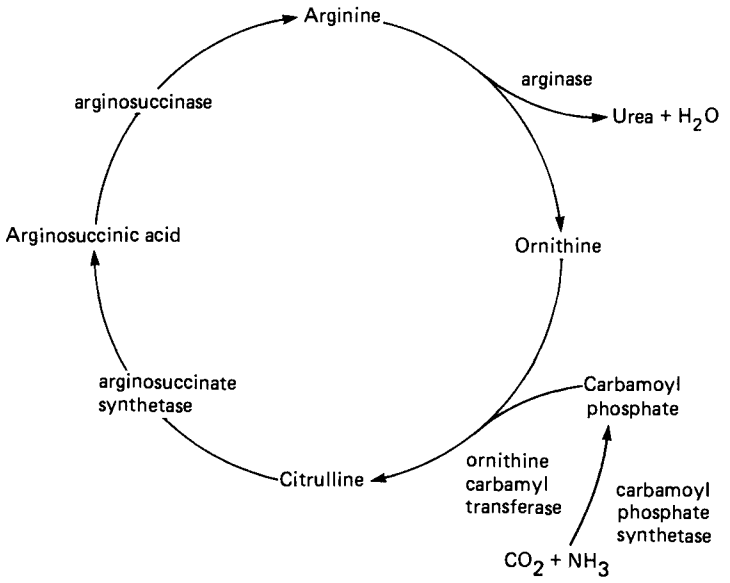


Figure 1.9 The formation of urea from amino acids

mop up the excess hydrogen ions. These are excreted in the urine as ammonium chloride. Normally there is very little ammonia in the blood, except in liver failure, when it is not converted into urea.

Uric acid

Uric acid is the end product of purine metabolism. Adenine is converted to hypoxanthine by the action of adenase, and guanine is similarly converted to xanthine. The enzyme xanthine oxidase converts both hypoxanthine to xanthine, and xanthine to uric acid. Some hypoxanthine is recycled for the synthesis of further nucleic acids by conversion to phosphoribosylpyrophosphate (PRPP), as shown in *Figure 1.10*. *Primary gout* is caused by an enzymatic defect blocking the pathway from hypoxanthine to PRPP and resulting in the deposition of uric acid pyrophosphate in joints and tissue as gouty tophi. Hyperuricaemia can also lead to renal damage and the formation of uric acid calculi.

Creatine and creatinine

The structure and metabolism of creatine are shown in *Figure 1.11*. Creatine is synthesized in the liver from amino acids, and transported by the blood before being taken up by muscle to form

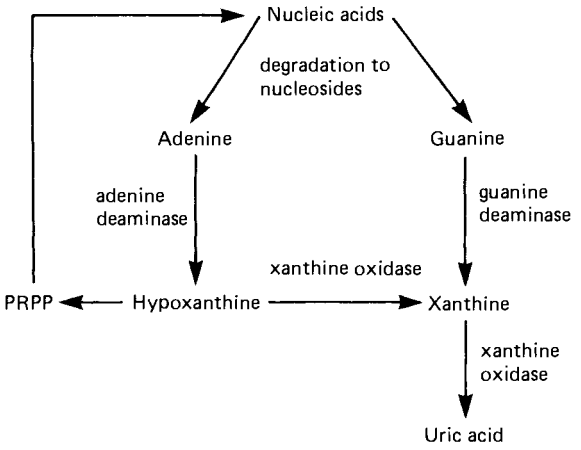


Figure 1.10 Uric acid metabolism. PRPP = phosphoribosylpyrophosphate

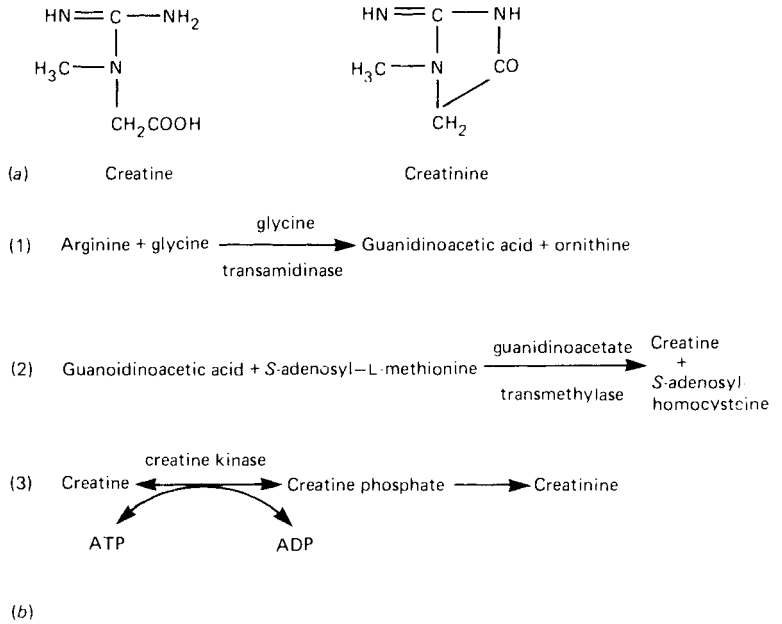


Figure 1.11 (*a*) The structure and (*b*) metabolism of creatine

creatine phosphate. The excess (usually very little) is excreted in the urine. During muscular contraction, creatine is converted to creatinine (the anhydride of creatine), which is also excreted in the urine. The normal daily creatinine excretion is proportional to the muscle mass. In adults it is approximately 9–18 mmol/day, and is unaffected by variations in diet. Creatinine is both filtered by the glomerulus and excreted by the renal tubules. Its excretion is therefore a useful indicator of glomerular function, and thus the severity of renal disease.

In muscular disease the urinary creatine is raised while urinary creatinine is lowered.

Amino acids

Amino acids are substances containing one or more amino (NH_2) groups or a cyclic imino (NH) group and a carboxylic acid (COOH) group. Their general structure can be summarized as $R.CH.NH_2.COOH$, where R is a variant. Those of biological interest are the α-amino acids, in which the amino group is attached to the carbon atom adjacent to the carboxylic acid group. The *essential amino acids*, which cannot be synthesized and must therefore be included in the diet, are phenylalanine, valine, tryptophan, threonine, lysine, leucine, isoleucine and methionine. In addition, arginine and histidine are also essential during growth. All other amino acid requirements can be met by interconversion and synthesis within the body.

All body proteins, hormones and creatine are synthesized from the amino acid pool. Amino acids which are not used in this process are excreted in the urine. There is selective reabsorption of necessary amino acids by the renal tubules. Excessive renal excretion may be due to several causes:

1. *Overflow aminoaciduria.* This refers to the increased excretion of specific amino acids due to their increased production – for example of phenylalanine in phenylketonuria, which is due to a deficiency of phenylalanine hydroxylase.
2. *Renal tubular aminoaciduria.* This is caused by increased excretion of specific amino acids, or groups of amino acids, due to a defect in renal tubular reabsorption. In cystinuria, for example, there is increased excretion of cystine, ornithine, arginine and lysine.
3. *Generalized aminoaciduria.* In the adult Fanconi syndrome the renal tubules do not reabsorb amino acids, glucose and phosphate, thus leading to excessive urinary excretion of these sub-

stances. Generalized aminoaciduria also occurs in Wilson's disease (a disorder of copper metabolism), in which copper is deposited in the kidneys, damaging the renal tubules and thus allowing amino acids and other substances to be excreted in excess.

The laboratory investigation of aminoaciduria is usually by chromatographic studies.

Proteins

These comprise a group of substances of extremely large molecular weight, ranging from a few thousand to millions. They consist of chains of α-amino acids linked by peptide bonds (—CO.NH—) between the carboxyl group of one unit and the amino group of another. They always contain carbon, hydrogen, oxygen and nitrogen, the nitrogen content being relatively constant in proportion to the total weight. In addition to the above elements, proteins may contain sulphur.

Proteins may be broadly classified according to their solubility. *Scleroproteins* are fibrous and insoluble in water. They include keratin (hair) as well as the supportive and connective tissue proteins collagen and elastin. *Globular proteins*, by contrast, are soluble in water or salt solutions. Examples of globular proteins are *histones*, the basic proteins of the cell nuclei which are bound to nucleic acid; *albumin*, which is important in the maintenance of osmotic pressure and as a transport medium when bound to other substances; and *globulins*, which are slightly soluble in water but more soluble in salt solutions.

Proteins may be linked to a non-protein substance, in which case the protein is termed a conjugated protein; the non-protein fraction is known as the prosthetic group. Their structure is extremely complex. The primary structure of proteins is determined by the amino acid sequence. The repeated chains are coiled to form a spiral (the secondary structure), which is further folded around itself to give the tertiary structure.

Chemically, proteins are very sensitive, and the addition of many laboratory chemicals causes irreversible loss of their original properties, that is, they become denatured. They are pH sensitive and precipitate at the isoelectric point (when the net charge of the component amino acids is zero). They are formed in the liver and reticuloendothelial system from amino acids absorbed from the intestinal brush border cells by active transport. Those not required are deaminated in the liver, the nitrogen removed forming ammonia, which is then converted to urea and removed by the kidneys.

The principal functions of proteins are: (1) to act as a carrier by binding to other substances for transportation; (2) to provide immunological protection against foreign substances by forming antibodies; and (3) to promote metabolic chemical reactions by their action as enzymes.

Plasma proteins

Albumin

Albumin comprises 50–60 per cent of the total plasma protein and is the main protein produced by the liver. Its predominant functions are to maintain osmotic pressure and to act as a carrier for transport by reversibly binding to other substances (e.g. bilirubin, lipids, hormones, metals, drugs).

Oedema is caused by loss of albumin, as plasma concentrations below 30 g/ℓ disturb the osmotic balance. Analbuminaemia is an inherited condition affecting albumin synthesis. In bisalbuminaemia, another inherited disorder, two albumin bands are seen on electrophoretic separation.

Globulins

α_1-Globulins The main component of the α_1-fraction is α_1-antitrypsin, which inhibits the action of proteases. Deficiencies of this globulin are associated with liver diseases in children, and with pulmonary emphysema. Glycoprotein and HDL are also in this fraction.

α_2-Globulins The main component of this fraction is α_2-macroglobulin, a large molecule with a molecular weight of 900 000. It inhibits proteases and binds to some enzymes. It is increased in the nephrotic syndrome. Haptoglobin and caeruloplasmin are also α_2-globulins.

α-Fetoprotein The protein is normally found in fetal plasma and tissue, but its concentration decreases rapidly after birth. Its importance in clinical chemistry is that approximately half of the patients with hepatic carcinoma have high fetoprotein levels.

β-Globulins These include VLDL, transferrin, and the components of complement.

γ-Globulins Most of the *immunoglobulins* (Ig) are found in this fraction, although some are also present in the β- and α_2-regions. Their structure is shown in *Figure 1.12*. Each molecule consists of

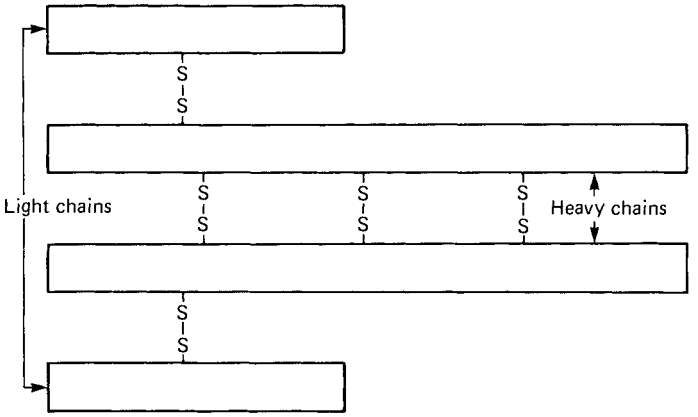


Figure 1.12 The general structure of immunoglobulins

two light (L) polypeptide chains (molecular weight about 20 000) linked by disulphide bridges to two heavy (H) polypeptide chains (molecular weight about 55 000). There are two types of light chains, and they are common to all classes of immunoglobulins. Their molecular weights are shown below:

IgG (γ) 150 000–160 000
IgA (α) 160 000
IgM (μ) 1 000 000
IgD (δ) 190 000
IgE (ε) 200 000

The most abundant immunoglobulins are IgG, which are formed in response to the presence of toxins and bacterial lysins. Newborn infants have adult levels in the cord blood, but this decreases very sharply at birth. The naturally occurring blood group antigens (IgM) increase rapidly after birth. IgA immunoglobulins are antibacterial and are produced to combat local infections. IgE immunoglobulins are produced as a result of local hypersensitivity (allergic response).

Cryoglobulins Cryoglobulins are immunoglobulins which precipitate at +4 °C and redissolve at 37 °C. They are associated with hypergammaglobulinaemia.

Laboratory investigation of plasma proteins usually includes:

1. Determination of plasma protein and albumin concentrations
2. Differentiation of protein fractions by electrophoresis

3. Determination of specific protein concentrations
4. Determination of immunoglobulin fractions.

Enzymes

These are a large group of proteins which act as organic catalysts by accelerating biological reactions by as much as a million times. Their molecular weight ranges from 13 000 (ribonuclease) to 1 000 000 (pyruvate decarboxylase). Enzymes are synthesized in the ribosomes of the endoplasmic reticulum in a continuous cycle of building up, inactivation and breaking down, in which a constant enzyme level is maintained. Some (e.g. the intestinal digestive enzymes) are normally present outside the cells, but others are normally located inside the cell and are only released into the extracellular fluid on cell damage.

Enzymes may consist of two parts, a protein (apoenzyme) and a cofactor (prosthetic group). The substance on which the enzyme acts is called the substrate. The substrate specificity and rate of reaction are both determined by the protein part of the enzyme. Some enzymes also require the presence of an organic non-protein coenzyme or an inorganic activator in order to become active. For example, lactate dehydrogenase (LDH) requires NAD as a coenzyme, and amylase requires chloride ions as an activator.

Other conditions necessary for full catalytic activity include the presence of sufficient substrate, and optimum pH and temperature for the reaction. While enzyme activity increases by a factor of 1.5 to 3.0 for each rise in temperature of 10 °C, enzymes are denatured by very high temperatures.

Enzyme reactions are often highly specific, the substrate combining with the enzyme at specific active binding sites in a manner often likened to a lock and key mechanism, where only a specific shape will fit. Initially the enzyme combines with the substrate to form an enzyme–substrate complex. Once the reaction is completed, the product or products are released, and the enzyme is freed for catalysis of further reactions (*Figure 1.13*). Close structural analogues may combine with the enzyme instead of the substrate, thus inhibiting the enzyme activity. This is termed *competitive inhibition.* In *allosteric inhibition* the inhibiting substance binds to the enzyme at a site other than the active site, but in such a way as to prevent the substrate from combining with the enzyme (*Figure 1.14*). Both these forms of inhibition may be overcome by the addition of sufficient substrate to swamp the inhibitor. Other forms of inhibition are independent of the substrate concentration. Heavy metals, for

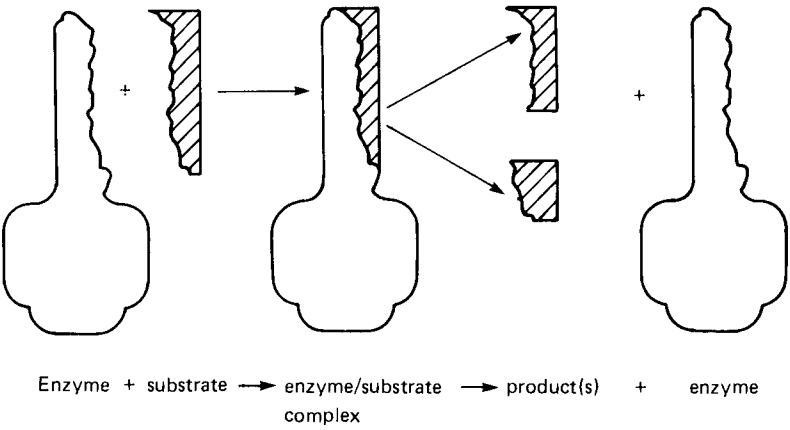


Figure 1.13 The mechanism of enzyme action

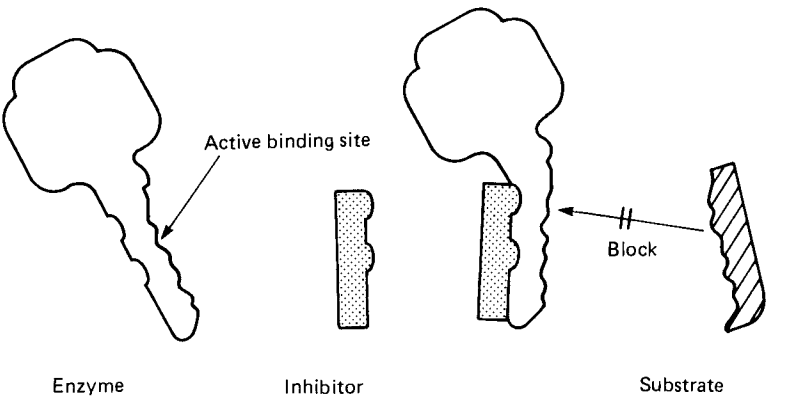


Figure 1.14 Allosteric inhibition of enzymes

example, inactivate sulphydryl groups (—SH), and the products of these reactions inhibit further activity.

Classification of enzymes
Enzymes are classified according to (a) the substrate acted upon and (b) the reaction catalysed. For example, malonate dehydrogenase catalyses the dehydrogenation of L-malonate to oxaloacetate.

Oxidoreductases This group of enzymes is responsible for the promotion of oxidation and reduction reactions. For example,

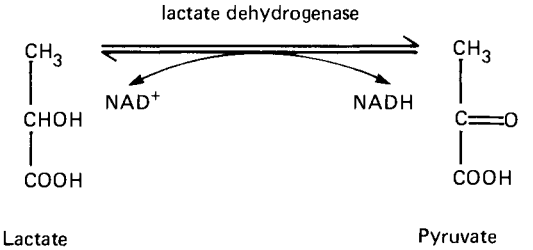


Figure 1.15 The action of lactate dehydrogenase (L-lactate, NAD-oxidoreductase)

lactate dehydrogenase catalyses the oxidation of the CHOH group of lactic acid to produce pyruvic acid. Dehydrogenases also require the presence of the NAD^+/NADH coenzyme (*Figure 1.15*).

Transferases In this class, groups are transferred from the substrate molecule and attached to another substance. For example, transaminases (aminotransferases) transfer nitrogen from an α-amino group to an α-oxo group, as shown for aspartate transaminase (AST) in *Figure 1.16*. The amino group from aspartic acid is exchanged for the oxo group of α-oxoglutaric acid, giving oxaloacetic acid and glutamic acid. Creatine kinase (CK) is another important transferase; this enzyme catalyses the transfer of phosphate from creatine phosphate to ADP, giving creatine and ATP.

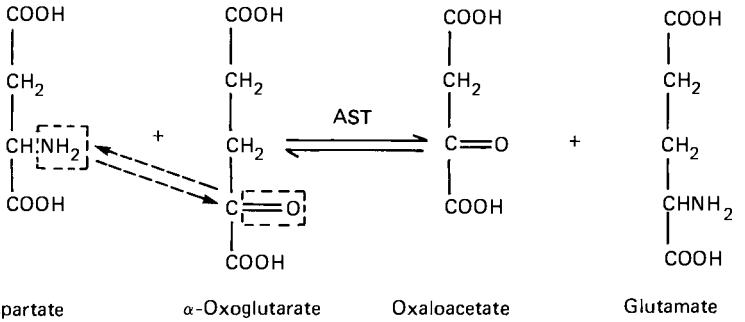
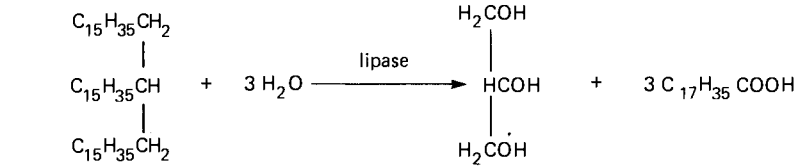


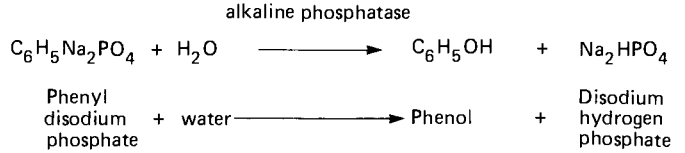
Figure 1.16 The transfer of nitrogen by aspartate aminotransferase (aspartate transaminase; AST)

Hydrolases Hydrolases bring about the hydrolysis of many different substrates. For example, lipase (glycerol-ester hydrolase) hydrolyses glycerol tristearate to glycerol and stearic acid, and phosphatases hydrolyse organic monophosphoric esters to produce inorganic phosphate (*Figure 1.17*). Amylase hydrolyses the α-1,4-



Glycerol tristearate + water ⟶ Glycerol + Stearic acid

(*a*)



(*b*)

Figure 1.17 The hydrolysis of ester linkages by (*a*) lipase (glycerol-ester hydrolase) and (*b*) alkaline phosphatase

glucoside links in the polysaccharide chain to produce smaller sugar units.

Lyases The substrate molecule is degraded by lyase activity to remove groups from it. Aldolase (ketose-1-phosphate aldehyde-lyase) converts fructose 1,6-diphosphate into glyceraldehyde phosphate and dihydroxyacetone phosphate (*Figure 1.18*).

Isomerases These enzymes rearrange groupings within the substrate molecule. For example, phosphoglucomutase catalyses the conversion of glucose 1-phosphate to glucose 6-phosphate.

Ligases These enzymes perform the opposite function to lyases by promoting the formation of bonds.

Enzymes in clinical chemistry

Enzyme studies in clinical chemistry are valuable as a diagnostic aid and in following the course of disease. In many cases enzymes are not specific to particular organs so that a battery of enzyme determinations may have to be carried out. In addition, some

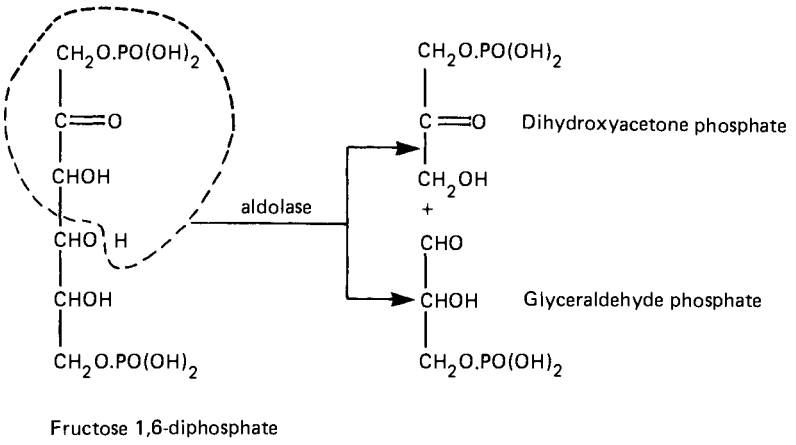


Figure 1.18 The enzymatic degradation of fructose 1,6-diphosphate by aldolase (ketose-1-phosphate aldehyde lyase)

enzymes will show an increase in activity before others, so that failure to perform group tests could result in failure to observe an abnormality. Several enzymes are organ-specific, and the study of isoenzymes is often of value in either determining the specific organ from which the enzyme originated, or excluding specific organs from this possibility. Isoenzymes are defined as having similar catalytic activity but different chemical and physical properties. They may be isolated by the following techniques:

1. Electrophoresis
2. Heat stability or lability tests
3. Substrate specificity
4. Resistance to inhibitors

Lactate dehydrogenase may be separated into five electrophoretic fractions, that nearest to the anode (positive pole) being the heart isoenzyme, and that nearest the cathode (negative pole) the liver isoenzyme.

In the heat stability test of Wroblewski, the heart LDH fraction is more stable than the liver fraction when subjected to a temperature of 60 °C for one hour.

The liver alkaline phosphatase isoenzyme 5′-nucleotidase is specifically confined to the hydrolysis of 5′-phosphate groups, unlike other alkaline phosphatase fractions. This isoenzyme is also not inhibited by EDTA, which contrasts to other alkaline phosphatase isoenzymes.

Inborn errors of metabolism

In 1908 Garrod postulated that a 'wild' gene was responsible for the presence or absence of a particular enzyme, and that in the homozygous mutant this was congenitally absent. The mutation thus resulted in the formation of an abnormal protein molecule. As metabolic pathways are enzyme-controlled, a specific enzyme deficiency will result in a metabolic defect. Collectively these are known as *inborn errors of metabolism*. If the responsible gene occurs only on the X or Y chromosome it is said to be 'sex-linked', but if it affects any other chromosome (autosome) it is said to be 'autosomal'. The gene is 'recessive' if the disorder is only observed in homozygotes, but 'dominant' if it is also observed in heterozygotes.

Some inborn errors are not pathologically important, but the early detection of others may be vital to life or to prevent severe permanent damage to the patient.

Errors of amino acid metabolism

Phenylketonuria This is reported to account for 0.5 per cent of all cases of mental deficiency in the United Kingdom, and the urine of all babies in this country is routinely screened for β-phenylalanine. The disorder is caused by the absence of phenylalanine hydroxylase, which converts phenylalanine to tyrosine (*Figures 1.19* and *1.20*). Permanent damage can be prevented by placing the infant on a phenylalanine-free diet, and early detection is therefore vital.

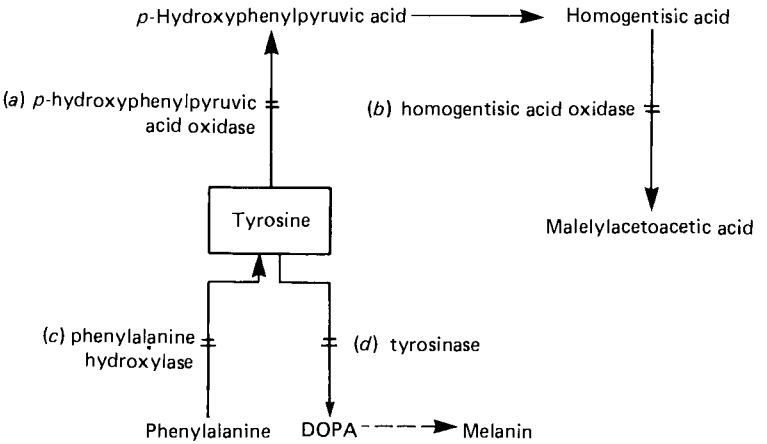
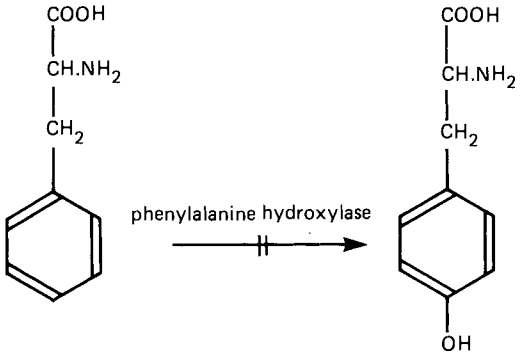


Figure 1.19 The inborn errors of tyrosine metabolism. (*a*) Tyrosinosis; (*b*) alkaptonuria; (*c*) phenylketonuria; (*d*) albinism



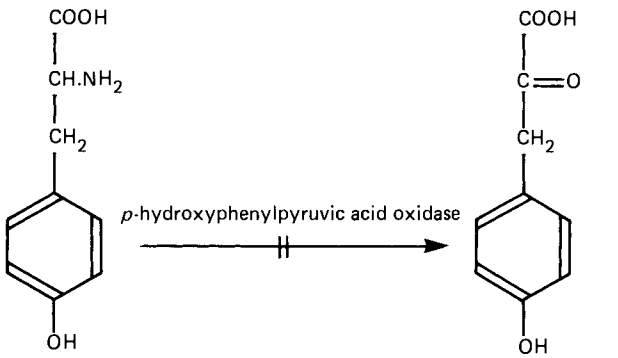
Phenylalanine Tyrosine

Figure 1.20 The enzymatic defect in phenylketonuria

Laboratory investigation includes:

1. Urinary screening for β-phenylalanine
2. Guthrie microbiological assay
3. Chemical determination of β-phenylalanine in blood.

Tyrosinosis This is due to a deficiency of liver *p*-hydroxyphenylpyruvic acid oxidase (*Figure 1.21*) and the consequent accumulation of tyrosine, which damages the kidneys, causing generalized aminoaciduria.



Tyrosine *p*-Hydroxyphenylpyruvic acid

Figure 1.21 The enzymatic defect in tyrosinosis

Laboratory investigation includes:

1. Urinary amino acid chromatography
2. Determination of blood tyrosine.

Alkaptonuria Laboratory interest in this deficiency is due mainly to the abnormal discolouration of the urine, as the disease appears to be otherwise harmless. The defect is due to a deficiency of homogentisic acid oxidase which converts homogentisic acid to 4-maleylacetoacetic acid (*Figure 1.22; see also Figure 1.19*). The resultant accumulation of alkapton in the cartilage of affected individuals leads to a tendency to arthritis in middle age.

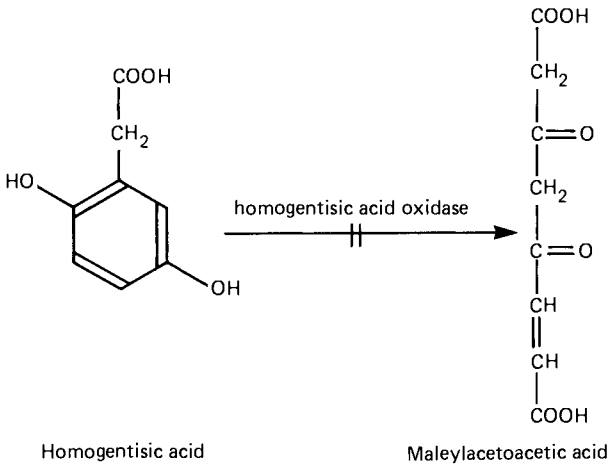


Figure 1.22 The enzymatic defect in alkaptonuria

The laboratory investigation of alkaptonuria includes testing for abnormal pigmentation of the urine, and screening tests for urinary reducing substances:

1. If the fresh urine is allowed to stand in a cylinder or test tube, it will darken progressively from the top downwards.
2. Both Benedict's test and the ferric chloride test for reducing substances give positive reactions, whereas Thormählen's test is negative. The latter is useful in differentiating from melanogen, which gives a positive Thormählen test.

Albinism This is caused by a deficiency of tyrosinase (*o*-diphenol

oxidase), which catalyses the oxidation of tyrosin first to dihydroxyphenylalanine (DOPA) and then through a series of intermediate products to melanin (*Figure 1.23*). Normally melanin is produced by stimulation of the melanocytes of the innermost epidermal layer by the sun and combined with protein to form a protective layer. Albinos lack the normal pigment in skin, hair and iris, and are thus very sensitive to light.

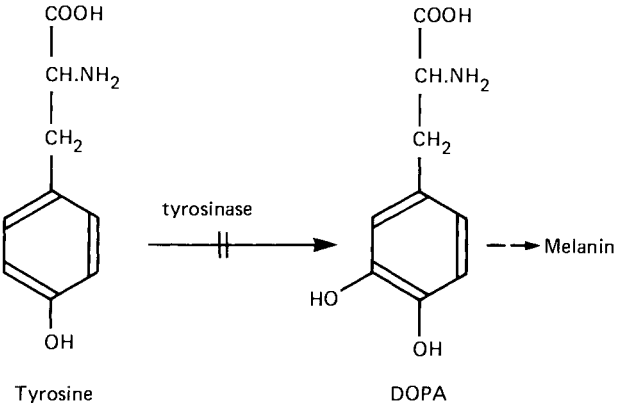


Figure 1.23 The enzymatic defect in albinism

Albinism is not normally investigated by the clinical chemistry laboratory.

The four conditions described above are all inborn errors of tyrosine metabolism, and are summarized in *Figure 1.19*.

Maple syrup disease (leucinosis) In this disease there is a deficiency of branched-chain oxo-acid decarboxylase resulting in excessive urinary excretion of branched-chain amino acids. If undetected, severe mental retardation develops. This, however, can be prevented by early treatment with a diet free of branched-chain amino acids (*Figure 1.24*).

The diagnosis is confirmed by the chromatographic detection of leucine, isoleucine and valine in the urine.

Hartnup disease This is caused by an inability to absorb tryptophan from the renal tubules and the intestine. The resultant tryptophan deficiency blocks the metabolism of nicotinic acid, which, if not detected, will lead to mental retardation and stunted growth.

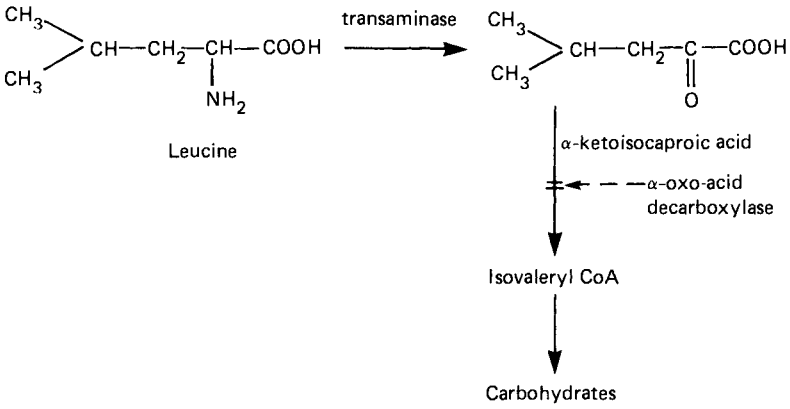


Figure 1.24 The enzymatic defect in maple syrup disease (leucinosis)

The diagnosis is confirmed by the chromatographic detection of urinary indoles and neutral amino acids.

Cystinosis and cystinuria Cystine is metabolized to coenzyme A and taurine. In cystinosis an inborn error in this metabolic pathway leads to accumulation of cystine in the tissues, resulting in damage to the renal system and consequent generalized aminoaciduria. This is an overflow aminoaciduria, and plasma cystine concentrations are therefore also elevated.

Cystinuria, on the other hand, is not itself harmful, although there is a tendency for renal calculi to form because of the relative insolubility of cystine. The inability of the renal tubules to reabsorb the dibasic amino acids cystine, ornithine, arginine and lysine, which is responsible for this condition, leads to their excessive excretion in the urine. The aim of treatment is therefore to ensure that these amino acids are dissolved. Copious amounts of water are usually the first course, followed by sodium bicarbonate if this proves to be insufficient. If this too is unsuccessful, oral D-penicillamine can be given in order to convert cystine to penicillamine-cystine disulphide, which is more soluble.

Laboratory investigation includes:

1. Amino acid chromatography
2. Screening tests for urinary cystine
3. Analysis of renal calculi.

Porphyrias

A further group of inborn errors of metabolism, most of which are inherited as mendelian autosomal dominant traits, are the porphyrias. The name derives from the increased excretion of porphyrins and their precursors, which are important in the synthesis of haemoglobin.

Haemoglobin is metabolized predominantly in the liver and bone marrow, and the porphyrias are therefore either hepatic or erythropoietic in origin.

The first stage in the synthesis of haemoglobin is the formation of δ-aminolaevulinic acid (DALA) from succinate and acetate, catalysed by the enzyme DALA synthase. Two molecules of DALA combine under the influence of DALA dehydratase to form porphobilinogen (PBG). A linear tetrapyrrole is formed by the amalgamation of four molecules of PBG, linked together by methene ($—CH_2—$) bridges. Action of uroporphyrinogen III synthase and cosynthase causes the two ends of the intermediate linear tetrapyrrole to unite, forming the cyclic tetrapyrrole uroporphyrin III. Uroporphyrinogen III decarboxylase removes the carboxyl ($—COO^-$) group from the substituted acetyl groups, leaving a methyl ($—CH_3$) substituent and forming coproporphyrinogen III. Coproporphyrinogen III oxidase and decarboxylase convert two of the substituent propionyl groups to vinyl ($—CH.CH_2$) groups, forming protoporphyrin IX, which is loosely bound to ferrous iron by the action of ferrochelatase to form the final prosthetic substance haem. This combines with the protein to form haemoglobin. The main stages in the synthesis of haem are illustrated in *Figure 1.25*.

Hepatic porphyrias There are four main types. *Acute intermittent hepatic porphyria* is caused by a deficiency of uroporphyrinogen III synthase and an accompanying increase in DALA synthetase, resulting in excess production of DALA and porphobilinogen. *Cutaneous hepatic porphyria* (porphyria cutanea tarda) is usually acquired, but there may be a hereditary tendency. It is characteristically precipitated by alcohol, and to a lesser extent by barbiturates. It has also been traced to the agricultural use of hexachlorobenzene. The deficient enzyme is uroporphyrinogen III decarboxylase (*see Figure 1.25*), and the urinary excretion of uroporphyrins is therefore increased. Liver function tests are also abnormal. *Porphyria variegata* shows the features of both diseases. It is sometimes referred to as the 'Dutch' type of the disease, and has been traced back to an early Dutch settler in South Africa. *Hepatic coproporphyria* is characterized by an increased excretion of coproporphyrins in both

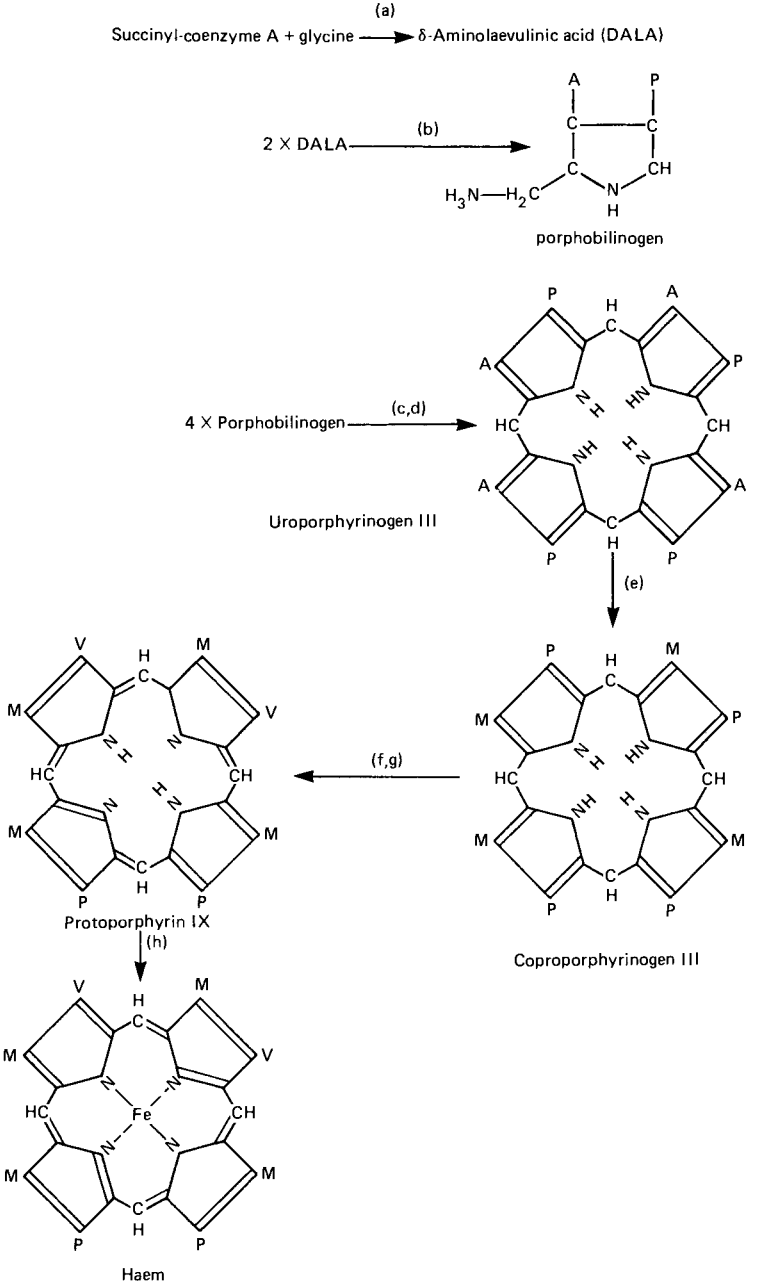


Figure 1.25 The synthesis of haem and the stages at which inborn errors of metabolism may occur. A = acetate; P = propionate; M = methyl; V = vinyl. Key to enzymes: (*a*) DALA synthase; (*b*) DALA dehydratase; (*c*) uroporphyrinogen III synthase; (*d*) uroporphyrinogen III cosynthase; (*e*) uroporphyrinogen III decarboxylase; (*f*) coproporphyrinogen III oxidase; (*g*) coproporphyrinogen decarboxylase; (*h*) ferrochelatase

urine and faeces. The defective enzyme in this disease is coproporphyrinogen oxidase.

Erythropoietic porphyrias *Congenital erythropoietic porphyria* was the first to be identified, although it is by far the most rare. It was described by Schulz in 1874, and is characterized by a very severe sensitivity to sunlight, which often leads to scarring, disfigurement, and even blindness. There is marked pigmentation of both skin and teeth. Unlike the other porphyrias, this disease is transmitted by a recessive gene, which accounts for its extreme rarity. The enzyme uroporphyrinogen III cosynthase (*see Figure 1.25*) is absent, and thus prevents the formation of the normal series III isomers. Uroporphyrin I and coproporphyrin I are both found in the urine. *Erythropoietic protoporphyria* results from an inability to chelate ferrous iron with protoporphyrin IX because of a ferrochelatase deficiency (*see Figure 1.25*). Protoporphyrin IX is therefore found in the urine.

The laboratory investigation of porphyrias and porphyrinurias includes screening tests for porphobilinogen and porphyrins, and the determination of DALA, uroporphyrins and coproporphyrins.

Chapter 2

Chemical endocrinology

Introduction

Most organic secretions are transported via ducts to the site of their action. This is called exocrine (external) secretion. The organs discussed in this section are endocrine (internally secreting, ductless) glands. Their substances are secreted directly into the circulation in very small quantities and, until recently, were too small for accurate chemical analysis. They have a wide variety of regulatory functions throughout the body and are called *hormones* from the Greek word hormaein 'to stir up, stimulate or promote'. Chemically they are either steroids or proteins. They stimulate or promote functions in the body at sites other than that of their production.

The pituitary

The pituitary (or hypophysis) is a very small organ weighing only about 0.5 g and measuring about 1 cm at its widest point. It consists of two lobes, the anterior, which accounts for about 70 per cent of the total organ mass, and the posterior. They are situated underneath the midbrain and are connected to the hypothalamus by a short pituitary stalk. Embryologically the two lobes are quite distinct and represent two separate organs.

The anterior pituitary

This produces basically two types of hormone: those having a *direct action* on the body and those stimulating the production of hormones in other organs (*tropic hormones*).

The first group of hormones includes growth hormone (GH) and prolactin. These are both produced by the acidophilic cells and have structural similarities. GH is the most abundant of all the anterior pituitary hormones, being present in about 1000 times the concentration of the other hormones. It increases both the transport of

amino acids into the cells and their synthesis into proteins, thus stimulating growth. Increased production in childhood gives rise to pituitary giantism, while increased production in adult life produces acromegaly (large extremities). GH also increases the breakdown of fats, so decreasing carbohydrate catabolism; in excess, it gives rise to diabetes mellitus by its action as an insulin antagonist. Decreased production of GH in childhood leads to pituitary dwarfism. Prolactin is responsible for the stimulation of lactation after delivery, but its production at other times is suppressed by the action of oestrogens.

The tropic hormones are named after their target organs, e.g. thyroid stimulating hormone (TSH), follicle stimulating hormone (FSH), leutinizing hormone (LH), adrenocorticotropic hormone (ACTH) and melanocyte stimulating hormone (MSH).

TSH and FSH are similar in that they have a common α-chain in their structure. There are also similarities in the structure of ACTH and MSH. The tropic hormones are all produced by the basophilic cells. TSH stimulates development and growth of the thyroid gland, and ACTH the production of cortisol by the adrenal cortex. FSH stimulates the maturation of the Graafian follicles and promotes oestrogen production in females. In males it promotes development of the seminiferous tubules and spermatogenesis. LH stimulates production of progesterone and oestrogens by the corpus luteum in females, and the production of testosterone in males. MSH stimulates melanocytes to produce skin pigments. A characteristic of hypopituitarism is skin pallor, and of hyperpituitarism, hyperpigmentation.

The synthesis and release of hormones from the anterior pituitary is controlled by stimulating and inhibiting hormones released from the hypothalamus, and by circulating levels of the hormones released by the target organs (negative feedback control). For example, increased concentration of circulating cortisol inhibits the production of the tropic hormone ACTH (*Figure 2.1*).

The posterior pituitary

Hormones from this source are actually produced in the hypothalamus and transported to and stored in the nerve endings of the posterior lobe. Antidiuretic hormone (ADH) acts on the distal renal tubules, increasing their permeability and allowing increased water retention. Diabetes insipidus is caused by ADH deficiency leading to excretion of copious amounts of water. The production of ADH is stimulated by an increase in plasma osmolality and an increase in blood volume. It is inhibited by ethanol.

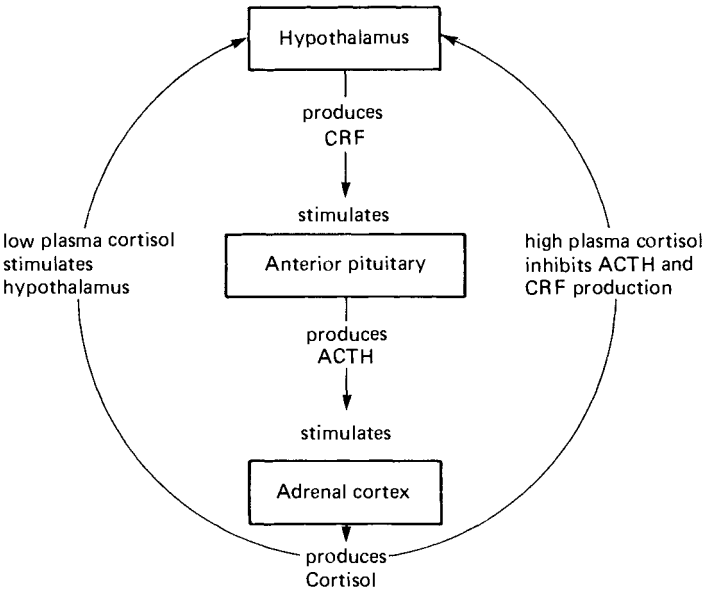


Figure 2.1 The adrenal–pituitary-hypothalamic axis. Negative feedback control of circulating hormones

Oxytoxin is a hormone produced by the posterior pituitary. It is concerned with lactation and uterine contraction.

The thyroid

In response to stimulation by TSH the thyroid produces tetraiodothyronine (thyroxine or T_4) and tri-iodothyronine (T_3) from tyrosine. The gland is situated in the neck below the larynx and consists of two lobes linked by an isthmus. The cells are arranged into groups, or acini, with central follicles which contain colloid. T_3 and T_4 combine with colloid to form a high molecular weight (670 000) protein, called thyroglobulin, which is normally stored in the follicles. The hormones are released from thyroglobulin before entering the blood stream, where they are rapidly bound to carrier proteins, mainly thyroid-binding globulin (TBG), and remain bound until they are taken up by the tissues. Only the free forms are biologically active and, of these, T_3 is the most potent. T_4 however, is the most abundant, with a concentration about 60 times that of T_3.

The effect of the thyroid hormones is to increase the metabolic rate by:

1. stimulating oxygen consumption of nerve tissue;
2. increasing the rate of carbohydrate absorption from the intestine;
3. stimulating cholesterol synthesis.

The secretion of thyroid hormones is regulated by negative feedback control. Free T_3 and T_4 inhibit the release of thyroid releasing hormone (TRH) from the hypothalamus and TSH from the anterior pituitary.

Thyroid insufficiency is found in myxoedema and Hashimoto's disease. Increased secretion of thyroid hormones is found in Grave's disease.

The thyroid also secretes calcitonin, which is synthesized in the cells of the tissue between the follicles. Its action is to lower the blood calcium concentration by inhibiting its active transport from bone to the extracellular fluid.

The parathyroid glands

The parathyroids are two small reddish-yellow glands situated on the posterior aspect of each thyroid lobe. They secrete a single-chain polypeptide hormone (MW 9000) called parathormone (PTH), in response to low circulating levels of ionized calcium. The release of PTH is controlled by a negative feedback mechanism, so that high plasma calcium ion concentrations inhibit PTH secretion. PTH increases circulating calcium and decreases circulating phosphate levels by:

1. mobilizing calcium from bone;
2. increasing renal tubular reabsorption of calcium;
3. decreasing renal tubular reabsorption of inorganic phosphate.

The circulating level of ionized calcium is maintained within very narrow limits, which is essential for bone formation, blood coagulation and the maintenance of cell permeability. Low plasma concentrations have a pronounced neuromuscular effect, as demonstrated by the marked twitching in patients with tetany (hypoparathyroidism).

Hyperparathyroidism leads to the formation of renal calculi due to the increased urinary excretion of calcium.

The pancreas

This is a large organ, 12–15 cm in length, situated on the posterior abdominal wall. It is both an endocrine and an exocrine gland. Its endocrine function is carried out by specialized cells in the *islets of Langerhans*, named after their discoverer Paul Langerhans, who first described them in 1869. These are pale areas with an abundant capillary blood supply, surrounded by a reticulum. The α-cells form about 20 per cent of the total islet cells, and contain acidophilic granules which secrete the hormone glucagon. About 75 per cent of the islet cells are the granular β-cells which secrete the hormone insulin. The function of the remainder of the islet cells is not clearly understood.

Insulin

This was first isolated from beef by Banting and Best, and first used successfully in the treatment of diabetes mellitus in 1922. It was also the first protein to have its structure completely determined, by Race and Sanger in 1954. Each molecule consists of two amino acid chains (A and B) linked by internal disulphide bridges. The chains consist of a total of 51 amino acid residues, 21 on the A chain and 30 on the B chain. Insulin is produced in the cellular endoplasmic reticulum and at first is a long single chain of amino acids (*preproinsulin*). This is reduced in size by the removal of 23 amino acid residues, and the chain becomes folded to form *proinsulin*. Hydrolysis of the connecting peptide converts this to the active hormone (*Figure 2.2*), which is stored in granular form in the β-cells and released when the granule membrane fuses with the cellular membrane. Insulin is released rapidly in response to high blood glucose concentrations in the pancreas and its production is controlled by a feedback mechanism. It is thought to bind very tightly to a glycoprotein receptor at the tissue cell membrane, and that this binding in some way triggers its actions. The primary metabolic effect of insulin is to facilitate diffusion of glucose across the cell membrane by altering its permeability, thus allowing glucose, bound to a carrier, to be taken up by the cell. It also inhibits the lipolytic activity of some hormones by decreasing the amount of cyclic AMP available for the breakdown of fats.

Insulin deficiency causes diabetes mellitus, which is characterized clinically by fatigue, loss of weight, the excretion of large volumes of urine, often containing glucose and excessive thirst (polydipsia). Biochemically it is characterized by hyperglycaemia and glycosuria. Because of the manifold metabolic effects of insulin, diabetes

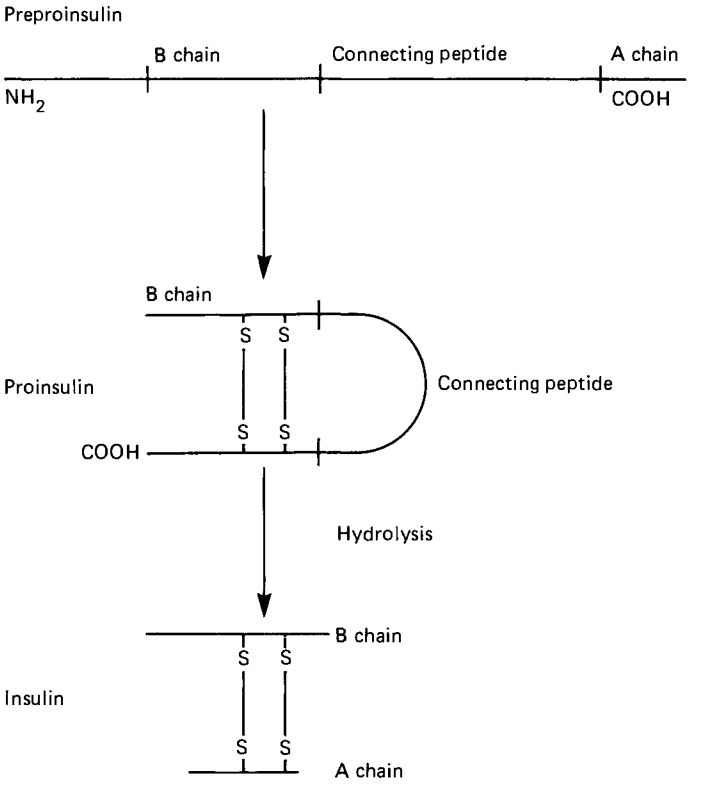


Figure 2.2 The synthesis of insulin

mellitus is a very complex disturbance involving both carbohydrate and lipid metabolism. Essentially, dehydration is caused by the excretion of very large volumes of water, which necessarily accompany the excretion of glucose when the renal threshold is exceeded. The loss of glucose leads to a depletion of necessary carbohydrate, and therefore increased breakdown of fats to glucose (gluconeogenesis). Increased gluconeogenesis leads to increased formation of ketone bodies during β-oxidation, and thus more dehydration. If untreated, coma, followed by death, may ensue.

Glucagon

This consists of a single polypeptide chain of 29 amino acid residues and has a molecular weight of about 35 000. In contrast to insulin,

the molecule does not contain cystine. The synthesis and pattern of release into the circulation are similar to those of insulin. Glucagon is an insulin antagonist, i.e. its functions are directly opposite to those of insulin. It promotes insulin production.

The adrenal (suprarenal) glands

These are two small glands, one situated on top of each kidney, hence the name suprarenal. Each consists of an outer cortex which secretes the adrenal cortical hormones and an inner medulla which secretes the catecholamine (pressor) hormones.

The adrenal medulla

The granular chromaffin cells of the medulla produce two hormones from the metabolism of tyrosine. Tyrosine itself is a derivative of catechol, and as both hormones possess amino groups they are catecholamines (*see Figure 6.11*). Tyrosine is first hydroxylated and decarboxylated to form noradrenaline, which is then methylated to form adrenaline. The hormones are stored in the cell granules until acetylcholine increases the permeability of the cell wall, allowing the fusion of granules and cellular membrane to release the hormone into the circulation.

The principal action of noradrenaline is that of a vasoconstrictor, while that of adrenaline is to stimulate cardiac output. They also break down liver and muscle glycogen, promote liver gluconeogenesis and mobilize fatty acids. Increased production of catecholamine hormones is found in tumours of chromaffin tissue (phaeochromocytomas). As shown in *Figure 6.11*, the principal metabolite is 3-methoxy-4-hydroxymandelic acid (VMA). The diagnosis is confirmed by the demonstration of increased levels of VMA in the urine.

The adrenal cortex

This consists of three layers of cells, the zona glomerulosa, fasciculata, and reticularis. The narrow outer zona glomerulosa consists of small cells which secrete aldosterone, the most potent mineralocorticoid. Its principal effect is to increase reabsorption of sodium (and with it passive reabsorption of water) from the distal renal tubules in exchange for potassium or hydrogen ions. This process is regulated by the renin–angiotensin system (*Figure 2.3*), which in turn is controlled by the blood pressure.

Once the blood pressure falls below a critical level, the cells of the

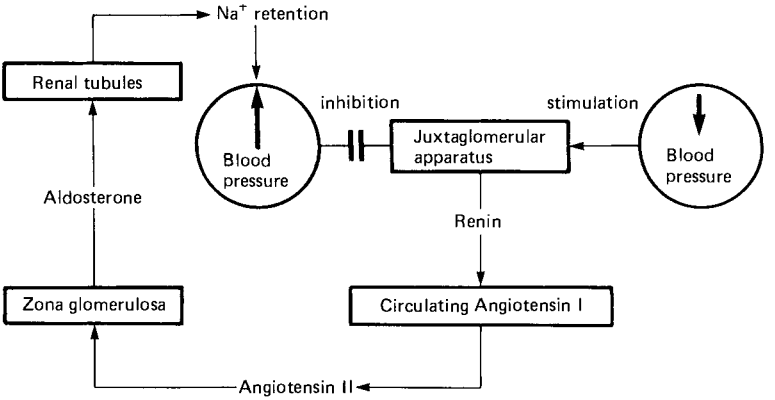


Figure 2.3 The mechanism of the renin–angiotensin system

juxtaglomerular apparatus (the point where the distal renal tubule touches the afferent renal arteriole) secrete renin directly into the blood stream. This proteolytic enzyme converts circulating angiotensin I into the active angiotensin II, which stimulates the cells of the zona glomerulosa to secrete aldosterone. Angiotensin II is a powerful vasoconstrictor which, as such, raises the blood pressure. Aldosterone in turn, by causing retention of sodium, increases both the osmotic and the blood pressure, which stretches the cells of the juxtaglomerular apparatus and prevents further release of renin.

The zona fasciculata is a much wider area, which produces glucocorticoids in response to stimulation by ACTH from the anterior pituitary. The production of glucocorticoids is regulated by a feedback mechanism which depends on the concentration of circulating cortisol, the principal glucocorticoid.

The hypothalamus is sensitive to the circulating level of cortisol and, when this is low, secretes corticotropin releasing factor (CRF). This stimulates the anterior pituitary to produce ACTH, which in turn activates the zona fasciculata and zona reticularis of the adrenal cortex to synthesize steroid hormones. This system, however, has no control over aldosterone. High circulating levels of cortisol inhibit the release of CRF and so no stimulation is given to the adrenal cortex (*see Figure 2.1*). Glucocorticoids promote the conversion of non-carbohydrate substances to glucose (gluconeogenesis). Their principal site of action is the liver, where they promote conversion of amino acids to glucose by activating cyclic AMP. They also activate AMP in the mobilization of fatty acids, and have a role in erythropoiesis (the production of red blood cells) and in the response to

allergy and stress. The negative feedback mechanism controlling cortisol production is shown in *Figure 2.1*.

The zona reticularis (the innermost layer) is composed of cells similar to those of the zona fasciculata, but containing less lipid. It is thought that they are responsible for the secretion of adrenal androgens and oestrogens. These hormones are more fully discussed below.

Steroid hormone synthesis in the adrenal cortex stems from the combination of cholesterol with acetate to form pregnenolone, which, by a series of enzyme-activated steps, is converted to the active hormone. The principal steps are shown in *Figure 2.4*. The hormones are metabolized in the liver to the tetrahydro derivatives and conjugated with glucuronic or sulphuric acid before being excreted in the urine.

Overactivity of the adrenal cortex may lead to primary aldosteronism, with increased reabsorption of sodium and water from the distal renal tubules causing oedema, or to Cushing's syndrome, where increased cortisol production increases gluconeogenesis and thus causes muscle wasting. Increased androgen production gives rise to the adrenogenital syndrome, a disorder which causes precocious secondary sexual development in children and hirsutism in adult females. The opposite effects are observed with underactivity. In Addison's disease there is inadequate renal tubular reabsorption of sodium and water, causing severe plasma electrolyte disturbance with decreased sodium and increased potassium levels, and hypoglycaemia due to the decreased formation of glucose from amino acids by gluconeogenesis.

Adrenocortical function is usually investigated by tests designed to stimulate or suppress the secretion of steroid hormones, either by direct action on the adrenal cortex or by an effect on the governing feedback mechanism.

The testes

Testicular development is controlled by follicle stimulating hormone, and the production of testicular androgens (the principal one of which is testosterone) by luteinizing hormone. Both these gonadotropic hormones are secreted by the anterior pituitary. Dehydroepiandrosterone (DHA), the androgen secreted by the adrenal cortex, is secreted in response to ACTH. Androgens are conjugated with sulphuric acid in the liver and excreted as 17-oxosteroids in the urine (*Figure 2.5*).

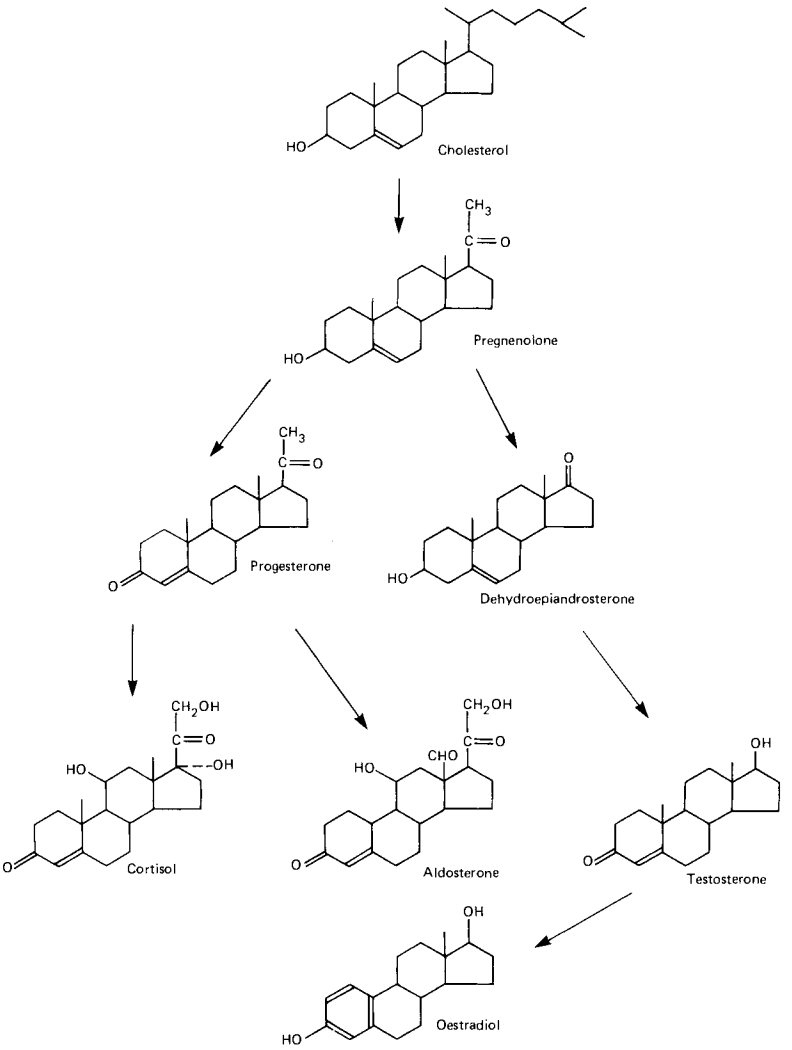


Figure 2.4 The synthesis of steroid hormones

The ovaries

FSH from the anterior pituitary stimulates development of the Graafian follicle, which promotes secretion of the oestrogen 17β-oestradiol. As the production of this hormone increases throughout

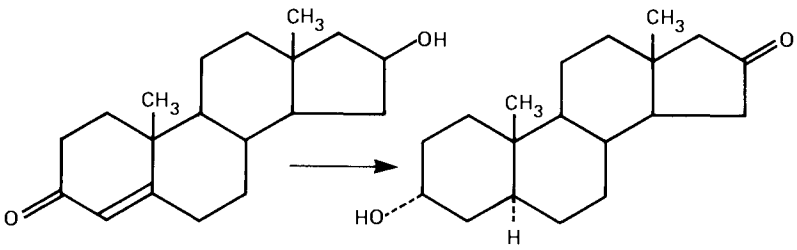


Figure 2.5 The formation of aetiocholanolone (a 17-oxosteroid) from the androgen testoterone

the follicular phase it depresses the production of FSH and stimulates that of LH by the anterior pituitary, which in turn stimulates ovulation. If fertilization occurs, the ovule is implanted in the endometrium, forming the corpus luteum. Chorionic gonadotropin from the developing corpus luteum released into the maternal circulation is sufficient to stimulate adequate steroid production by the mother until the developing corpus luteum is able to produce sufficient 17β-oestradiol and progesterone itself.

Both oestrogen and progesterone production rise during pregnancy, reaching a maximum at parturition. During pregnancy, oestrogens affect the reproductive organs to facilitate birth, and cause proliferation of the fetal cells. They also control some of the secondary sexual characteristics. Progesterone stimulates the placental cells to store glycogen, fats and amino acids for the developing fetus. The cycle of ovarian hormones is shown in *Figure 2.6*.

Gastrointestinal hormones

Gastrin

This hormone is secreted by the granular cells of the gastric and duodenal mucosa, and its production is controlled by a negative feedback mechanism dependent on the acidity of the stomach contents. Gastrin stimulates growth of the gastric mucosa and the secretion of both acid and pepsin by the mucosal cells. It also stimulates the production of insulin and glucagon, and possibly calcitonin. Gastrin levels are characteristically raised in pernicious anaemia, where there is achlorhydria, and lowered in the presence of gastric ulcers, where the gastric acidity is high.

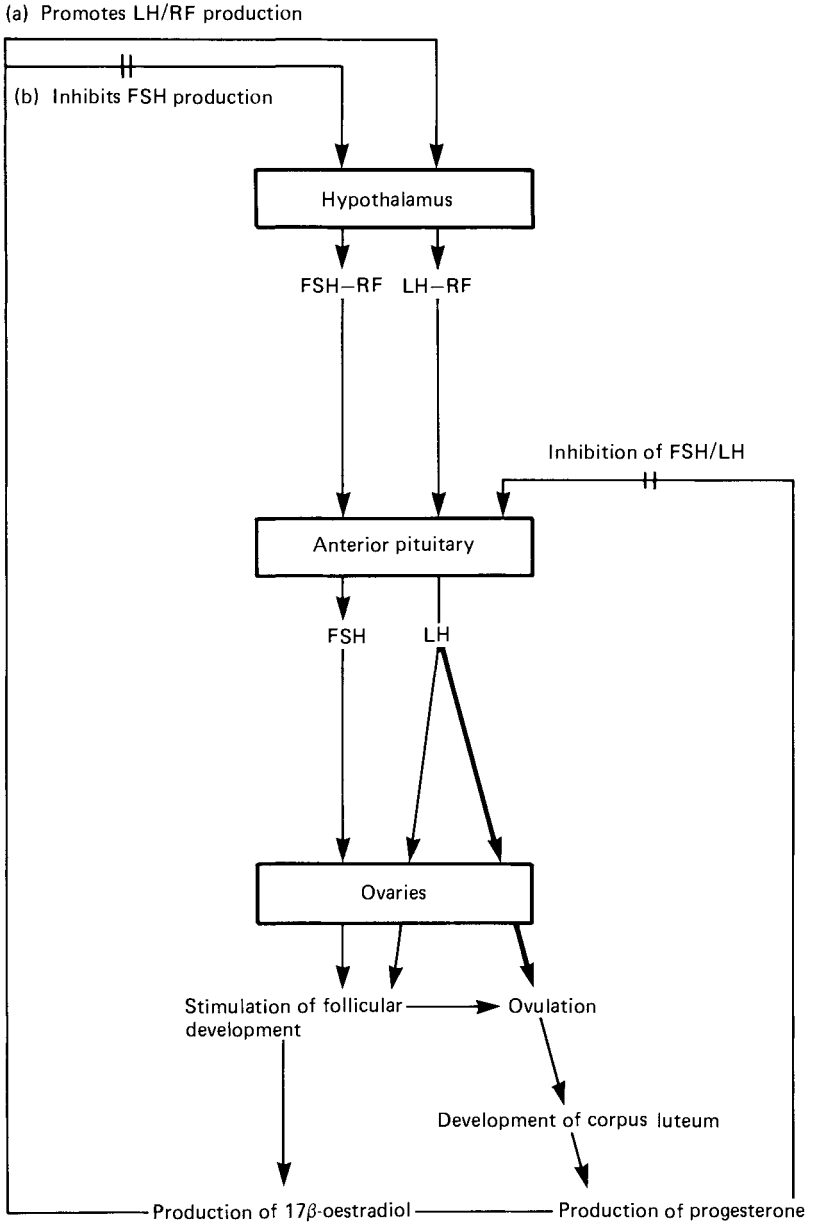


Figure 2.6 The cycle of ovarian hormones. LH = luteinizing hormone; FSH = follicle stimulating hormone; FSH-RF = follicle stimulating hormone releasing factor

Cholecystokinin-pancreozymin (CCK)
CCK is secreted by the cells of the upper small intestine. Its structure is similar to that of gastrin, but the molecule is larger. It initiates the constriction of the gallbladder, thus controlling biliary secretion into the small intestine, and the secretion of pancreatic enzymes. It is secreted in response to the presence of high molecular weight fatty acids in the intestine.

Secretin
This hormone is similar to glucagon in structure and is secreted by the upper intestinal mucosal cells. It increases biliary and pancreatic secretion of bicarbonate.

Assessment of hormonal function

Hormonal function can be assessed in the laboratory in several ways:

1. Direct measurement of the hormone concentration, usually by radioisotope techniques (e.g. thyroxine).
2. Measurement of the parameters affected by the hormonal action (e.g. plasma osmolality as a measure of ADH function).
3. Measurement of the effects of stimulation or suppression tests (e.g. the ACTH stimulation of the adrenal cortex, and the dexamethasone suppression test).

Principles of the techniques involved in the above tests are discussed elsewhere.

Chapter 3

Liver function

Anatomy

The liver is the largest organ in the body, weighing about 1.5 kg. It is roughly wedge-shaped and lies in the upper abdomen just below the diaphragm. It has two main lobes, the thin, flat left lobe, and a much larger right lobe, part of which is further subdivided to form the quadrate and caudate lobes. The lobes are divided into lobules, each of which consists of radiating layers of cells separated by the biliary canaliculi into which the bile is discharged. These canaliculi join to form the larger bile ducts which drain into the main lobules. The bile ducts unite to form the hepatic duct, which becomes the common bile duct when joined by the cystic duct from the gallbladder. This is a pear-shaped sac on the under surface of the right lobe which is lined with columnar epithelial cells and stores bile secreted by the liver. The common bile duct fuses with the pancreatic duct before entering the duodenum. The basic anatomy of the liver is shown in *Figure 3.1.*

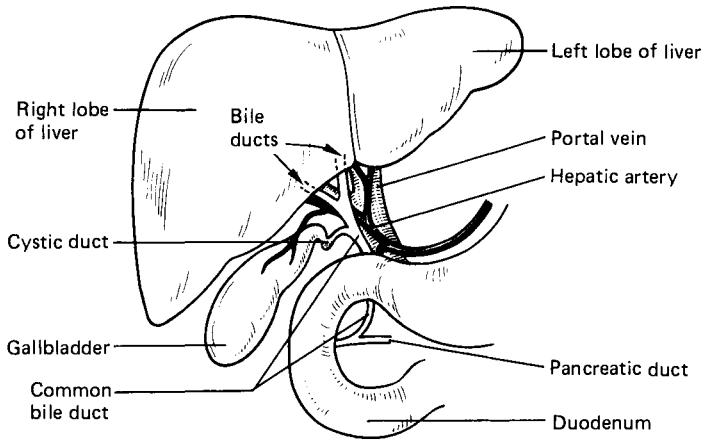


Figure 3.1 Basic anatomy of the liver and gallbladder

Function

The principal functions of the liver are the formation of bile, the synthesis and metabolism of proteins and amino acids, the storage of carbohydrates, and the removal of toxic substances from the body.

The formation of bile

Approximately 0.5–1 ℓ of bile is formed daily, and its flow is stimulated by the gastrointestinal hormone secretin. It is a complex mixture of lecithin, cholesterol, inorganic and bile salts, and bile pigment.

Bile acids such as cholic acid (derived from the conversion of cholesterol in the bile) enter the intestine conjugated with glycine or taurine as sodium glycocholate and sodium taurocholate. In the intestine they combine with fatty acids and triglycerides to form an emulsion which increases the absorption of fats. They also activate pancreatic lipase. Bile salt deficiency leads to impaired fat absorption and steatorrhoea (increased fat in the faeces).

The bile pigment bilirubin, which is responsible for the yellow discolouration of skin and sclera in jaundice (*see* below), is formed from the breakdown of haemoglobin in the reticuloendothelial system. It is transported to the liver bound to albumin, where it is conjugated with glucuronic acid by the enzyme glucuronyltransferase. The conjugated bilirubin is secreted into the biliary canaliculi and excreted in the bile, where it is converted by bacterial action to urobilinogen, in which form it is excreted in the faeces, although a small fraction is reabsorbed and excreted in the urine. On excretion it is rapidly oxidized to urobilin. A summary of the metabolism of bilirubin is shown in *Figure 3.2*.

Jaundice

In *haemolytic anaemia* there is, as the name implies, excessive destruction of red blood cells. The liver therefore receives larger amounts of bilirubin than it can conjugate and remove, and this is reflected in the increased concentrations of unconjugated (free) bilirubin in the plasma. In *Gilbert's syndrome* the increase in unconjugated bilirubin is not due to haemolysis but to reduced uptake of bilirubin by the liver cells, although liver glucuronyltransferase is also reduced. Activity of this enzyme may be enhanced by treatment with phenobarbitone. *Crigler–Najjar syndrome* is a rare disease caused by a severe deficiency or absence of glucuronyltrans-

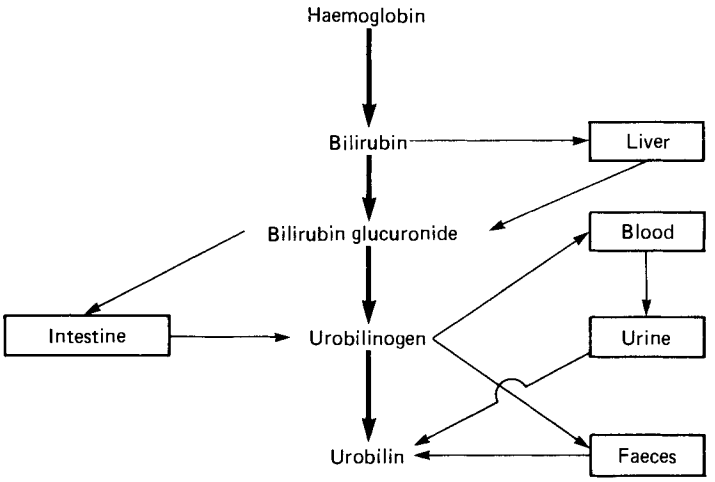


Figure 3.2 The metabolism of bilirubin (*see text for details*)

ferase and consequent accumulation of unconjugated bilirubin. Unconjugated bilirubin is not water-soluble, and therefore cannot be excreted. It is toxic to the tissues. *Dubin–Johnson syndrome* is characterized by increased concentrations of conjugated bilirubin in the plasma due to an inability to transport the conjugated bilirubin into the biliary canaliculi. In *obstructive jaundice* the increase is always in conjugated bilirubin. Biliary tract obstruction may be caused by gallstones or by pressure from the pancreas on the bile duct, as in carcinoma of the head of the pancreas.

In all forms of non-obstructive jaundice where there is an excess of conjugated bilirubin, there is also increased excretion of urobilinogen.

The synthesis and metabolism of amino acids and proteins

The liver is the only site of albumin synthesis, and most of the other plasma proteins, with the exception of the γ-globulins, are also synthesized in the liver. In addition, the liver is responsible for the production of α-fetoprotein before and shortly after birth. This protein, not normally present in adults, has been demonstrated in the serum of patients with hepatic carcinoma. The main functions of albumin are the regulation of osmotic pressure and to act as carrier of many substances throughout the body.

Amino acids are degraded in the liver by deamination and transamination, resulting in the formation of ammonia and urea

(*Figure 3.3*). Liver damage leads to inadequate removal of ammonia, and thus low blood urea and high blood ammonia concentrations.

The storage of carbohydrates

The liver acts as a buffer in the supply of carbohydrates for fuel. When the blood glucose level is high, glucose is converted to glycogen and stored, while when the blood glucose is low, glycogen is released from this store and hydrolysed to glucose (*see Figure 1.1* for this part of the Embden–Meyerhof–Parnas pathway). Deficiencies of the enzymes involved in the production and breakdown of glycogen in the liver are known collectively as the *glycogen storage diseases*; the most common of these (but still rare) is Von Gierke's disease (glucose-6-phosphatase deficiency).

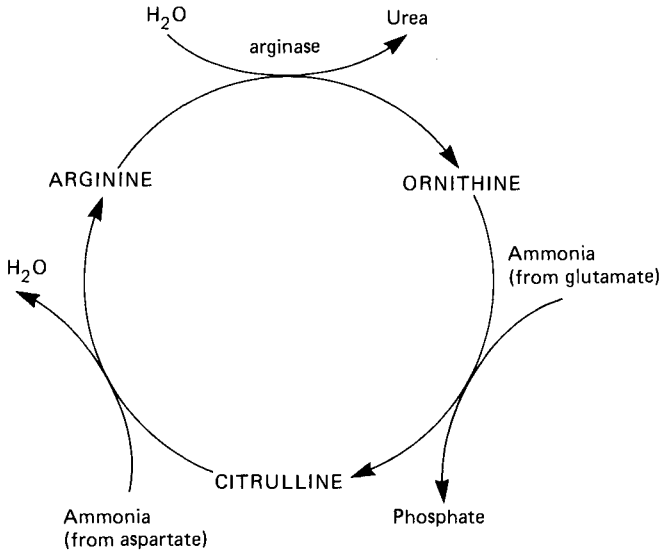


Figure 3.3 The formation of urea from ammonia

The removal of toxic substances from the body

Many substances are removed from the body by conversion to water-soluble substances in the liver so that they can be excreted by the kidneys. This may be done by oxidation, reduction, hydroxylation, and conjugation with glucuronic or sulphuric acid.

Other liver functions

1. Approximately one-fifth of the synthesis and breakdown of red blood cells takes place in the liver.
2. Vitamins A, D, K, B_{12} and folic acid are stored in the liver, and nicotinic acid is formed here from tryptophan.
3. Chylomicrons are taken up by the liver and converted to smaller units.
4. Cholesterol is synthesized and used with protein in the formation of VLDL by the liver.

Assessment of liver function

Because of the wide range of functions performed by the liver, a battery of tests is needed if liver function is to be assessed adequately, unless specific enzyme deficiencies are suspected. For example, a set of liver function tests may include determination of bilirubin and its metabolites, serum alkaline phosphatase, albumin, alanine aminotransferase and urea. The galactose tolerance test will provide information regarding carbohydrate metabolism, and the bromsulphthalein test information regarding excretory function. For discussion of individual tests please *see* Chapter 6.

Chapter 4

Renal function and acid–base metabolism

Anatomy

There are approximately one million functional units (nephrons) in each kidney (*Figure 4.1*). The afferent renal arteriole gives rise to a tuft of capillaries (glomerulus) which is embedded in the blind loop of the nephron (Bowman's capsule) and whose membrane allows small substances to pass from the blood into the capsule. Extending from the capsule is the proximal convoluted tubule terminating in a straight portion (the pars recta) which leads into the thick descending limb of the loop of Henlé. The thin section of the descending limb extends towards the renal pelvis. The point at which the distal renal tubule touches the afferent arteriole is termed the macula densa and marks the beginning of the distal convoluted tubule, which enters the collecting duct. This duct joins the collecting tubule

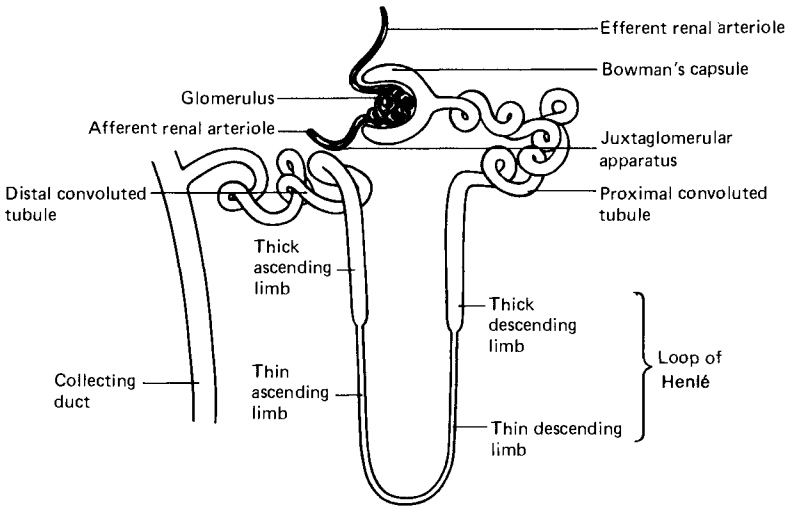


Figure 4.1 Functional anatomy of the nephron

leading to the renal pelvis and ureter. The cells of the afferent renal arteriole at the macula densa form the juxtaglomerular apparatus and secrete renin.

Function

The kidneys have several important functions: glomerular filtration; tubular reabsorption of selected substances; tubular excretion; the production of renin; and secretion of erythropoietin.

Glomerular filtration

The hydrostatic pressure of the blood entering the glomerulus is higher than that of the fluid within the renal tubule, so that fluid from the blood is forced into the capsule, producing an ultrafiltrate which contains not only waste products, but also many substances which are still required by the body and thus need to be recovered. The function of the remainder of the nephron is to reabsorb these substances and to secrete those which are not required. Approximately 1.25 litres of blood flow through the kidney each minute. The amount of filtrate passing through the renal tubule is the *renal plasma flow* (RPF) and is expressed as the volume (ml) of filtrate formed per minute. The *effective renal plasma flow* (ERPF) is usually measured by determining the amount of *p*-aminohippuric acid (PAH) cleared by the kidneys per minute. This substance is both filtered by the glomerulus and secreted by the tubules. The normal ERPF is approximately 625 ml/minute. The *glomerular filtration rate* (GFR) is the rate at which a substance which is neither secreted nor absorbed by the renal tubules (e.g. inulin) is cleared from the plasma. The normal GFR is approximately 125 ml/minute. The ratio of GFR to ERPF is known as the *filtration fraction* and approximates to the amount of filtrate absorbed. This is usually about 0.18.

Renal tubular reabsorption and excretion

Many substances including sodium, potassium, phosphate, chloride, amino acids and sugars, are reabsorbed from the proximal tubule together with large volumes of water, so that as the filtrate approaches the loop of the descending limb its osmolality increases, gradually forming a concentration gradient within the limb. Some of these are 'threshold' substances, i.e. they are not reabsorbed when the blood concentration reaches the threshold value. For example,

once the blood glucose concentration exceeds 10 mmol/ℓ, glucose is excreted in the urine.

Hydrogen and potassium ions are secreted by the tubular cells in exchange for the sodium ions reabsorbed. These combine with phosphate and bicarbonate ions. Ammonia is also secreted by the tubular cells. This combines with hydrogen ions to form ammonium ions which are excreted as ammonium chloride. The renal tubular secretion of hydrogen and ammonia in exchange for sodium is shown in *Figure 4.2*. The ascending limb of Henle's loop is impermeable to sodium and water but not to chloride, so that in its passage through this portion the osmolality of the tubular fluid decreases.

By the time the filtrate reaches the distal tubule about 80 per cent of its original water content, i.e. that entering the glomerulus, has been reabsorbed. Final adjustments to the fluid volume are made under the influence of the hormones ADH and aldosterone. ADH alters the permeability of the distal renal tubules by making the cells permeable to water. Release of this hormone is regulated by a feedback mechanism dependent on the blood volume. Aldosterone permits the distal tubules to reabsorb sodium. In response to a lowered blood pressure in the afferent renal arteriole the granular cells of the juxtaglomerular apparatus secrete renin, which activates angiotensin in the blood to stimulate the adrenal cortex to release aldosterone.

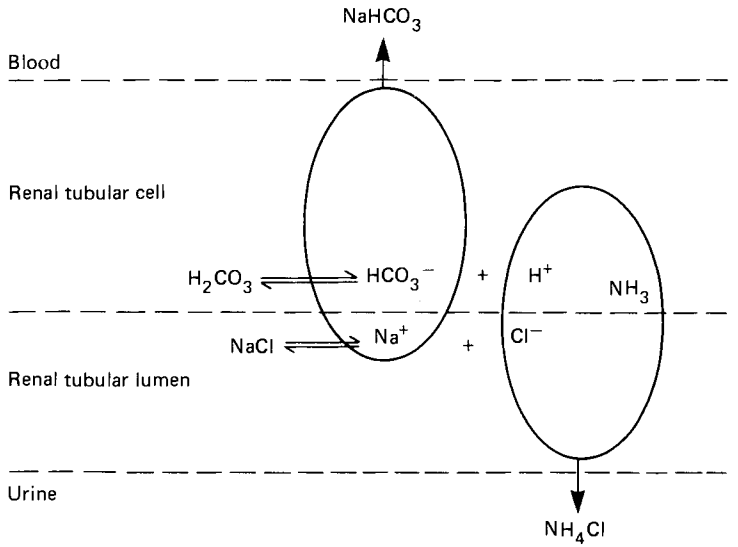


Figure 4.2 The renal tubular excretion of hydrogen and ammonia

Assessment of renal function

Damage to the nephrons increases the permeability of the glomerular membrane, allowing molecules, including protein, to pass into the tubules. Albumin, because of its relatively small molecular weight, is the first protein to cross the membranes and therefore usually the first to be found in the urine. Albuminuria and a decreased serum albumin concentration are therefore characteristic findings in renal disease. In addition to protein, many other substances are found in the urine following renal damage. Uraemia is a characteristic feature of renal disease, and reflects the inability of the kidney to excrete the waste products of metabolism. Creatinine parallels the rise in urea but is less specific, and unreliable when determined at low levels. The creatinine clearance is a valuable indicator of glomerular function. The determination of plasma electrolytes and blood gases can be used to assess the degree of renal acidosis produced by a reduction in bicarbonate and the corresponding alteration of other electrolytes. The efficiency of the renal tubules to produce acid is determined by the ammonium chloride test.

Hydrogen ion concentration

Many substances dissociate to form ions when in solution. Those which are highly ionized are usually inorganic, and those which are weakly ionized are usually organic.

Acids are defined as substances which liberate hydrogen ions (hydrogen donors) and bases as substances which accept hydrogen ions. Water dissociates to a very small extent to form hydroxonium and hydroxyl ions, as shown in the equation below:

$$2\,H_2O \rightleftarrows H_3O^+ + OH^-$$

For convenience the hydroxonium ions are usually referred to as hydrogen ions, and the equation is therefore commonly written as below, although strictly speaking this is incorrect:

$$H_2O \rightleftarrows H^+ + OH^-$$

Using Ostwald's dilution law the dissociation constant for water is calculated as follows:

$$K_w = \frac{[OH^-] \times [H^+]}{[H_2O]} = 10^{-14}\ \text{mol}/\ell$$

As the degree of dissociation is very small indeed and most of the

water is in the molecular form, the concentration of water molecules may be ignored, so that

$$K_w = [OH^-] \times [H^+] = 10^{-14} \text{ mol}/\ell$$

It follows that if the concentration of one ion is known, that of the other can be readily calculated.

Sørensen eliminated the problem of using negative exponents by introducing the concept of the pH value, defined as the logarithm of the reciprocal of the hydrogen ion concentration, i.e.:

$$pH = \log \frac{1}{[H^+]}$$

By definition a molar solution (i.e. a solution containing 1 mol of that substance per litre) of a completely dissociated monovalent acid has a hydrogen ion concentration of 1.0 mol/ℓ, and therefore

$$pH = \log \frac{1}{1.0} = 0$$

A 0.1 molar solution would contain 0.1 mol/ℓ and have a pH of

$$\log \frac{1}{0.1} = 1.0$$

Similarly, a molar solution of a completely dissociated base has a hydroxyl ion concentration of 1.0 mol/ℓ. Since

$$[OH^-] \times [H^+] = 10^{-14} \text{ and } [OH^-] = 1.0$$

it follows that

$$[H^+] = 10^{-14} \text{ and } pH = \log \frac{1}{10^{-14}} = 14$$

The pH of an 0.1 molar solution of the same base can be calculated in the same way. Since the hydroxyl ion concentration is 10^{-1} mol/ℓ,

$$[H^+] = 10^{-13} \text{ mol}/\ell \text{ and } pH = \log \frac{1}{10^{-13}} = 13$$

As the example above shows, each change in the pH value of 1 unit denotes a tenfold change in the ionic concentration of the solution.

A solution is said to be neutral when the concentration of hydrogen ions and hydroxyl ions is the same, i.e. 10^{-7} mol/ℓ. At that point

$$pH = \frac{1}{10^{-7}} = 7.0$$

For simplicity, we have so far ignored the fact that substances do

not dissociate completely, and that the degree of dissociation has to be taken into account when calculating the pH (*see later*).

Let us now consider the case of an organic acid (e.g. acetic acid) which is not highly ionized, i.e. a weak acid. In solution it will ionize as follows:

$$CH_3COOH + H_2O \rightleftarrows CH_3COO^- + H_3O^+$$

However, as water is only very slightly ionized (*see above*), its effect on the equation may be disregarded, and for convenience the hydroxonium ion will again be regarded as the hydrogen ion. The equation may then be rewritten as

$$CH_3COOH \rightleftarrows CH_3COO^- + H^+$$

According to the law of mass action

$$K_a = \frac{[CH_3COO^-] \times [H^+]}{[CH_3COOH]}$$

where K_a is the dissociation constant for acetic acid.

If the degree of dissociation is α and the concentration of acetic acid is c, the equation may be rewritten as

$$K_a = \frac{(\alpha c)(\alpha c)}{(1-\alpha)c} = \frac{(\alpha c)^2}{(1-\alpha)c}$$

Since, compared with unity, the value of α is very small, this may be disregarded in the denominator so that

$$K_a = \frac{(\alpha c)^2}{c} \text{ or } \alpha^2 = \frac{K_a}{c} \text{ or } \alpha = \sqrt{\frac{K_a}{c}}$$

The dissociation constant for acetic acid is 1.75×10^{-5}. At a concentration of 1 mol/ℓ the degree of dissociation is therefore

$$\alpha = \sqrt{\frac{1.75 \times 10^{-5}}{1.0}} = \sqrt{\frac{17.5 \times 10^{-6}}{1.0}} = 4.183 \times 10^{-3}$$

and the hydrogen ion concentration

$$[H^+] = 4.183 \times 10^{-3} \times 1.0 \text{ mol}/\ell$$

The pH of this solution is therefore

$$pH = \log \frac{1}{4.183 \times 10^{-3}} = 0.0 - 0.6215 + 3 = 2.38$$

Analogous calculation of the pH of 0.1 molar and 0.01 molar solutions of acetic acid gives values of 2.88 and 3.38 respectively, indicating that a tenfold change in concentration of solutions that are only weakly dissociated does not bring about as significant a

change in the pH as equivalent changes in the concentration of strong, highly dissociated organic acids. This property forms the basis of pH buffering.

Buffer solutions

In the body, as in many test reactions, it is necessary to keep the pH within narrow limits despite the addition of strong acids and alkalis. A buffer solution consists of a mixture of a weak acid and its salt. Its buffering action is due to the mopping up of hydroxyl ions by the weak acid to produce its salt and water, e.g.

$$NaOH + CH_3COOH \rightleftarrows CH_3COONa + H_2O$$

and the mopping up of excess hydrogen ions by combining with the salt to form a weak acid, e.g.

$$HCl + CH_3COONa \rightleftarrows CH_3COOH + NaCl$$

The pH of a buffer solution is proportional to the ratio of the molar concentrations of the weak acid and its salt. Increasing the salt concentration increases the pH of the buffer. The concentration of undissociated acid molecules almost equals that of the molecular concentration (as it is weak) and the dissociated salt ions almost equal that of the molar salt concentration. With this in mind, and returning to the law of mass action, we can write for the buffer solution containing acetic acid and sodium acetate:

$$K_a = \frac{[CH_3COO^-] \times [H^+]}{[CH_3COOH]} \text{ or } [H^+] = \frac{K_a[CH_3COOH]}{[CH_3COO^-]}$$

and more generally

$$[H^+] = \frac{K_a[\text{acid}]}{[\text{salt}]} \text{ or } pH = \log 1/K_a \frac{[\text{salt}]}{[\text{acid}]}$$

By analogy with the definition of the pH value we can formulate that

$$pK_a = \log \frac{1}{K_a}$$

The pH of a buffer solution is therefore

$$pH = pK_a + \log \frac{[\text{salt}]}{[\text{acid}]}$$

This is known as the Henderson–Hasselbalch equation.

The body has two important buffer systems to control the acid-base balance:

1. The carbonate-bicarbonate buffering system:

$$H_2CO_3 \rightleftarrows H^+ + HCO_3^-$$

2. The haemoglobin (Hb) buffering system:

$$HHb \rightleftarrows H^+ + Hb^-$$

In addition, excess bicarbonate can be removed via the lungs (as expired CO_2) through an increase in the rate of ventilation.

The normal pH of blood is 7.4. An increase in pH above 7.45 is termed *alkalosis* and may be respiratory, i.e. due to a primary decrease in the P_{CO_2}, or metabolic, due to a primary increase in bicarbonate. A decrease below pH 7.35 is termed *acidosis* and may also be either respiratory, due to a primary increase in P_{CO_2}, or metabolic, due to a primary decrease in bicarbonate. In order to compensate for changes in the acid–base balance, the body responds to a primary respiratory alkalosis with a secondary metabolic acidosis, or vice versa, and to a primary metabolic acidosis with a secondary respiratory alkalosis.

The buffering power of plasma depends mainly on the carbonate-bicarbonate system. Since carbonic acid is only a very tiny fraction of the system, and is also in equilibrium with the dissolved CO_2 in the plasma, this may be ignored, so that the Henderson–Hasselbalch equation now becomes:

$$pH = pK + \log\frac{[HCO_3^-]}{[CO_2^-]}$$

In the laboratory investigation of acid–base disturbances, pH, P_{CO_2} and bicarbonate determinations may all be used.

Chapter 5

Analytical techniques in clinical chemistry

Potentiometric determination

This technique depends on the measurement of differences in electric potential (U) between two electrodes in an electrochemical cell. In such a cell, electrons can be accepted or donated by an inert metallic conductor. There is a three-dimensional relationship between the potential, the current and the concentration (or more correctly, the activity) of the solution, so that if two of these factors are known or can be measured, the third may be derived from them. This relationship is expressed for U by the *Nernst equation,* which, in its simplest form, is

$$U = U^0 + 0.0591 \log(\text{ionic activity})$$

where U is the maximum difference in potential when the current is zero, and U^0 the standard electrode potential.

The value of 0.0591 is derived from the ratio $(RT/nF) \times 2.3$, where R is the gas constant, T the temperature, n the valency, and F the Faraday constant. One electrode in the cell acts as the reference and is of a known and constant potential, while the other is the measuring electrode. Reference electrodes are usually either silver/silver chloride or mercury/mercurous chloride (calomel) in composition.

A system for pH measurement is shown in *Figure 5.1.* The measuring electrode consists of a glass tube with a very thin-walled glass bulb at the base. Inside the tube is a buffer solution and a silver/silver chloride wire connected to an electrical lead. The second electrode usually consists of a mercury/mercurous chloride electrode in saturated potassium chloride solution. The two electrodes are connected by an electrical circuit with a measuring unit between them. Hydrogen ions in solution normally move in a random manner; however, when the current is applied a potential difference is set up between the two electrodes and the hydroxyl ions migrate towards the anode (positive electrode) and the hydrogen ions towards the cathode (negative electrode). The current is carried by

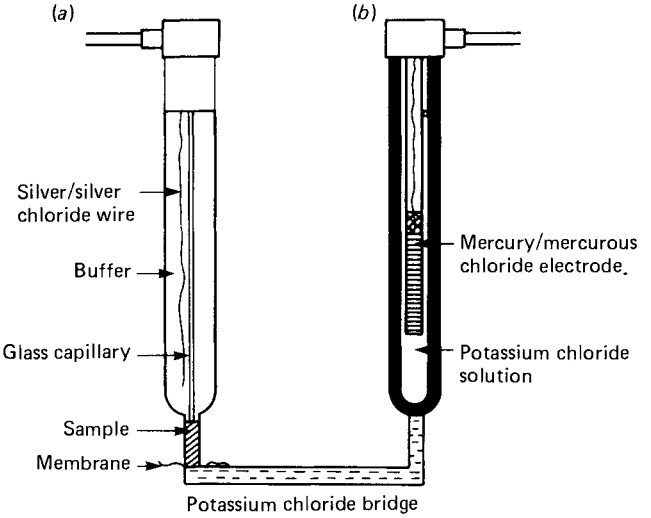


Figure 5.1 An electrode system for the determination of pH. (*a*) Measuring electrode. (*b*) Reference electrode. The membrane allows passage of ions but prevents contamination of the potassium chloride (KCl) with the sample. The KCl bridge forms electrical contact between the electrodes

the positively charged hydrogen ions and is measured on the galvanometer.

For pH measurement, the primary standard is 0.05M potassium hydrogen phthalate, defined by the British Standards Institute as having a pH of 4.0 at 15 °C.

The Severinghaus PCO_2 electrode uses a normal glass pH electrode, but a gas-permeable membrane to separate the sample from the reference bicarbonate solution in which the reference electrode is placed. Diffusion of CO_2 from the sample across the membrane decreases the pH of the reference solution in proportion to the concentration of PCO_2.

The Clark PO_2 electrode consists of a platinum cathode separated from the sample by a gas-permeable membrane. The anode is a silver/silver chloride electrode. Oxygen from the sample diffuses across the membrane and causes a difference in potential between the two electrodes. The amount of current produced is directly proportional to the PO_2 concentration.

Direct potentiometric measurements which do not involve dilution of the sample may give different results to those obtained by other methods or those involving dilution, because they measure the concentration in the plasma water and not that in the total plasma

volume. In normal plasma the ratio of the final volume to the initial volume is very nearly the same for plasma and plasma water, and the difference in results is very small and of no consequence; however, in sera with high lipid or protein concentrations the results show marked differences, with indirect measurements giving apparently lower values.

The use of ion-specific or ion-selective electrodes has become increasingly popular in the field of clinical chemistry. They have the advantage that only very small samples are needed, and that these are not altered in any way. The use of ion-selective membranes allows direct measurement of selected ions (e.g. calcium ions) or indirect measurement of other substances; for example, the hydrogen peroxide electrode is used for the determination of cholesterol and glucose by measuring the hydrogen peroxide formed from the appropriate enzyme-controlled chemical reaction.

Spectroscopy

Spectroscopy is the observation of specific absorption bands in coloured solutions. A simple spectroscope will permit qualitative investigation of blood and urine pigments, and with some refinement the instrument may be used to make semiquantitative measurements.

Light passing through a prism is split into its component parts, and a coloured solution placed between the eye of the observer and a suitable light source will absorb some of the light rays. As fluorescent light produces specific emission lines which interfere with the observation, sunlight or tungsten light, which do not interfere, should be used. Dark bands appear at the point(s) where the light has been absorbed. Many substances have specific absorption bands and these may be used in their identification.

The Hartridge reversion spectroscope has two reversed spectra and is calibrated in ångström (10 Å = 1 nm). When the equivalent absorption bands of both spectra are in exact alignment the reading on the micrometer (in ångström) is that of the absorption band (*Figure 5.2*).

In addition to the simple reading of absorption bands, readings before and after treatment with reducing agents are often of value in the identification of pigments. By convention, the bands are identified by Greek letters, commencing with the band nearest to the red end of the spectrum, which will be the α-band.

Artificial standards may also be prepared and their absorbance compared with that of the test solution.

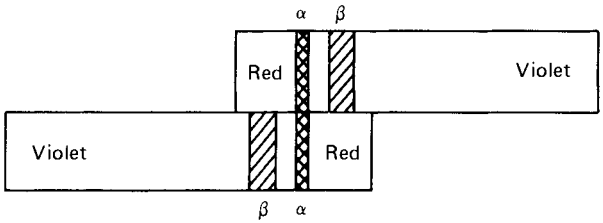


Figure 5.2 The alignment of absorption bands of oxyhaemoglobin using the Hartridge reversion spectroscope. The α-bands are aligned at 5780 Å (578 nm). The β-bands are at 5400 Å (540 nm) and are not aligned

Blood specimens which are diluted in water often give hazy absorption bands because of incomplete rupture of all membranes; very dilute ammonia solution may be used as a diluent in order to rupture the cell membranes. It must be remembered, however, that ammonia is a reducing agent, and in sufficient concentration, may alter the spectra obtained.

Spectrophotometry

Spectrophotometry is used for quantitative analysis of many substances which are either coloured themselves or which produce a colour when allowed to react with other substances. The colour of a substance is due to the wavelengths of light which are transmitted through it or reflected by it. Light falling on a coloured solution is either absorbed by it or transmitted through it. Absorption curves show that the wavelengths of maximum absorption and light transmission are complementary; for example, a red solution transmits more red light than light of any other colour, and absorbs more green light. The reverse is true for a green solution. The more intense the colour of a solution, the greater the amount of light absorbed. In many instances the amount of light absorbed is proportional to the concentration of the coloured substance.

According to Beer and Lambert's laws the amount of light absorbed by successive layers of equal thickness of a solution is the same and proportional to the number of light-absorbing molecules in the light path. If, therefore, the light absorption could be measured directly, using matched cuvettes (cells of the same thickness and with the same optical characteristics), a plot of varying concentrations against absorption would yield a straight line. The concentration of a solution of unknown concentration is directly proportional to that of a similarly treated known standard:

$$\frac{C_t}{A_t}=\frac{C_s}{A_s} \text{ or } C_t=\frac{C_s \times A_t}{A_s}$$

where C_t is the concentration of the test, C_s the concentration of the standard, A_t the absorption of the test, and A_s the absorption of the standard. This statement holds only when Beer and Lambert's laws are obeyed. It is therefore necessary to verify this experimentally, and, if it applies to a given solution, to determine the range over which it applies.

Spectrophotometers measure the light which is transmitted, not that which is absorbed. Transmitted light falling onto a photoelectric cell containing a photosensitive element produces a current which is proportional to the amount of light transmitted. The current is then relayed to a galvanometer, where it is measured. If a colourless reference solution is placed in front of the light source, no light will be absorbed, and the amount of light transmitted (T) is 100 per cent. The amount of light transmitted by coloured solutions may be compared to that of similarly treated standard solutions. Plotted against concentration, this produces a logarithmic curve (*Figure 5.3*). Since this arrangement is not very convenient, most instruments are calibrated in absorbance, which is the logarithm of the ratio of the incident light (I_0) to the transmitted light (I_t).

$$\text{Absorbance } (E)=\log\frac{I_0}{I_t}=\log\frac{100}{\%T}=2-\log\ \%T$$

Where Beer's law is obeyed, this produces a linear relationship when plotted against concentration (*Figure 5.3*).

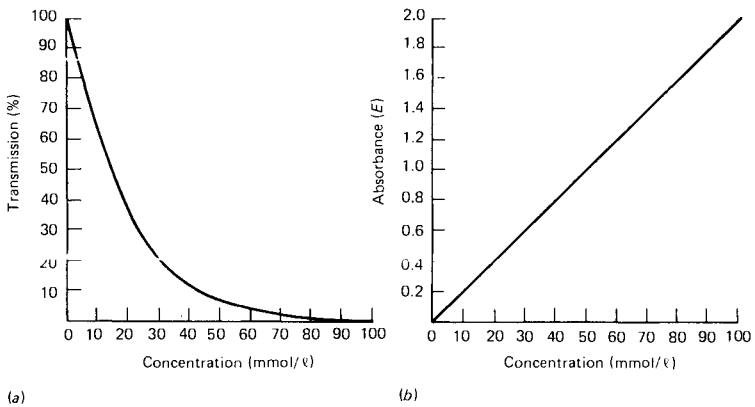


Figure 5.3 The relationship between (*a*) transmission and concentration and (*b*) absorbance and concentration (*see text for details*)

In addition to the light absorbed and transmitted, some light is reflected, but as this is contant for a given instrument it can be ignored, provided the solution is clear and the conditions under which the test and standard are read are identical.

In the simplest type of spectrophotometer (*Figure 5.4*), light passes from a source through a slit and filter to the solution in a cuvette. The transmitted light falling on the photocell produces a current which is measured by a galvanometer.

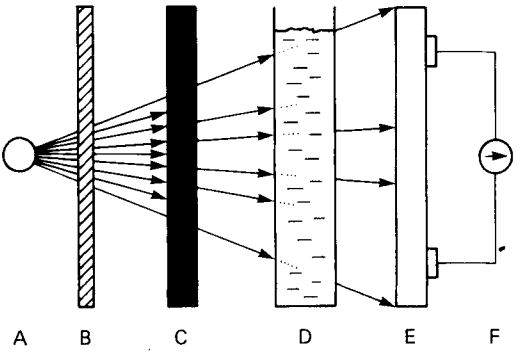


Figure 5.4 Optical diagram of a simple spectrophotometer (colorimeter). A = light source; B = adjustable slit; C = glass filter; D = cuvette holding coloured solution; E = photoelectric cell; F = galvanometer

Spectrophotometers differ in their ability to resolve the bands of light and produce (so-called) monochromatic light. This is illustrated in *Figure 5.5*, which shows the absorption curves of an oxyhaemoglobin solution obtained from three different instruments, using (a) a series of spectral filters, (b) a continuous wedge filter, and (c) a diffraction grating. If white light were allowed to pass through the coloured solution, only a very small proportion of the incident light would be absorbed, and therefore the relative absorption (sensitivity) would be low. In order to achieve maximum absorbance, unwanted light must be eliminated. The simplest method of doing this is to use coloured filters, which are usually made of glass. The optimum filter is usually one of the complementary colour to that of the solution being measured, unless there is interference from the reagents themselves or from other substances in the test solution. *Figure 5.6* shows the relationship between coloured solutions and the wavelength of light absorbed.

In order to produce the desired spectral characteristics some glass

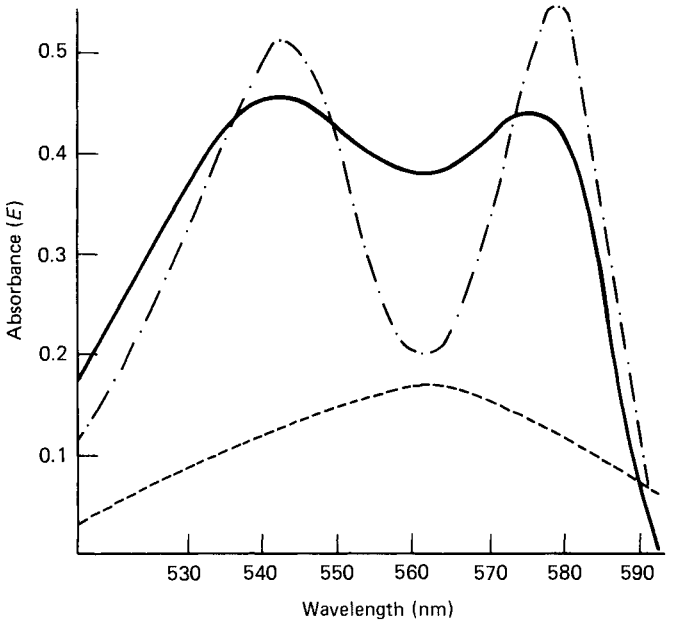


Figure 5.5 Absorption curves of oxyhaemoglobin obtained with three different instruments using a series of different glass filters (----); a continuous wedge filter (——); a diffraction grating (—·—·)

filters are made of two layers of glass sandwiched together. With simple filters, the *band pass width* (*Figure 5.7*) is often too wide for a particular purpose, and in order to improve resolution, this can be reduced to the order of 10–15 nm by using interference filters, i.e. successive layers of partially reflecting metallic film interspaced by a dielectric substance between two protective glass absorption filters. Internal reflection of light between the metal films reduces the wavelength of the transmitted light.

Diffraction gratings are produced from a master grating and consist of a series of grooves ruled extremely closely together and reproduced in epoxy resin which is aluminized and mounted on an optically flat surface. Each successive wave on the wave front has a different distance to travel to reach the grating, and so the deflected rays will be scattered (*Figure 5.8*). Rotation of the grating in relation to the slit permits selection of the wavelength of light allowed to pass through. Prisms are also used to select light of narrow band pass width, as their resolving power is very good. Light is diffracted by the prism so that the rays become separated. Selection, as with diffraction gratings, is by rotation of the prism in relation to the slit.

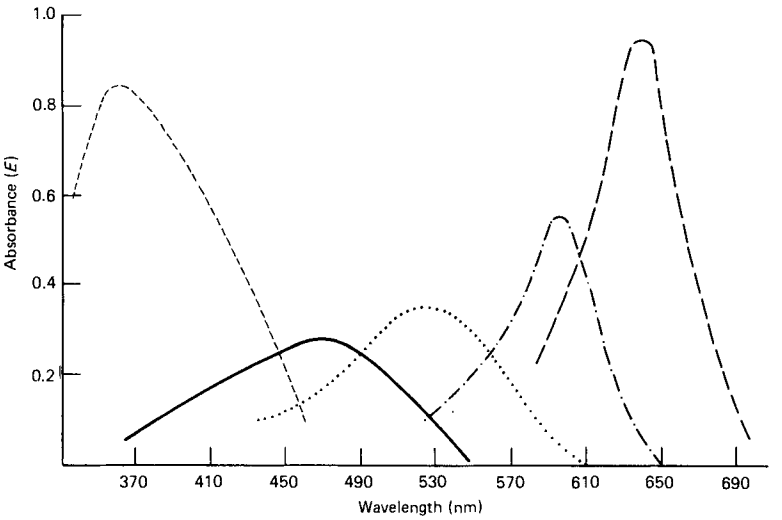


Figure 5.6 The relationship between the colour of a solution and the wavelength of light absorbed. (---) yellow; (——) orange; (.....) red; (–·–·–) purple; (– –) green

The accuracy of wavelength calibration may be checked and set against special holmium and didymium (mixture of neodymium and preneodymium) filters. In the visible spectrum, these have very narrow peaks at the wavelengths indicated below:

Holmium: 418 nm, 453 nm, 536 nm, 637 nm
Didymium: 573 nm, 586 nm, 685 nm

The light source of a spectrophotometer depends on the wavelength at which readings are required. For recordings in the visible spectrum a tungsten or mercury vapour lamp is required. For ultraviolet absorption a hydrogen lamp is required. Most spectrophotometers use a hydrogen lamp for readings from 200 to 350 nm and a tungsten source for readings above 350 nm. The simplest photodetectors (photoelectric cells) used in colorimetry consist of a copper or iron base plate covered on one surface by a layer of selenium on top of which is coated a thin layer of a light-transmitting metal. At the perimeter of this layer is a metal collecting ring (*Figure 5.9*). Light falling onto the cell penetrates the thin metallic layer covering the selenium, causing the selenium to liberate electrons in proportion to the amount of light reaching it. These electrons pass from the transparent metallic layer to the collecting ring before being directed through the instrument to a galvanometer

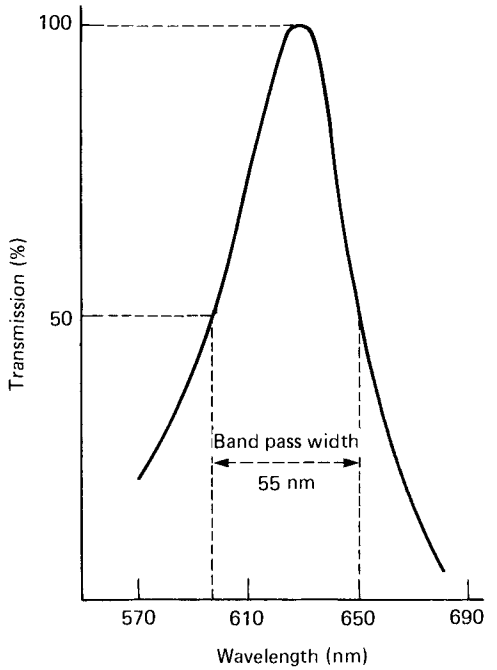


Figure 5.7 Transmission spectrum of a filter showing the band pass width and nominal wavelength. (In the example shown the nominal wavelength is 625 nm and the band pass width is 55 nm)

which records the amount of current produced. When very narrow band path widths are used, as with diffraction gratings or prisms, considerably less light is transmitted, so that much more sensitive photodetectors are required, and phototubes or photomultiplier tubes are used. These are evacuated tubes filled with an inert gas at a very low pressure and which contain a thin caesium or potassium oxide layer from which electrons are emitted as light penetrates the tube. These electrons are collected by a metal anode and multiplied to give an infinitely greater number than was originally produced. The current is then measured by means of a galvanometer. A small contribution to the total current produced is generated by the continuous discharge of electrons from the phototube. This is the 'dark current' and for accurate readings needs to be subtracted from the total. Double-beam instruments, in which the light beam is split in order to pass through the test and reference solutions simultaneously, compensate automatically for the dark current as well as

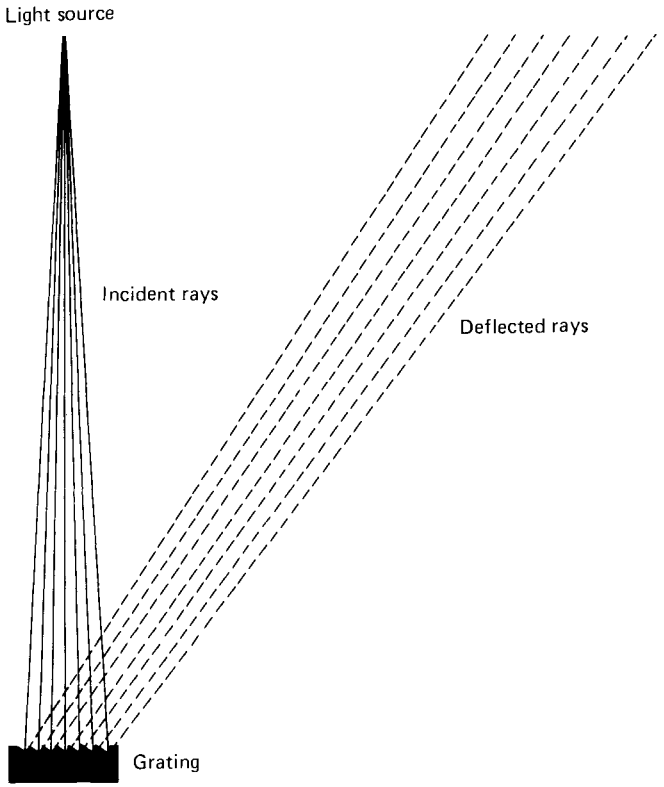


Figure 5.8 Diffraction grating

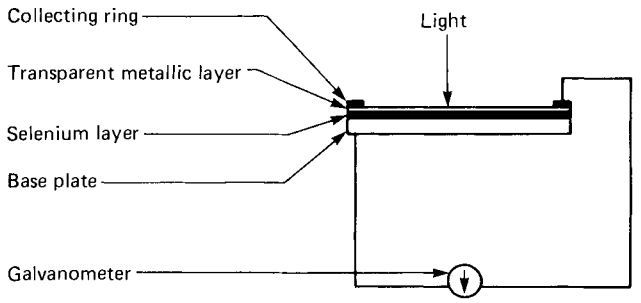


Figure 5.9 A simple photoelectric cell

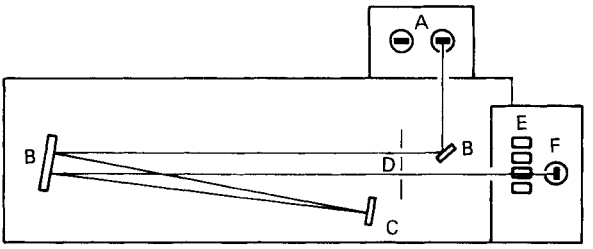


Figure 5.10 Diagram of a single-beam spectrophotometer. A = light source; B = mirror; C = diffraction grating; D = slit; E = sample cuvettes; F = phototube. Note: this instrument has two light sources (one for visible and one for ultraviolet light) and provision for four sample cuvettes

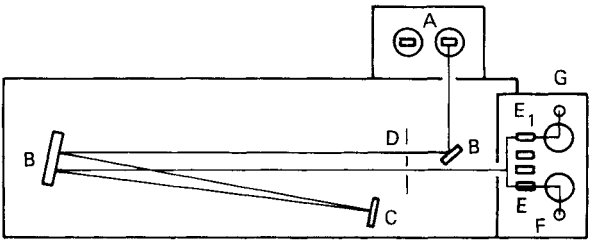


Figure 5.11 Diagram of a double-beam spectrophotometer. A = light source; B = mirror; C = diffraction grating; D = slit; E = sample cuvettes; E_1 = reference cuvette; F = sample phototube; G = reference phototube. Note the split beam between sample and reference tube

for any mains current fluctuation. *Figures 5.10* and *5.11* show optical diagrams for single- and double-beam spectrophotometers.

Flame emission spectrophotometry

This technique uses the specific light emissions which occur when excited electrons fall from a higher energy level back to their normal position (ground state). When heated strongly in a flame, the outer electrons of some elements become 'excited' and move outwards from their normal orbit to the next energy level. In so doing they absorb a specific amount of energy, which is related to the ground state of the electron and therefore specific to each element. The electrons are unable to remain in their new orbit and fall back to the ground state. As they return, the energy gained during excitation is released as emitted light of a specific wavelength. The excitation of lithium is shown diagramatically in *Figure 5.12*. As the amount of emission is proportional to the concentration of the element, a direct

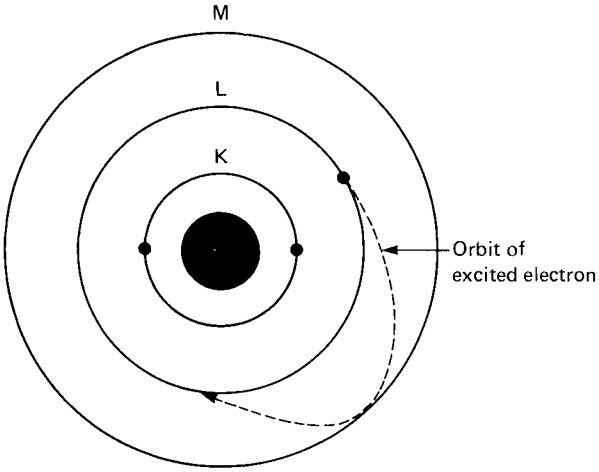


Figure 5.12 The excitation of lithium. The electron from the outer shell (L) migrates to a temporary higher energy level (M). As it returns to the ground state it loses this energy in the form of light emission. K, L and M represent different electron orbits (energy levels)

comparison between the emission of similarly treated standards and tests will determine the concentration of the test solution.

The essential components of a flame emission spectrophotometer are shown in *Figure 5.13*. The specimen is drawn into the system by air suction and atomized into a very fine mist in the spray chamber where the larger droplets and excess fluid pass to waste, whilst the fine spray is mixed with a suitable gas mixture (depending on the flame temperature required to achieve excitation) before being passed into the flame. The approximate flame temperatures of some commonly used mixtures are:

Coal gas–air	1800 °C
Propane–air	1900 °C
Acetylene–air	2000 °C

With monovalent elemental analysis, e.g. for sodium, potassium and lithium, the most commonly used mixture is propane–air.

The emitted light is selected by a suitable monochromator system (to remove wavelengths emitted by other interfering substances) and passed to a photodetector. An advantage of this technique is that it allows multielemental analysis by splitting the light beam and passing it through suitable filters for each desired analyte.

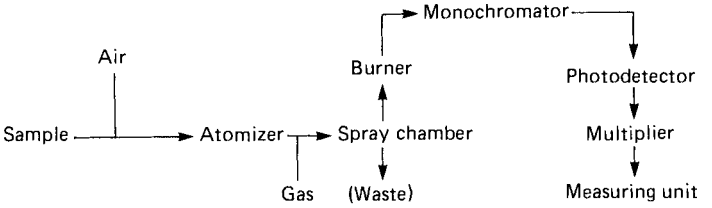


Figure 5.13 Basic components of a simple flame emission spectrophotometer

Two points are worthy of special note. The first is that an allowance has to be made for the difference in viscosity between aqueous standard solutions and plasma or serum samples, which contain a considerable amount of protein. Furthermore, care must be taken not to permit the dilution tubes and atomizer to become coated with a deposit through the continuous passage of protein-containing solutions, as this will increase the inherent error mentioned above. Secondly, it must be remembered that hyperproteinaemic and hyperlipaemic samples give erroneously low values because of the abnormal proportion of plasma volume taken up by proteins or lipids, neither of which are contained in the plasma water.

Variations in the flame characteristics and the atomization of samples may be overcome by the use of double-beam instruments using an internal standard. With this system, the diluent used for all the solutions to be analysed is a constant amount of another element, which is also analysed by the instrument but not contained in the test solutions. The light beam is split so that the added constituent becomes the reference analyte, and the potential difference between this and the test side is monitored. In this way, differences in gas pressure and atomization rate, as well as current fluctuations, are cancelled, and a steadier, more reliable result is obtained.

Atomic absorption spectrophotometry

The energy absorbed by atoms excited from the ground state is characteristic of those atoms. If light with the same wavelength characteristics as the radiation absorption of the element to be analysed is passed through a curtain of flame containing those atoms in the excited state, the amount of light energy absorbed will be proportional to the concentration of the element.

The light source is normally a hollow cathode lamp containing a small quantity of the appropriate element which emits light of the desired wavelength on the application of a high voltage. As the

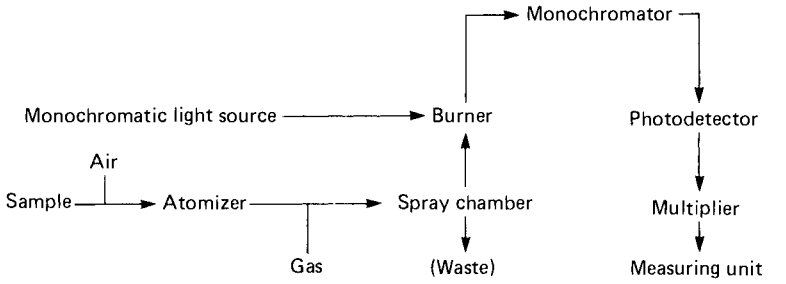


Figure 5.14 Basic components of a simple atomic absorption spectrophotometer

measurement is made at the ground state, this technique is more sensitive than conventional spectrophotometry. In addition, a larger number of elements can be analysed. The equipment is, however, more complex and expensive. The instrument (*Figure 5.14*) consists of a stable monochromatic light source (a mercury vapour or hollow cathode lamp) which directs a narrow beam of light across a flame into which the atomized sample has been aspirated. Absorption takes place according to the specific elements in the sample becoming excited. The beam is then directed to a monochromator system, as in a conventional spectrophotometer. Many instruments have a double-beam facility, i.e. the light beam is divided before entering the flame and the potential difference between the two is measured. In some instruments, the specimen is placed on a carbon rod or piece of tantalum instead of being passed through a flame. The temperature is then raised and the specimen atomized. This technique is more sensitive than that using a flame and allows analysis of trace metals, but the amount of interference is increased.

Interference in atomic absorption may be spectral, chemical, or due to ionization. Spectral interference, which is due to overlapping radiation from elements other than the test element, can be overcome by the selection of other spectral lines or by chemical separation before atomic absorption. Spectral interference from the flame itself can be eliminated by using an alternating current (a.c.) amplifier which will not accept the flame signals. Chemical interference is due to the formation of complexes with other substances. For example, phosphate interferes with the analysis of calcium by forming calcium phosphate, which reduces the method sensitivity. The addition of strontium or lanthanum, which have a greater affinity for phosphate, will prevent calcium from forming such complexes. Interference caused by ionization of free metallic atoms

is suppressed by the addition of a second, easily ionized metal (radiation buffer).

Fluorimetric analysis

Some molecules, when irradiated with a suitable light source, will become excited and absorb energy which, on return to the ground state, is released as light emission. However, as some of the energy absorbed is lost, the emitted light is of a longer wavelength than that which was absorbed. This phenomenon is known as *Stoke's shift* and the type of emission as *fluorescence*. Fluorimetry measures the emission from molecules in the ground state and is therefore much more sensitive than spectrophotometry, which measures excited molecules. The measuring instrument, however, is more complex. There is less interference than with spectrophotometry, as fewer substances fluoresce than simply absorb light. Fluorescence is most likely to occur in aromatic substances, especially if their structure is rigid. It is enhanced by chelation with metals, which gives added sensitivity to the technique.

The most generally useful source of excitation is a xenon lamp, as it has an emission spectrum throughout the visible spectrum as well as in the ultraviolet range, but zinc and mercury vapour lamps are also used. As shown in *Figure 5.15*, light from the source is passed through a monochromator (either a primary filter or a diffraction grating) which selects the wavelength of light for excitation, which is then passed to the test substance in the cuvette. The cuvette must have optical surfaces on all sides, as the fluorescence emitted is collected at right angles to the incident light. This is passed through a secondary filter or grating to select the fluorescent wavelength. The selected light passes to the photosensitive element, producing a current which is proportional to the amount of light falling upon it and thus to the concentration of the fluorescent material.

Nephelometric analysis

The measurement of the amount of turbidity caused by precipitation reactions has been used for semiquantitative analysis in clinical chemistry laboratories for many years, but until recently no suitable equipment was available to make full use of the relationship between the amount of light scattered and the concentration of the light-scattering particles. The amount of turbidity produced may be measured simply by visual comparison with a set of similarly treated standards or more precisely by the use of a spectrophotometer. The most sensitive and precise technique, however, is *laser nephelometry*.

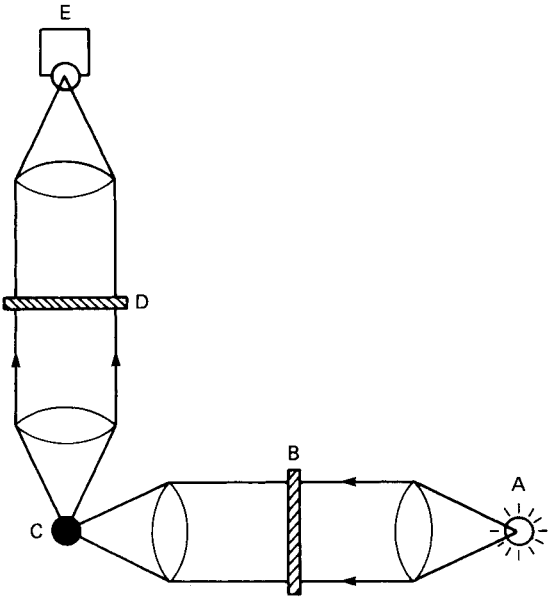


Figure 5.15 Optical diagram of a filter fluorimeter. A = light source; B = primary filter (monochromator); C = specimen; D = secondary filter; E = photomultiplier

This allows very much smaller quantities to be measured, and thus permits quantitative determination of the concentration of specific proteins by antibody–antigen precipitin methods.

In nephelometry the scattered light is measured at right angles to the incident light, as in fluorimetry. A laser (the name is an acronym from light amplification by stimulated emission of radiation) produces a very much more intense and narrow beam of light than other sources. This is achieved by bouncing excited atoms back and forth inside a glass laser core (*Figure 5.16*). This is surrounded by a glass tube, and both are filled with free helium and neon gas. At one end of the tube is a fully reflective mirror, while at the other there is a partially reflective mirror. On making electrical contact the helium atoms become excited and collide with the neon atoms, thus exciting them. The excited neon atoms emit photons of light, some of which escape via the partially reflective mirror. These are measured in the usual manner. Inside the core, the remaining excited atoms continue to bounce back and forth between the two mirrors, making further collisions and thus emitting more photons. In this way a light beam of very high intensity and very narrow band pass width is produced.

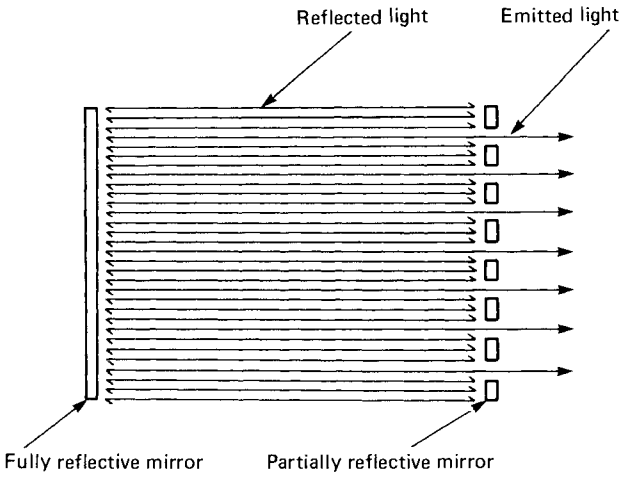


Figure 5.16 Diagram showing the production of a laser beam

The measurement of osmolality

Water placed on either side of a semipermeable membrane will flow freely through the membrane in either direction, but if the initial column height of water on one side of the membrane is greater than that on the other the direction of flow will be predominantly from the highest column until the levels on both sides have been equalized (*Figure 5.17a*). This passage of water is known as *osmosis*. If a solute is introduced into the system, i.e. if two solutions of different concentrations of the same solute are separated by a semipermeable membrane, water from the weaker solution will pass more quickly across the membrane than water from the stronger solution until both solutions have attained the same concentration (*Figure 5.17b*). The presence of a solute has therefore exerted a pressure against the membrane, equal and opposite to that of the column of water on either side. This pressure is known as the *osmotic pressure*. The amount of pressure exerted does not depend on the total weight of solute present but on the total number of particles in the solution. Equimolecular solutions of non-ionized substances will therefore exert the same osmotic pressure, but the pressure exerted by ionized solutions is governed by the total number of ions in solution.

The osmolality of a solution is a measure of the total number of particles in solution (and is therefore an indicator of the osmotic effect). Osmolality is expressed in mol/kg and is determined by the

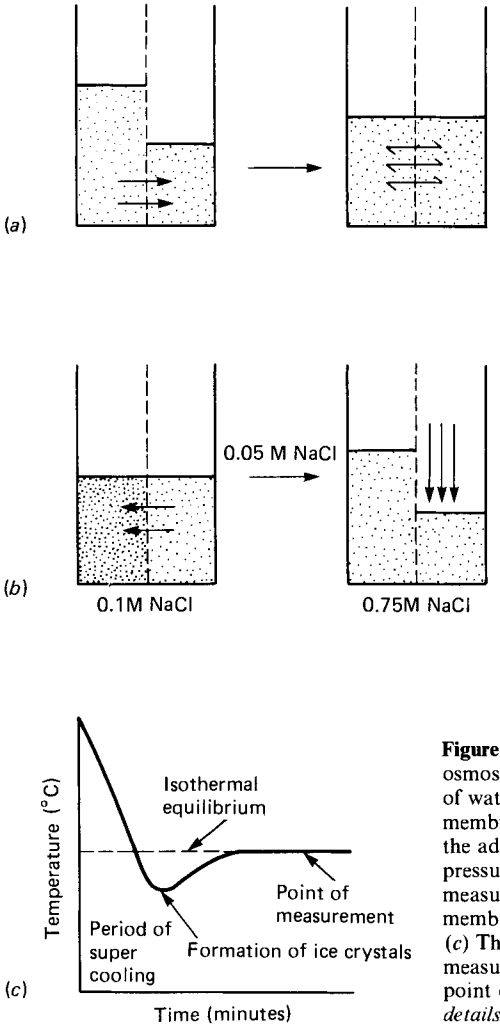


Figure 5.17 The principal effects of osmosis. (*a*) The osmotic movement of water across a semipermeable membrane. (*b*) The osmotic effect of the addition of a solute. The osmotic pressure exerted (arrows) can be measured on the opposite side of the membrane with a pressure gauge. (*c*) The osmolality is determined by measuring the decrease in freezing point of a solution (*see text for details*)

molar concentration × valency × osmolar coefficient (R). For example, 0.1 M NaCl has an osmolality of $0.1 \times 2 \times 0.9 = 0.18$ mol/kg. (In clinical laboratories this is normally expressed in mmol/kg, i.e. 180 mmol/kg.) Because glucose is not ionized in solution a 0.1 M solution has an osmolality of 0.1 mol/kg (100 mmol/kg).

Most of the osmotic effect in plasma is exerted by the sodium

concentration and is roughly equivalent to twice the plasma sodium concentration (in mmol/ℓ).

Osmolality is determined from the depression in freezing point, which also depends on the total number of particles in solution, in an instrument known as an *osmometer*. The samples and standards are placed into a refrigerated bath containing an antifreeze solution and an electric thermistor probe, which is sensitive to minute temperature changes, is placed into the test solution close to a vibrating rod or paddle. The thermistor probe is connected to a measuring unit which converts the temperature readings to osmolality units. Some modern instruments use a Peltier element, which is warm on one side and cold on the other, placed between a metal block containing apertures for the sample tube and the freezing needle on the cold side, and a cooling element on the warm side. A wheatstone bridge circuit is set up and the difference in temperature between freezing distilled water and the sample registers as a difference in resistance, which can be converted to osmolality units. The advantages of this system are that much smaller sample volumes are required, and that there is no need for cooling baths and antifreeze solutions. In both systems the sample is first supercooled to a point below its freezing point. Agitation then generates just sufficient heat to raise the temperature to the point of isothermal equilibrium – the 'hanging' point between freezing and thawing (*Figure 5.17c*). This temperature is maintained on a plateau for recording and conversion to osmolality units.

Immunological techniques

Precipitation reactions between antibodies and their corresponding antigens are of great value in the immunological determination of specific proteins. The precipitate produced by such a specific reaction is termed a *precipitin*. Until these methods became available, the techniques for the determination of plasma proteins were neither accurate nor specific.

The original immunodiffusion technique of Ouchterlony (*Figure 5.18a*) used plates containing an agar gel from which small wells of agar had been removed. The central well contained the specific antibody and the outer wells the test sera. On being allowed to stand, the antibody diffuses from the central well towards the perimeter, while the serum from the outer wells diffuses towards the centre. If any of the outer wells contains the antigen to the specific antibody, precipitation occurs along the line of intersection between the two diffusing substances. This qualitative technique was modified by

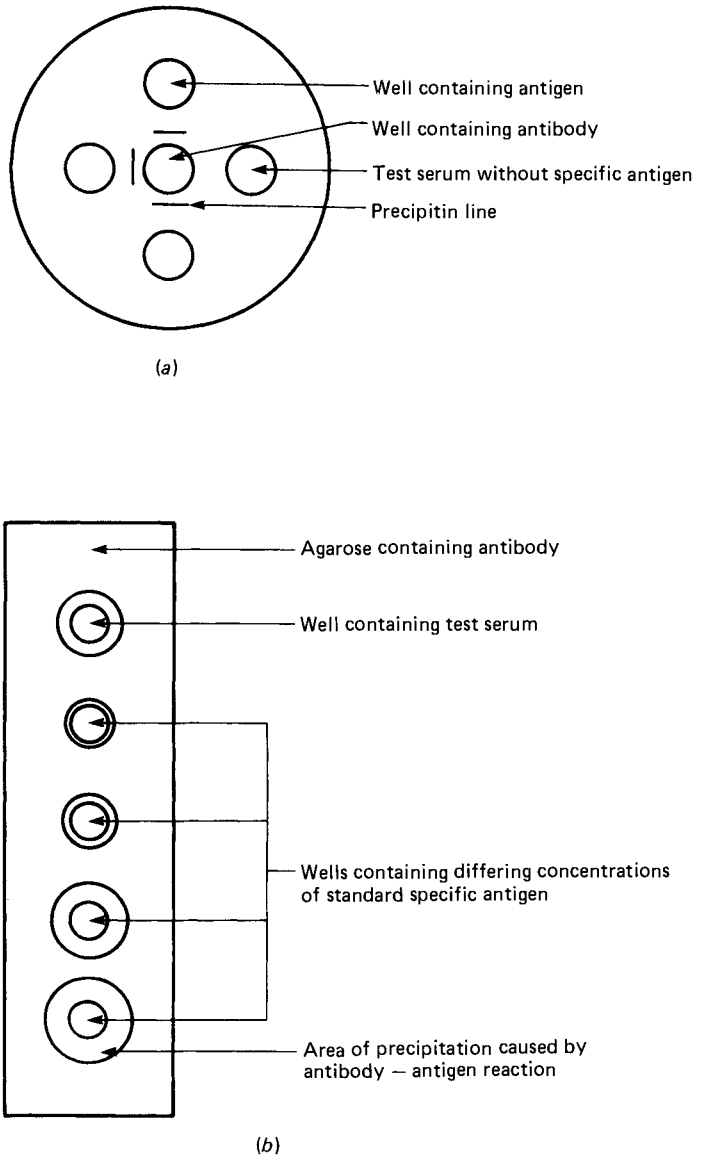


Figure 5.18 Immunodiffusion techniques using (*a*) the Ouchterlony and (*b*) the Mancini immunodiffusion plate.

Mancini to allow quantitative determination of specific proteins. The plates contain an agarose gel mixed with the specific antibody, and the wells, cut out as before, are filled with standard solutions of specific protein of known concentration and with the test serum (*Figure 5.18b*). If the serum contains the specific antigen, diffusion will result in the formation of a precipitin ring around the well. With increasing concentration of antigen this will spread further from the well. A comparison of the diameter of the rings produced from the standards and test sera will give a quantitative measure of the specific protein. By plotting either the logarithm of the antigen concentration against the diameter of the precipitin ring, or the antigen concentration against the square of the diameter, a linear relationship is found. The Clarke technique as modified by Laurell is a combination of electrophoresis in one direction followed by immunoelectrophoresis at right angles to the first separation. Serum is placed in the well of an agarose plate and an electrophoretic run is performed (*Figure 5.19a* and *b*). This is carefully placed alongside another agarose plate containing antibody, and re-run at 90° to the

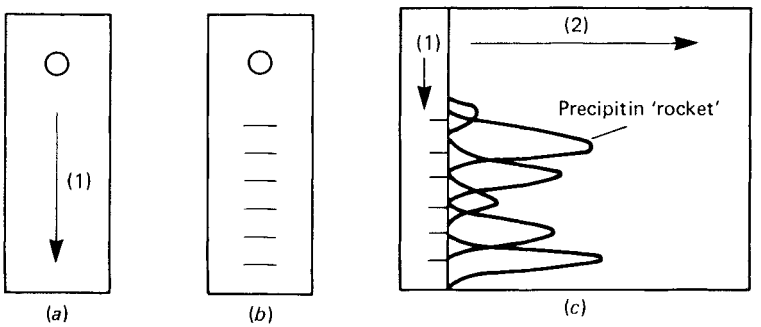


Figure 5.19 Stages in two-dimensional (rocket) immunoelectrophoresis. (*a*) Agarose plate with well containing test serum. (*b*) Completed first dimension electrophoretic run. (*c*) At right angles to first run, using an agarose plate containing antibody. The arrows indicate the direction of the two runs (*see text for details*)

first run (*Figure 5.19c*). A precipitin 'rocket' marks the area occupied by the antigen which has migrated into the antibody-containing agarose. The area of the rocket is proportional to the antigen concentration and may be compared to similarly treated known standards.

Chromatographic separation

Chromatography is the physical separation of substances by their different adsorption, or the partition of solutes between two imiscible phases, one of which is stationary and the other mobile. The stationary phase may be either a liquid or a solid, and the mobile phase either a gas or a liquid.

Adsorption chromatography uses a solid stationary phase with either a gas or liquid mobile phase. In gas–solid adsorption chromatography (GSC) the substance is injected into a carrier gas which is passed over a column of adsorbent material. Substances are adsorbed at different parts of the column according to their adsorbent properties. In liquid–solid adsorption chromatography a slurry of adsorbent, usually silica or alumina, is poured into a glass column which has a sieve or filter at the base and a tap or clip below to control the flow (*Figure 5.20b*). The analyte is poured into the column and the test substances are separated by adsorption onto the column. Elution is by the selective addition of suitable solvents for each separate substance.

Gel filtration is a variation of adsorption chromatography where very large molecules do not enter the pores of the stationary phase

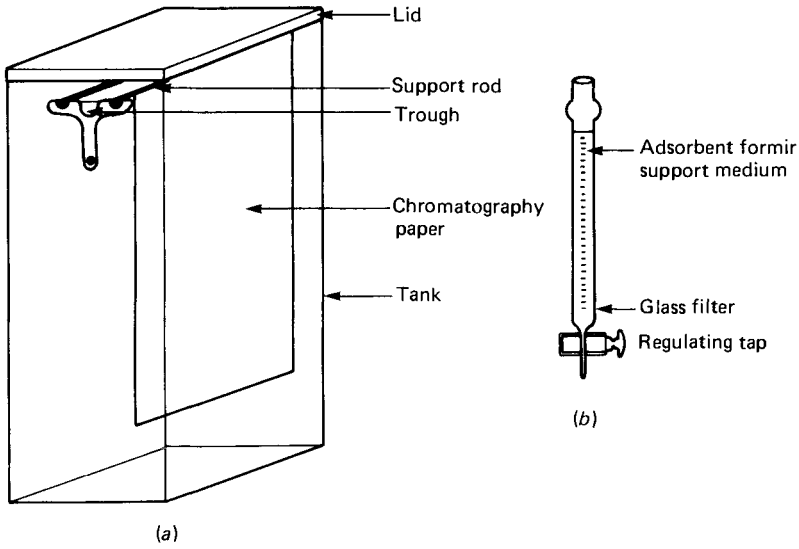


Figure 5.20 (*a*) Descending paper (partition) chromatography; (*b*) Glass column for liquid–solid adsorption chromatography.

and thus pass through the column faster than smaller molecules, which are eluted in decreasing order of molecular size, depending on their ability to enter the pores of the stationary phase.

In ion-exchange chromatography (another variation of solid–liquid chromatography) the column is a resin which will exchange ions with the substances passed through it.

Partition chromatography depends on the differing solubility of the substances in the mobile and stationary phases. Gas–liquid chromatography (GLC) is an example of such a system. Here the stationary phase is an involatile liquid coated onto a support medium. The substance for analysis is injected into a stream of carrier gas passing over the stationary phase. Substances are left on the column according to their degree of solubility, and eluted by the selective addition of suitable solvents. In paper and thin-layer chromatography the pores of the support medium (paper, silica gel or alumina) contain water and act as minute separating funnels, fractionating the sample presented to it as it rises or falls along the solvent front of the mobile phase. The different solutes are deposited along the support medium according to their solubility. The relative distance travelled by each substance from the point of application compared to the distance travelled by the solvent front is known as the R_f *value*. R_f values may be calculated for different substances in a variety of solvent systems and can be used to identify the substances. Standards should, however, always be run in addition, as it is very difficult to reproduce exactly the conditions of a chromatographic separation. In order to eliminate draughts and maintain a constant temperature, the chromatographic run is made in a closed equilibrated system. With paper, the run may be ascending, with the solvent mobile phase in the bottom of the tank, or descending, as illustrated in *Figure 5.20b*, where the solvent runs down the paper from a trough at the top of the tank. This is useful for the separation of substances which require long separation times, as the solvent can be allowed to drip off the end of the paper. Thin-layer chromatography (TLC) is normally only run using the ascending technique. The special advantages of TLC are that much smaller sample volumes and separation times are required than with paper, since the pores of the support medium are very much smaller. *Figure 5.21* shows a two-dimensional chromatographic separation in which, following a primary separation in the first solvent system, the chromatogram is dried and run in a second solvent system at 90° to the first. This technique is used in amino acid separations where a very large number of substances are to be separated. The sample is applied, as shown, in one corner of the paper. The final chromatogram is compared to a similarly treated 'map' prepared from known amino

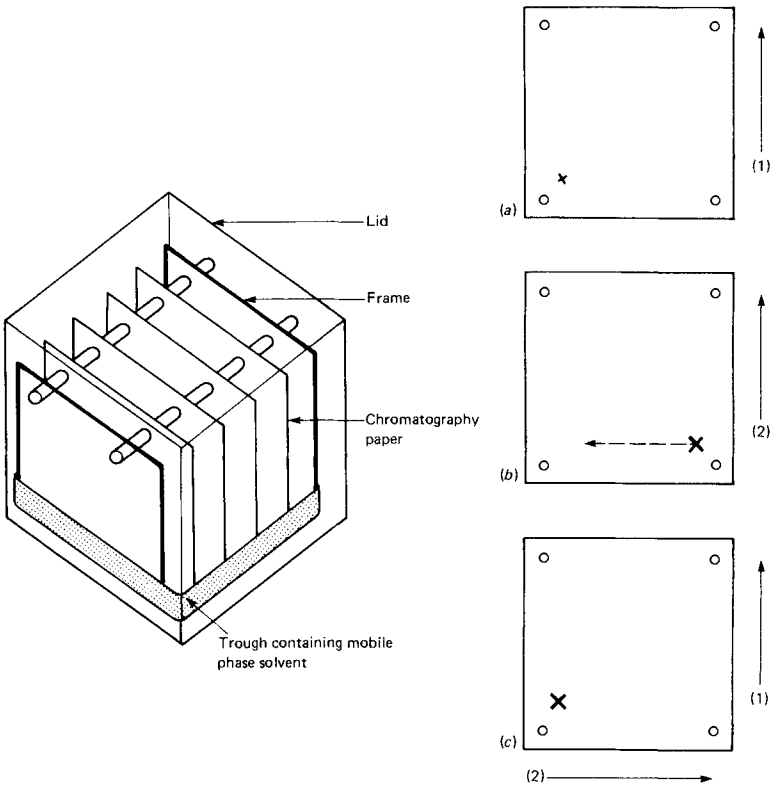


Figure 5.21 Two-dimensional chromatography. (*Left*) Shandon Universal chromatography tank showing ascending chromatogram in position. (*Right*) Diagram showing the two steps of the separation. (*a*) First run (1) in acetone:pyridine:ammonia solvent. (*b*) Second run (2) at 90° to the first run in isopropanol:formic acid:water. (*c*) Final appearance. (×) = point of sample application

acid standards in order to identify the amino acids present in the test sample.

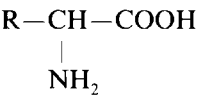
On completion of a chromatographic run, coloured substances will be readily seen without further treatment. Others may be visible under ultraviolet light or need the addition of a location reagent before they can be detected and identified. The location reagent is either sprayed onto the support medium or the medium may be dipped into a trough of the location reagent. The chromatogram then is air-dried and heated (if necessary) in order to develop the colour. Semiquantitative measurements can be made either by

elution of the spots from the medium and ordinary spectrophotometry or by reflectance densitometry of the spot on the support medium.

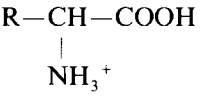
Protein electrophoresis

Proteins consist of very long chains of amino acids linked together by peptide bonds. It is the physical properties of these amino acids which are responsible for the migration of proteins during electrophoretic separation.

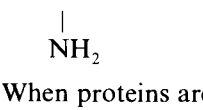
The simplest structural formula for an amino acid is



In solution they behave as *zwitterions*, becoming acidic or basic depending on the pH of the solution, and may be positively or negatively charged. For example, in acid solution the cation form predominates, giving a net excess of positive charges:



while in alkaline solution the anionic form predominates, giving a net excess of negative charges:



When proteins are placed on a supporting medium in a buffered solution, application of an electric current causes the cations (positively charged ions) to migrate towards the cathode (negative pole) and the anions (negatively charged ions) to migrate towards the anode (positively charged pole). As previously stated, very large numbers of amino acids are involved, and it is the *net* charges of the individual amino acids forming the proteins which govern the direction and rate of migration. This migration of substances in an electric field is known as *electrophoresis*. For each amino acid there is a pH at which no migration takes place, as the number of positive and negative charges on the molecule will be equal, and therefore cancel one another. This pH is known as the *isoelectric point*.

The weight and shape of the molecule also contribute to the rate,

but not the direction, of migration. Albumin, which has a relatively low molecular weight and is slender in shape, moves more easily than the globulins, which as their name implies, are globular in shape, and have higher molecular weights. The slowest moving proteins sometimes migrate in the reverse direction to that which is expected. This phenomenon, called *end electro-osmosis*, is caused by the mobility due to the charge being less than that in the reverse direction produced from the osmotic effect of the buffer.

The earliest method of electrophoresis was introduced by Tiselius, who used a U-shaped tube. This is a very sophisticated technique, known as *moving boundary electrophoresis,* and is not suitable for routine clinical analysis. The method used most frequently for clinical work is *zone electrophoresis*, which was originally carried out using filter paper as the support medium. This has now been largely superseded by cellulose acetate, which offers several distinct advantages over filter paper. Its pores are very much smaller and more uniform in size, and thus reduce migration time considerably. Many tests previously requiring an overnight paper run can now be carried out in less than an hour. There is also very much less dye retention than with filter paper, which adsorbs some of the dye used to stain the protein fractions. With cellulose acetate the dye is almost completely removed from the zones between the protein fractions, thus giving greatly improved resolution.

The sample is usually applied as a very thin line to a strip of acetate previously soaked in the same buffer which will later be used for the separation. This is stretched across the gap between the shoulders of a tank similar to that shown in *Figure 5.22* so that the starting line is about midway between the centre of the tank and the shoulder on the cathode side. A current of about 0.5 mA/cm width is then applied. Application in this portion of the strip will accelerate the rate of migration and minimize the effects of end electro-osmosis. Addition of a little bromophenol blue to the sample will provide a visual indication of the length of the electrophoretic run while it is still in progress. The excess dye will migrate in front of the albumin fraction, which in a routine run using a barbiturate buffer (pH 8.6) migrates furthest from the origin. The strip should be removed from the tank before the marker reaches the shoulder on the anode side of the tank.

Once removed, the strips should be treated without further delay in order to prevent diffusion of the separated bands. They should be layered onto a fixative until all the pores have become saturated with the solution before being immersed in it. It is common practice to combine fixing and staining in one single operation when aqueous dyes such as Ponceau 'S' are used by preparing the dye in 5 per cent

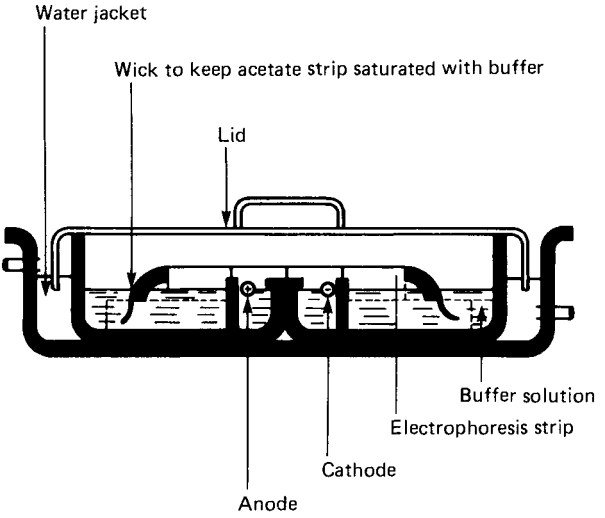


Figure 5.22 Shandon electrophoresis tank (modified from Kohn)

trichloracetic acid solution. The excess dye is removed by washing several times with 10 per cent acetic acid. For special purposes, such as the identification of lipoprotein fractions, specific dyes are used, e.g. oil red 'O' and Sudan black, which stain only the lipid-carrying protein.

The most generally accepted method of interpretation is visual comparison of the stained test strip with a similarly treated normal sample, to detect characteristic abnormal patterns produced by various clinical conditions. It is possible to clear the acetate strips with glacial acetic acid and to measure the colour intensity of the bands, or to cut out and elute the fractions prior to spectrophotometry. The stained strip may also be measured by direct densitometry. The latter, however, only records surface dye intensity, so that erroneously low values are obtained when scanning heavily stained bands. All methods are based on the assumption that the uptake of dye by the different protein fractions is proportional to their concentration. Unfortunately this is often not the case, as different protein fractions take up dyes to different extents.

The technique described above can identify only five protein fractions. However, by using support media which in addition to being microporous also act as a molecular sieve and retard the migration of larger molecules, many more fractions can be separated. This technique is known as *disc electrophoresis* and is usually

performed in glass tubes filled with starch gel or polyacrylamide gel as the support.

Electrophoresis is also used to separate and identify isoenzymes.

Radioisotope technique

Some atoms can exist in forms which, while having the same number of electrons, do not have the same atomic mass. They possess the same combining power (valency) with other elements, and share the same chemical properties. Such atoms of the same element are termed *isotopes*. Some of these emit radiation, and are termed radioisotopes. The amount of radiation emitted decreases with time, and the amount of decreasing radiation is constant with time. This rate of decrease in radiation is known as the *decay time*, and varies enormously with different elements. The time taken for the radiation to decrease to one half of the initial radiation is the *half-life*. The SI unit of radiation activity is the becquerel (Bq), which is defined as the rate of nuclear transformation (disintegration): 1 Bq = 1 nuclear transformation per second in the material. As this unit is extremely small, it is still conventional in clinical laboratories to use the curie (Ci) as a unit of radioactive decay ($1\ Ci = 3.7 \times 10^{10}$ Bq). Commonly, measurements are made in millicuries (mCi) or microcuries (μCi).

Several different forms of radiation are known to occur, but those measured in the clinical laboratory are confined to β and γ radiation. β-rays have a low penetration power and are used to a much lesser extent than γ-rays, which have a high penetration power and are waves of electromagnetic radiation similar to X-rays. β-rays are measured with a Geiger counter, whilst γ-rays are measured with a scintillation counter in which the radiation is absorbed by a phosphor, usually a sodium iodide crystal. The absorbed radiation causes light to be emitted by the phosphor. This emission is discontinuous, the effect being a 'twinkling' or scintillation. This is measured and compared to that obtained using known standards.

The principle of radioisotope dilution technique is based on the dilution of radioactivity which occurs when a known amount of a radioactive tracer is added to an unknown amount of the same (but unlabelled) isotope. The measured dilution of activity is due to dilution with the unlabelled substance and may be used to calculate the concentration of the isotope present in the original sample. Because radioactive isotopes are continuously decaying, standards and samples must always be prepared at the same time and from the same batch of labelled isotope so that the amount of decay will be the same.

The principle of radioimmunoassay (RIA) is based on the observation that when an antibody and an antigen react together a precipitate is formed (*Figure 5.23*). When a fixed amount of antibody (AB) to the protein to be determined is added to the specimen (antigen, AG) together with a known excess of labelled protein, the labelled antigen (LAG) and the unlabelled antigen (specimen) will compete for the available binding sites on the antibody. As there is

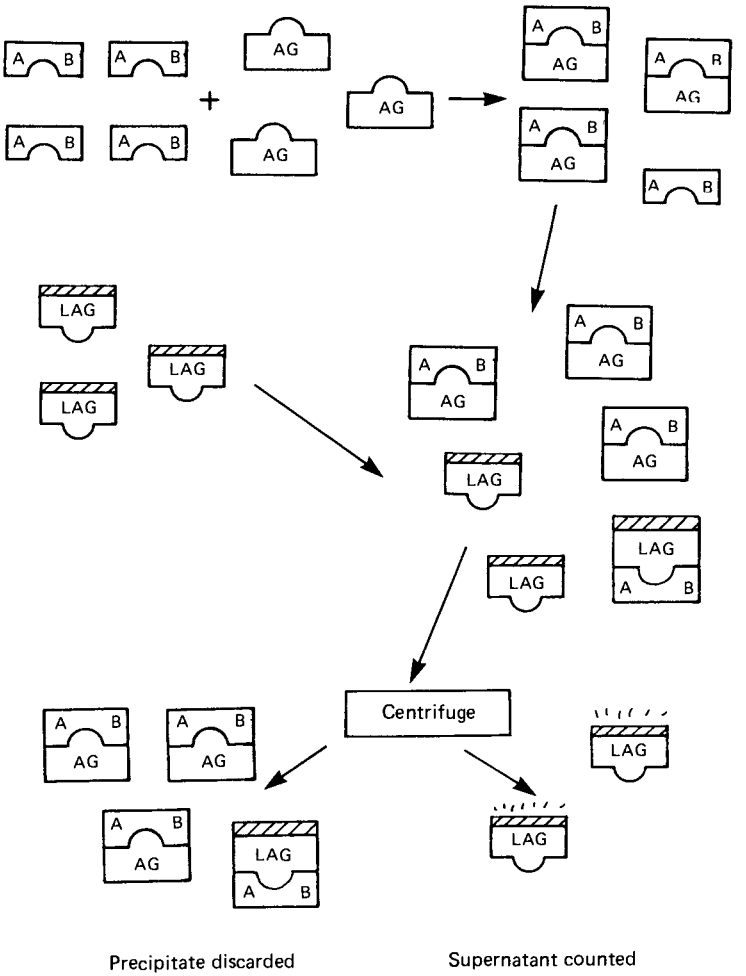


Figure 5.23 The principle of radioimmunoassay. AB = antibody; AG = antigen; LAG = labelled antigen (*see text for details*)

an excess of labelled antigen, some will remain 'free' (not bound to antibody) following the reaction period. Centrifugation of the mixture leaves a supernatant containing only the free antigen. The activity of the supernatant is indirectly related to the amount of antigen to be determined. The greater the activity (count), the less antigen has combined with the antibody.

Chapter 6

Basic principles of laboratory investigations

Bilirubin

The reader is referred to Chapter 3 for the formation and metabolism of bilirubin.

Although bilirubin is highly coloured and has a marked absorbance at 453 nm, direct determination at this wavelength is carried out only when serum levels are very high, as in neonatal jaundice, since at lower concentrations there may be significant interference from carotenes in the serum.

The commonest methods use a diazotization reaction in which bilirubin is coupled with *p*-sulphophenyldiazonium chloride to form an azo dye which is red in neutral solution and bluish-green in strongly acid or alkaline solution. The diazo reagent is unstable and must be prepared freshly before use from two reagents:

1. Van Den Bergh reagent 'A' (sulphanilic acid and hydrochloric acid)
2. Van Den Bergh reagent 'B' (sodium nitrite solution).

Conjugated bilirubin reacts directly with the diazo reagent and is thus termed *direct-reacting*, whereas the unconjugated bilirubin is bound to albumin and will not react until freed from its bond with albumin. This is termed *indirect-reacting* bilirubin. It may be freed by the addition of alcohol, and in the original methods ethanol was used. This, however, necessitated the removal of proteins. Methanol, as used by Malloy and Evelyn (1937), does not require protein precipitation. Other methods use an accelerator of urea or sodium benzoate to free the bilirubin for reaction (Jendrassik and Grof, 1938).

Bilirubin standards may be prepared from solutions in sodium bicarbonate or chloroform. The molar absorptivity of bilirubin is 60000 ± 800 at 453 nm.

Reference range
Serum bilirubin: less than 19 μmol/ℓ.

Pathology

An increase in bilirubin may be due to several factors:

1. Increased breakdown of red blood cells (haemolytic jaundice); this will be unconjugated (direct-reacting) bilirubin.
2. Obstruction of the gallbladder or bile duct (conjugated bilirubin).
3. Hepatic jaundice due to liver disease (conjugated or unconjugated bilirubin depending on the type and extent of the liver damage).

Bilirubin in urine

The 'Ictotest' (Ames) is based on a diazotization reaction with *p*-nitrobenzene-diazonium, *p*-toluene sulphonate and salicylsulphonic acid to give a blue colour.

Fouchet's test precipitates urinary sulphates with barium chloride, and the bilirubin is adsorbed onto the precipitate. The dried precipitate is treated with ferric chloride–trichloracetic acid reagent, which gives a blue colour in the presence of bilirubin.

Calcium

The blood level of calcium is controlled by three hormones:

1. 1,25-Dihydroxycalciferol (from vitamin D)
2. Parathormone
3. Calcitonin.

It circulates mainly bound to albumin, but it is only the ionized calcium which is active. It is essential for bone formation, for many of the clotting factors and for the maintenance of cell permeability.

Specimens for plasma calcium determinations should be collected from fasting patients, and without using a tourniquet in order to prevent venous stasis. Methods of analysis are numerous which perhaps is an indication of the difficulties with which the analyst has been beset until recent advances in technology. Older methods involved precipitation with ammonium oxalate and titration of the resultant oxalic acid with potassium permanganate, which was very time-consuming and required large blood samples. Gitelman (1967) developed a method in which calcium is chelated with cresolphthalein complexone, and then made alkaline with diethylamine to give a red complex. The addition of 8-hydroxyquinoline binds magnesium

and prevents the chelation of magnesium with the complexone. Gindler and King (1972), in a similar method which also uses 8-hydroxyquinoline, form a complex with calcium and methylthymol blue.

Several methods are based on complexiметric titration with ethylenediamine tetraacetic acid (EDTA). However, the end-point of the titration is difficult to read, which is illustrated by the large selection of indicators which have been used. It may, however, be monitored automatically. A typical method of this type uses the indicator murexide (ammonium purpurate). (Murexide is the substance formed in the test for uric acid in renal calculi.) Magnesium is prevented from interfering by making the solution strongly alkaline. The titration may also be monitored fluorimetrically (Diehl and Ellingshoe, 1956) using calcein as an indicator. Calcein combines with calcium ions to form a non-ionic calcium complex. The fluorescence displayed is quenched as calcium is displaced by EDTA during the titration.

Flame emission spectroscopy has been used but suffers from severe interference from sodium. This can, however, be eliminated by the addition of sodium to the standard solutions. It also requires a very hot flame, as calcium is divalent. Ionized calcium absorbs at 422 nm, and the atomic absorption method of analysis has been suggested as the reference method (Cali *et al.*,1972). Here the addition of lanthanum to form a complex with phosphates prevents their interference by combination with calcium ions.

Ionized calcium can be determined by the use of ion-selective electrodes and by membrane diffusion.

Urine specimens for calcium determination should be collected into acid containers in order to prevent the precipitation of calcium salts in alkaline solution.

Reference ranges

Plasma calcium: 2.19–2.50 mmol/ℓ
Urinary calcium: 2.5–7.7 mmol/24 hours

Pathology

Plasma calcium levels are increased in hyperparathyroidism, which causes increased release of calcium from the bones, and decreased in hypoparathyroidism, renal damage (where there is impaired phosphate excretion) and malabsorption.

Cholesterol

Of all the steroids found in the body, cholesterol is the most abundant. It is a precursor of the steroid hormones and an important constituent of lipoproteins. Ingested cholesterol is absorbed by the intestine in the presence of bile salts, and is incorporated into chylomicrons, in which form it is transported to the liver. Cholesterol is also synthesized in the liver. Excess cholesterol is excreted by the liver in the bile.

Total plasma cholesterol

The Δ^5-3-ketone grouping of the cholesterol molecule reacts with acetic anhydride and concentrated sulphuric acid (Lieberman-Burchard reaction) to give a green complex (Watson, 1960). The reaction is very sensitive to light, water, and temperature fluctuations, and the reagents can be very hazardous. Carefully performed, however, the method is extremely good and has been suggested as the reference method.

The Zlatkis reaction (Levine and Zak, 1964) is similar, and uses a ferric chloride, acetic acid and sulphuric acid mixture. This method is used in the Technicon 'Autoanalyser'.

The enzymatic determination of cholesterol (Trinder, 1969a) is based on the hydrolysis of cholesterol ester to free cholesterol, and the subsequent oxidation to Δ^4-cholesterone, with the production of hydrogen peroxide. The peroxide is determined by allowing it to react with 4-aminophenazone and phenol in the presence of peroxidase to form a coloured phenazone complex, or by direct measurement using a hydrogen peroxide specific electrode.

$$\text{Cholesterol ester} \xrightarrow{\text{cholesterol esterase}} \text{cholesterol} + \text{fatty acid}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{cholesterol oxidase}} \Delta^4\text{-cholesterone} + H_2O_2$$

$$H_2O_2 + \text{4-amino-phenazone} \xrightarrow{\text{peroxidase}} \text{coloured phenazone complex} + H_2O$$

HDL cholesterol

This can only be determined after prior removal of VLDL and LDL by precipitation with heparin and manganese chloride. One of the above methods is then used to determine the residual HDL cholesterol. If enzymatic determination is to be performed, the excess

manganese chloride must first be removed by chelation with EDTA (Albep *et al.*, 1978).

Reference range
The reference range for fasting plasma cholesterol is usually given as 4.0–6.7 mmol/ℓ. However, the desirable upper limit is 5.0 mmol/ℓ and the risk of coronary heart disease increases with fasting levels above this.

Pathology

Increased plasma cholesterol levels are found in hypercholesterolaemia (a condition which is associated with a high incidence of coronary heart disease), obstructive jaundice and diabetes. Several studies have shown HDL cholesterol to be lowered in patients with a predisposition to ischaemic heart disease, and considerable interest has focused on its determination.

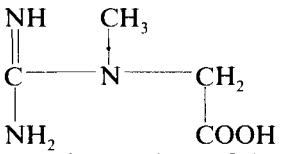
Creatine and creatinine

Creatine is formed in muscle from phosphocreatine by the action of creatine kinase (CK).

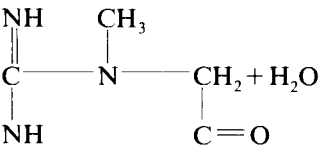
$$\text{Phosphocreatine} + \text{ADP} \xrightarrow{\text{creatine kinase}} \text{creatine} + \text{ATP}$$

Normally very little is found in the urine, but in conditions where there is increased muscular activity, both blood and urine concentrations are increased.

The determination of creatine has been largely superseded by that of the enzyme CK, which is more sensitive and accurate. However, it may be determined by measuring the creatinine concentration before and after conversion of creatine to its anhydride creatinine by vigorous heating in an acid solution. The difference is the amount of creatinine formed from creatine. As can be seen from the formulae below, 113 mg creatinine are formed from 131 mg creatine:



Creatine (mol. wt 131)



Creatinine (mol. wt 113)

Creatinine is formed from creatine and excreted in the urine. It is a good indicator of renal function, although the plasma creatinine takes longer to rise than the urea. As a clearance test (to determine the glomerular filtration rate) it is far superior to the urea clearance test.

The creatinine clearance is defined as the amount of plasma cleared of creatinine per minute, and is determined from the formula:

$$\text{Plasma creatinine clearance} = \frac{U \times V}{P} \text{ ml per minute}$$

where U is the urine creatinine concentration (μmol/ℓ), V is the volume of urine excreted per minute (ml), and P is the plasma creatinine concentration (μmol/ℓ).

Creatinine is determined mainly by its reaction with alkaline picrate (Jaffe reaction), which produces an orange colour. The reaction is not specific, as a variety of non-creatinine chromogens contribute to the final colour. Autoanalyser methods involving dialysis significantly reduce this interference since some of the non-creatinine chromogens are unable to cross the membrane. The use of Lloyd's reagent (Fuller's earth), Sephadex and ion-exchange resins to absorb the creatinine and decant the interfering chromogens gives improved results but slows the procedure so much that it becomes impracticable for routine use. Ether and iodine extraction techniques have also been used to remove the interference. Since reaction of 'true' creatinine with alkaline picrate is reported to be faster than that of the interfering chromogens, some methods rely on readings taken after a very short time interval in order to gain specificity. The alkaline picrate reagent itself is highly coloured in the region of maximum absorbance of the creatinine picrate, so that the readings are made at 520 mm, where there is maximum difference in absorbance between reagent and test, and greatest sensitivity.

Another chemical method involves the reaction with dinitrobenzoic acid and sodium carbonate to produce a red complex.

The advantages of enzymatic methods are their specificity for true creatinine and the very small sample volumes required. The main disadvantage is the relatively high cost compared to the alkaline picrate method. Franklyn Lim (1974) proposed a method which uses creatininase to produce ammonia, which is then allowed to react with α-oxoglutarate and NADPH, catalysed by the action of glutamate dehydrogenase (GD).

$$\text{Creatinine} \xrightarrow{\text{creatininase}} NH_4^+ + N\text{-methylhydantoin}$$

$$NH_4^+ + \alpha\text{-oxoglutarate} + NADPH \xrightarrow{GD} \text{L-glutamate} + NADP + H_2$$

The resultant oxidation of NADPH is monitored fluorimetrically. It can also be measured by monitoring the decrease in absorption at 340 mm.

Moss, Bondar and Buzzelli (1974) converted creatinine to creatine using the enzyme creatinine amidohydrolase, and produced a change in absorbance at 340 nm in a coupled enzyme reaction involving creatine kinase, pyruvate kinase (PK) and lactate dehydrogenase (LD).

Reference ranges
Plasma creatinine
  males: 53–106 μmol/ℓ
  females: 44–88 μmol/ℓ
Urinary creatinine: 6–13 mmol/24 hours
Creatinine clearance: 70–140 ml/minute

Drugs and poisons

Investigation of drugs and poisons in the clinical chemistry laboratory is a frequently discussed topic which is complicated by several factors other than the methodology itself. Legal and interpretative difficulties are an unfortunate and common problem. A brief discussion of some of these problems therefore seems appropriate.

Drugs can be defined as chemicals administered in order to alter the function of a living system in a variety of ways: (1) replacement for an absent or poorly functioning biological substance, as in steroid replacement therapy; (2) agonists, to promote or accelerate naturally occurring reactions; or (3) antagonists, to prevent reactions taking place.

Tests for such substances in the clinical chemistry laboratory are justified if the results can be used as an aid to treatment and prognosis. In general, the blood level of a drug is of more value than that in the urine. It must be borne in mind that these levels are influenced by many factors:

1. The amount of drug administered.
2. The rate of drug absorption. This may vary with the pH in the intestine or stomach, and with the time of ingestion (before or

after a meal). The absorptive efficiency of the intestinal villi is another contributing factor.

3. The rate at which the drug is distributed to the tissues. With injected drugs the response time is directly related to the site of injection and the site of action. Subcutaneous, intramuscular and intravenous injections will differ in their respective response times. Drugs are transported in the body bound to albumin, in which form they are inactive. Only the free form is active.
4. The rate at which the drug is removed from the tissues. Most are conjugated with glucuronic acid by the liver and excreted as the glucuronide by the kidneys.

Similar doses of the same drug will not necessarily produce the same response in different patients. Drug intolerance may be due to a lowered plasma albumin concentration, allowing a greater concentration of the free drug to be available for action, or to renal damage resulting in inefficient clearance of the conjugated metabolite. Similarly liver damage causes a reduction in the ability to conjugate the drug metabolite. In some cases, many times the normal dose of drug may be required to produce the desired effect because of acquired tolerance from past use. Specific enzyme deficiencies may cause idiosyncratic sensitivity to substances such as suxamethonium, and hypersensitivity (caused by antibody production) may give severe reactions in others.

The lack of information about the time of ingestion of the drug, or the amount taken, may give rise to misleading results and render the analysis useless. It is therefore vital to collect as much information as possible before embarking on long and complex investigations.

The mortality statistics for England and Wales (1983) show that the most common causes of death due to drug poisoning were:

Analgesics – 36% of poisoning deaths (salicylates 7%)
Sedatives and hypnotics – 23% (barbiturates 15%)
Psychotic agents – 19%
Antidepressants – 11%

The most commonly occurring analyses requested for determination by the clinical chemistry laboratories are the determination of plasma salicylates and paracetamol, and blood barbiturates.

Barbiturates

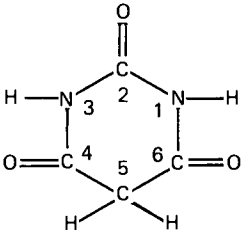
Barbituric acid was first synthesized by Bayer in 1864 from malonic acid and urea. This substance has no hypnotic properties, which are due to the effect of substituents at C-5. The hypnotic activity of

barbiturates was first observed in 1903 by Emil Fischer, who used diethyl barbituric acid on dogs. Interest in this group of substances, once aroused, became so intense that by 1955 the United States was producing over 350 000 kg of barbiturates a year. The vast quantity of drugs available, and the ease with which they can be obtained, has led to considerable misuse and thus demand for detection and determination. Efforts are being made to curb the prescription of barbiturates in favour of benzodiazepine drugs, which are reportedly as effective but present fewer problems. Because there is such a large variety of barbiturates, with widely varying rates of metabolism, their determination has fallen into some disrepute. This is due largely to the inability of many laboratories to identify the specific barbiturate with sufficient certainty, and therefore give a reliable quantitative result. It has also been shown that a decreasing blood barbiturate concentration does not necessarily indicate an improvement in the prognosis.

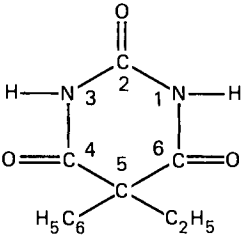
Hypnotic activity is associated with the substituents at C-5, and the three groups of barbiturates are distinguished by the type of activity (*Figure 6.1*). Phenobarbitone is a long-acting barbiturate with 5-phenyl,5-ethyl groups at C-5. Amylobarbitone is intermediate-acting, and has 5-isopentyl,5-ethyl substituents. Thiopentone is short-acting and has 5-ethyl,5-(1-methylbutyl) groups at C-5.

The best method for the detection and determination of barbiturates is gas–liquid or HPLC chromatography, as the barbiturate can be positively identified by its retention time, and the quantity accurately measured. The technique, however, is not available to all laboratories, and is not suitable for emergency work outside normal working hours. The most commonly used technique is that of Goldbaum, modified by Broughton (1956), which measures the ultraviolet absorption properties of the two ionized forms at pH 10 and pH 13 (*Figure 6.2*). At pH 10, barbiturates have an absorption peak at 240 nm, while at pH 13 they have an absorption peak at 254 nm and a trough at 234 nm. Scanning of the absorption spectra will detect the presence of a barbiturate, and measurement of the wavelength of maximum difference (260 nm) will give a quantitative value. Interference from salicylates is removed by a phosphate buffer wash at pH 7.4. Heating the alkaline extract hydrolyses intermediate- and long-acting barbiturates to urea and malonic acid, while the shorter acting ones remain unaffected. Spectrophotometric examination of the extract before and after heating at 100°C for 15 minutes may therefore be used as a means of partial identification.

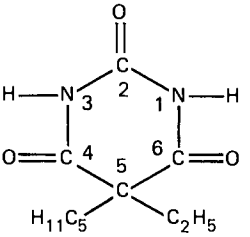
Lubran (1961) introduced a barbiturate–mercury reaction with diphenylcarbazone which has an absorbance at 555 nm. The method, however, is not easy to perform with good precision,



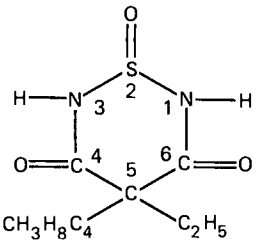
Barbituric acid (non-active)



Phenobarbitone (long-acting)



Amylobarbitone (intermediate-acting)



Thiopentone (short-acting)

Figure 6.1 Barbiturates

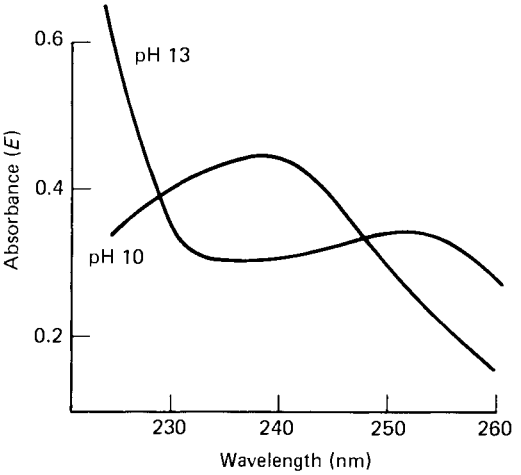


Figure 6.2 Absorption curve of barbiturates at pH 10 and 13

although this is said to be improved by extraction into dichloromethane.

Enzyme-multiplied immunoassay technique (EMIT) has been used for the detection and estimation of a variety of drugs, including barbiturates. The method is rapid and requires little in the way of special equipment, but is expensive. The specimen containing the free drug is added to the specific antibody, followed by a known excess of enzyme-bound drug. Both the free and the enzyme-bound drug bind to the antibody, but the enzyme bound to the antibody is inactivated by the bond. The excess enzyme-bound drug is allowed to act on its specific substrate. Measurement of the enzyme reaction therefore gives a measure of the free drug in the original specimen. If there is a large amount of free drug, this will take up most of the available binding sites, leaving a relatively large excess of enzyme-bound drug available for activity. Conversely, a small amount of free drug leaves a large number of binding sites available for the enzyme-bound drug, and thus a smaller excess of active enzyme. The principle of this technique is shown in *Figure 6.3*.

Radioimmunoassay has also been used to determine barbiturate concentrations by mixing drug labelled with iodine-125 with goat antigen and measuring the activity after incubation. Chromatography has been used extensively as a qualitative test for barbiturates, both with paper and thin-layer plates.

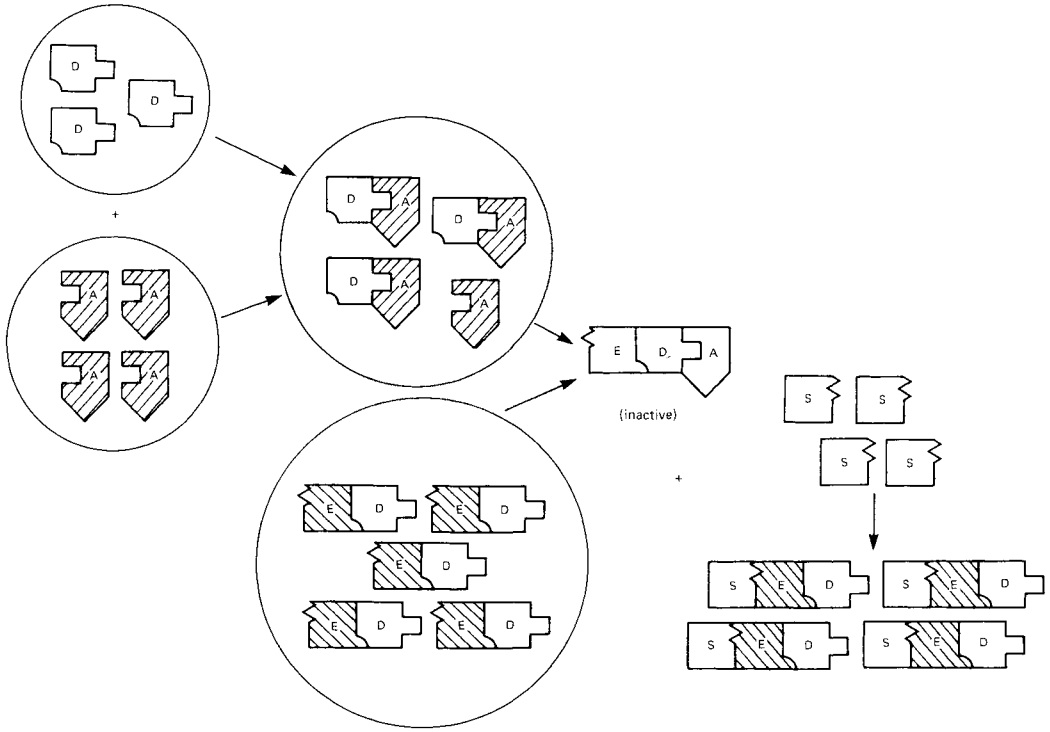
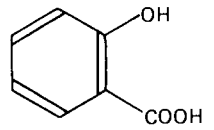
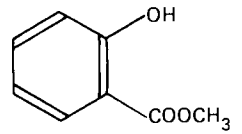


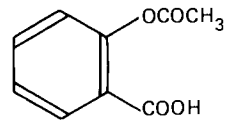
Figure 6.3 The principle of drug analysis by enzyme-multiplied immunoassay technique (EMIT). D = drug; A = antibody; S = substrate; ED = enzyme-bound drug (*see text for details*)



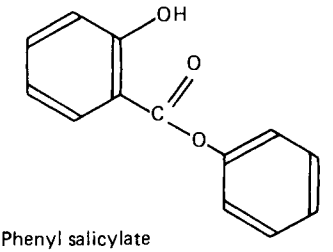
Salicylic acid



Methyl salicylate
(Wintergreen)



Acetylsalicylic acid
(aspirin)



Phenyl salicylate

Figure 6.4 The structure of salicylic acid and three of its esters

Salicylates

Salicylates are effective in relieving pain and reducing fever, and are used in esterified form (*Figure 6.4*). Salicylic acid itself has an irritant effect on the gastric mucosa as it is highly acidic. Its esters, however, are less acidic and not appreciably hydrolysed by the gastric juice. Salicylates have been used since the eleventh century, when Avi-

cenna used the bark of the willow (*Salix alba*) to treat fever, and aspirin (acetylsalicylic acid) has been commercially available since 1899. Overdosage of salicylates can produce complex acid–base and renal disturbances. Alkaline diuresis is normally carried out in adults where the plasma salicylate exceeds 50 mg per 100 ml.

The detection and determination of salicylates in blood depends on their reaction with acidified ferric nitrate, which gives a purple complex. In order to minimize interference from proteins, these can be precipitated at the same time (Trinder, 1954). The reaction with alkaline Folin-Ciocalteau reagent results in the formation of a blue complex. This method, however, suffers from interference from phenolic amino acids, which also produce a blue colour.

In urine, salicylates are qualitatively detected by their reaction with 10 per cent ferric chloride (Gerhardt's test), which gives a purple colour. Acetone, however, gives the same colour and must be removed by prior boiling of the urine, which will volatilize the acetone but not remove the salicylates. Salicylates give an atypical reaction with 'Phenistix' (Ames), which are used for the determination of *p*-aminosalicylic acid, a test based on the principle described above.

Paracetamol

Paracetamol (*N*-acetyl-*p*-aminophenol) is also an analgesic, and is the major metabolite of phenacetin. Overdosage can cause necrosis of the liver, and a major clinical problem is that it produces no early warning signs or coma to alert the clinician. Paracetamol levels must be related to a 'treatment line' which depends on the interval between ingestion and the taking of the blood sample for analysis. Serious liver damage may be expected if the plasma level is over 30 mg/100 ml four hours following ingestion, whilst levels below 12 mg/100 ml are unlikely to adversely affect the patient.

The best analytical methods are those involving high pressure liquid chromatography (HPLC) or gas chromatography (GLC), but these may not be generally available, especially for emergency use. Glynn and Kendall (1975) used the yellow diazo complex produced with sulphanilic acid and sodium nitrite in alkaline solution. Another colorimetric screening procedure uses the reaction with acidified ferric tris(pyridyl)triazine to form a coloured complex after extraction into ethyl acetate (Lin and Oka, 1980). The Cambridge Life Sciences Company have produced a kit for an enzymatic assay based on the conversion of paracetamol to acetate and *p*-aminophenol using the enzyme aryl acylamidase (Price, Hammond and Scawen, 1983). The *p*-aminophenol reacts with

alkaline *o*-cresol in the presence of copper and ammonium ions to form a coloured complex.

Urine screening for drugs of abuse

Many patients depend on a combination of drugs, which are best detected by a comprehensive screening procedure. It is usually more convenient to use urine instead of blood for this qualitative procedure. Identification is based on extraction of the drug at a suitable pH, and chromatographic separation of the concentrated extract, followed by detection with a location reagent.

Alkaloids

These are nitrogenous bases occurring in plants, especially in the poppy family (Papaveraceae), which have narcotic (sleep-inducing) properties. The exudate from crude opium contains about 23 alkaloids, the most abundant of which is morphine. Codeine is the phenolic methyl ether of morphine.

For screening purposes, ammonium carbonate is added to the urine, and this is then extracted with chloroform. The chloroform extract is evaporated to dryness and the residue dissolved in a little methanol. The concentrated extract is separated by thin layer chromatography using an ethyl acetate, methanol and ammonia mixture as the solvent. The dried chromatogram is sprayed with iodoplatinate reagent to locate the alkaloids, which show up as bluish-black spots on a pink background. The reaction is said to be enhanced by the addition of a small quantity of hydrochloric acid to the reagent.

Amphetamines

These are volatile basic drugs which act by inhibiting the enzyme monoamine oxidase. They stimulate the adrenal cortex and medulla, and increase cardiac output. They are not metabolized, but are excreted unchanged in the urine.

The screening test is carried out in urine made alkaline with sodium hydroxide and then extracted with chloroform. The extract is evaporated almost to dryness and the concentrated extract separated by thin layer chromatography using methanol and ammonia as the solvent. The dried chromatogram is sprayed with fast blue 'B' dye (tetraazotized *o*-dianisidine) to locate the amphetamines.

Barbiturates

These are extracted into chloroform from urine made acid with sulphuric acid, and then separated by chromatography as in the test

described for alkaloids. The dried chromatogram is sprayed with fast blue 'B' dye for location of the barbiturates.

Paraquat
This is a highly toxic quaternary base used extensively as a weed-killer. It causes severe ulceration of the skin and mucous membranes, and finally liver, heart and pulmonary damage. Urine may be screened for paraquat by the addition of sodium bicarbonate and sodium dithionite (Kerr, 1968) to produce a bluish purple colour.

Heavy metals
Most trace metals are best detected and measured by atomic absorption techniques. Mercury and lead, however, may be coupled with dithizone (diphenylthiocarbazone) to produce a green and red complex respectively. When testing for lead it is most important to ensure that all water used is lead-free, and that none of the equipment is contaminated by this element.

Enzyme determination

In contrast to most other substances, enzymes are not measured in terms of weight or concentration but in units of activity, defined as the rate at which they cause a reaction to proceed.

The term enzyme is derived from the source of the first one discovered, and means 'in yeast'. They are a large group of proteins which act as biological catalysts by promoting and regulating many chemical reactions in living organisms.

Enzymes first combine with a substrate at specific binding sites on their molecule to form an enzyme–substrate complex, which is subsequently broken down into the free enzyme (which becomes available for more catalytic reactions) and the product(s) of the reaction (*see Figure 1.13*).

The definition and value of enzyme units depends on the method used for their determination, and a wide variety of units have been defined. This has led to considerable confusion, especially in the comparison of one unit value to another for the same enzyme. An attempt to resolve this problem was the introduction of the international unit (i.u.) of enzyme activity, defined as the amount of enzyme which will catalyse the reaction of 1 μmol of substrate per minute under defined and controlled conditions. Unfortunately, enzymes have different affinities for substrates so that it is not always possible to compare units from one method with those produced by another. This is usually the result of the different properties of the isoenzymes

(*see* Chapter 1). This difference in behaviour is very useful in the separation and identification of isoenzymes. Small structural differences in enzymes are reflected in their different electrophoretic patterns, and other differences can be detected by their response to inhibitors or heat.

In enzyme determinations, the ratio of substrate to enzyme is very important. If insufficient substrate is available to occupy all the available binding sites on the enzyme molecule, erroneously low values will be obtained. The pH and temperature of the reaction are also very important and can influence the direction and rate of the reaction.

Many enzymes require the presence of other substances in order to become active. Inorganic *activators* are essential for the activity of many of them. For example, magnesium and manganese ions are necessary for alkaline phosphatase activity. Care must therefore be taken not to use anticoagulants which will remove the essential ions. Organic *cofactors* are also essential for some enzyme reactions. For example, dehydrogenases require a nicotinamide adenine dinucleotide (NAD^+) coenzyme in order to function. This group of coenzymes is extremely useful in the kinetic determination of enzyme activity.

Many methods of determination are based on a 'two point' technique, in which the amount of product is measured before and after a timed reaction, the difference being due to the enzyme activity. In the reaction A + B = C + D, either the concentration of C or D, both before and after a given incubation period, or the rate of disappearance of A or B may be measured. In either case, two determinations have to be made, and for each of these the reaction has to be stopped. A more sophisticated method is to monitor the reaction while it is still in progress (kinetic determination). Many enzymes require either NAD^+ or $NADP^+$ in order to function. These coenzymes share the same absorption characteristics in that the reduced forms both have a maximum absorbance at 260 nm and also absorb at 340 nm, while the oxidized forms absorb only at 260 nm and not at 340 nm. The rate of appearance or disappearance of the absorption at 340 nm is therefore a measure of the rate of enzyme activity. The molar absorptivity (e) of $NADH^+$ is 6.22×10^3 at 340 nm. This is determined by the formula:

$$e = \frac{E}{c \times d}$$

where E = absorbance, c = concentration of $NADH^+$ (moles/ℓ) and d = length of light path (cm). By relating the difference in absorbance

at 340 nm to the reaction equation, the enzyme activity can be determined. In the example below, NADH is required for the conversion of pyruvate to lactate by the enzyme lactate dehydrogenase:

$$\text{Pyruvate} + \text{NADH} \xrightarrow{\text{lactate dehydrogenase}} \text{lactate} + \text{NAD}^+$$

For each molecule of substrate converted, one molecule of coenzyme is converted to the oxidized form. The absorption curves for these coenzymes are shown in *Figure 6.5*. Enzyme reactions which do not themselves require the above coenzymes may be linked to those which do, in order to allow kinetic determination.

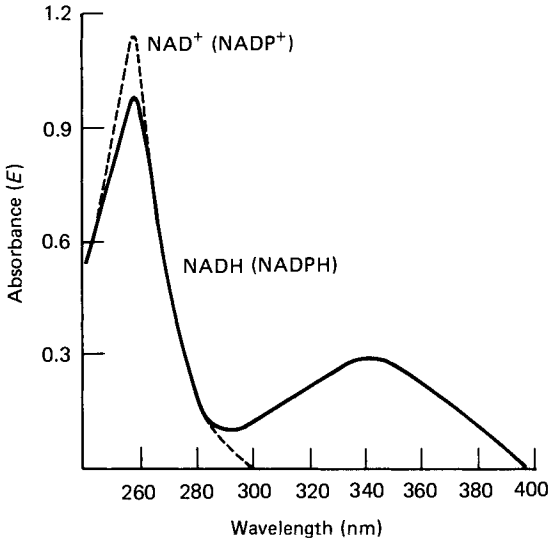


Figure 6.5 Ultraviolet absorption spectra of NADH and NAD^+

Amylase

This enzyme, which is found predominantly in pancreatic juice and saliva, hydrolyses starch to dextrin and reducing monosaccharides. The main value of its determination is in the diagnosis of acute pancreatitis, where cellular damage allows release of the enzyme into the peritoneum and the circulation. Chloride ions are an essential activator.

$$\text{Starch} \xrightarrow[Cl^-]{\text{amylase}} \text{maltose}$$

Older methods for the determination of amylase are based on the incubation of a starch–saline solution with serial dilutions of serum. The activity is determined by finding the highest dilution of serum which will digest the starch–saline solution. (The presence of starch is detected by the addition of dilute iodine solution.)

A water-insoluble cross-linked starch polymer developed by the Pharmacia company is now widely used as the substrate. Incubation of α-amylase with the polymer hydrolyses the starch, forming a water-soluble blue complex which can be measured at 620 nm. This method compares well with saccharogenic methods, which measure the amount of sugar produced.

Klein, Foreman and Searcy (1970) have proposed a similar method using insoluble Cibachron Blue F3GA-amylose as substrate. This is hydrolysed to simple saccharides which are still bound to the dye, but soluble in water, thus giving a blue solution which is compared to a standard of free Cibachron Blue.

The saliva (S) and pancreatic (P) isoenzymes can be differentiated by preincubation of the substrate with a protein inhibitor derived from wheat. The S-isoenzyme is inhibited but the P-isoenzyme is not. Determination of α-amylase before and after inhibition allows calculation of the P:S ratio, which is normally 1.8:1 (O'Donnell, Fitzgerald and McGeeney, 1977).

The DuPont Automatic Clinical Analyser (ACA) uses gel filtration to remove any glucose present in the serum. This is followed by a chain reaction, starting with maltopentose substrate and involving α-amylase, α-glucosidase, hexokinase and glucose-6-phosphate dehydrogenase (G-6-PDH) to produce NADH.

$$\text{Maltopentose} \xrightarrow{\alpha\text{-amylase}} \text{maltotriose} + \text{maltose}$$

$$\text{Maltotriose} + \text{maltose} \xrightarrow{\alpha\text{-glucosidase}} \text{glucose}$$

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{hexokinase}} \text{glucose 6-phosphate} + \text{ADP}$$

$$\text{Glucose 6-phosphate} + \text{NAD}^+ \xrightarrow{\text{G-6-PDH}} \text{6-phosphogluconate} + \text{NADH}$$

The NADH is measured by the increase in absorbance at 340 nm (Schmidt, 1961).

Reference range
Serum α-amylase: 70–300 i.u./ℓ.

Aldolase

Although this enzyme is widely distributed throughout the tissues, it is found mainly in skeletal and heart muscle. It catalyses the breakdown of fructose 1,6-diphosphate to two triose phosphate molecules (*see Figure 1.18*). Its activity is increased in myocardial infarction, reaching maximum values 24–48 hours after the infarction and returning to normal after about 5 days. The rise, however, is slight compared to that of other enzymes, and is rarely used for the detection of this condition. Very marked increases in activity are seen in muscular dystrophy, especially of the Duchenne type. Lower values are observed in Tay–Sachs disease.

Determination may be kinetic or colorimetric. One kinetic method (Warburg and Christian, 1943) uses a chain reaction involving aldolase, triose phosphate isomerase and glyceraldehydephosphate dehydrogenase (GAPDH) to produce NADH, thus giving an increase in absorbance at 340 nm.

$$\text{Fructose 1,6-diphosphate} \xrightarrow{\text{aldolase}} \text{glyceraldehyde phosphate} + \text{dihydroxyacetone phosphate}$$

$$\text{Dihydroxyacetone phosphate} \xrightarrow{\text{triose phosphate isomerase}} \text{glyceraldehyde phosphate}$$

$$\text{Glyceraldehyde phosphate} + NAD^+ \xrightarrow{\text{GAPDH}} \text{3-phosphogluconate} + NADH$$

The dihydroxyacetone phosphate produced by aldolase activity may also be allowed to react with NADH, using the enzyme glycerophosphate dehydrogenase (GDH). This reaction produces NAD^+ and thus a decrease in absorbance at 340 nm.

$$\text{Dihydroxyacetone phosphate} + NADH \xrightarrow{\text{GDH}} \text{glyceraldehyde phosphate} + NAD^+$$

The colorimetric method of Sibley and Fleisher (1954) uses dinitrophenylhydrazine to produce coloured hydrazones with the oxo acids formed from aldolase activity.

Reference range
Serum aldolase: 1.5–7.2 i.u./ℓ.

Creatine kinase

Creatine kinase (CK) catalyses the transfer of phosphate from creatine phosphate to ADP, forming creatine and ATP. It is most abundant in cardiac, skeletal and uterine muscle, and is the most sensitive enzyme for the laboratory investigation of muscular dystrophy, where serum values can reach 60 times the normal level. It is also raised in myocardial infarction, reaching maximum activity 24 hours after infarction, and returning to normal in 2–3 days. The enzyme is activated by magnesium, manganese and calcium ions, and inhibited by EDTA, citrate, fluoride and heparin. Its activity can be determined by colorimetric, fluorimetric or kinetic methods.

The colorimetric method of Ennor and Rosenberg (1954) is based on the Ennor–Stokes reaction, in which creatine is allowed to react with α-napthol and diacetyl to form a red complex. Modifications of this method include the addition of cysteine to the reaction mixture to enhance the linearity, and chloromercuribenzoate to prevent interference in the colour reaction from the added cysteine.

The fluorimetric assay (Sax and Moore, 1967) uses the reaction of creatine with alkaline triketohydrindene hydrate (ninhydrin).

The kinetic method (Rosalski, 1967) uses a chain reaction involving creatine kinase, hexokinase and glucose-6-phosphate dehydrogenase to produce NADPH, which gives an increase in absorbance at 340 nm.

$$\text{Creatine phosphate} + \text{ADP} \xrightarrow{\text{creatine kinase}} \text{creatine} + \text{ATP}$$

$$\text{ATP} + \text{glucose} \xrightarrow{\text{hexokinase}} \text{glucose 6-phosphate} + \text{ADP}$$

$$\text{Glucose 6-phosphate} + \text{NADP}^{+} \xrightarrow{\text{G-6-PDH}} \text{NADPH} + \text{6-phosphogluconate}$$

Reference range
Serum creatine phosphokinase: less than 70 i.u./ℓ

Lactate dehydrogenase

Lactate dehydrogenase is found in liver, kidney, muscle and heart tissue. It catalyses the oxidation of lactate to pyruvate:

$$\text{Lactate} + \text{NAD}^+ \xrightarrow{\text{LDH}} \text{pyruvate} + \text{NADH}$$

Serum activity is increased in myocardial infarction, where it may reach up to three times the normal value and remain raised for about 8 days. Very high increases are observed in pernicious anaemia, and activity is also increased in liver and renal disease, but in these conditions it is of less diagnostic importance than other determinations.

Isoenzyme studies of lactate dehydrogenase have proved very useful in clinical chemistry. Five fractions have been isolated by electrophoresis. The fastest moving (LD_1) contains most of the heart fraction, while the slowest moving (LD_5) contains most of the liver isoenzyme. The heart fraction is more resistant to heat and inhibition by urea than the liver fraction. The enzyme is inhibited by pyruvate, zinc and *p*-chloromercuribenzoate. Its activity is determined by the spectrophotometric (kinetic) method of Wroblewski and La Due (1955) which measures the increase in absorbance at 340 nm.

Some enzymes are substrate-specific and will only bind with one substrate. The substrate specificity of the heart isoenzyme is demonstrated in the use of α-hydroxybutyrate as substrate for the determination of α-hydroxybutyrate dehydrogenase (αHBD), which it reduces more readily than lactate. In myocardial infarction αHBD shows a 3–5-fold increase in the activity and remains elevated for up to two weeks. αHBD can also be determined kinetically using the following reaction, which results in a decrease in the absorbance at 340 nm (Rosalski and Wilkinson, 1964):

$$\alpha\text{-Oxobutyrate} + \text{NADH} \xrightarrow{\alpha\text{HBD}} \alpha\text{-hydroxybutyrate} + \text{NAD}^+$$

Reference range
Serum lactate dehydrogenase: less than 240 i.u./ℓ
Serum α-hydroxybutyrate dehydrogenase: 53–144 i.u./ℓ.

Phosphatases

This is a group of enzymes responsible for the hydrolysis of organic monophosphoric esters:

$$\text{R.PO}_4 \xrightarrow[(\text{Mg}^{2+})]{\text{phosphatase}} \text{R} + \text{PO}_4^{3-}$$

Magnesium ions are an essential activator, and EDTA, which chelates magnesium, will therefore inhibit the activity.

Some phosphatases are most active at a pH of about 5.0 (acid phosphatases), while others prefer a pH of about 10.0 (alkaline phosphatases).

Acid phosphatases

Most of the serum acid phosphatase is derived from the prostate, but small amounts are found in other tissues, notably the red blood cells. The clinical value of differentiating acid phosphatase isoenzymes is that it allows determination of prostatic acid phosphatase without interference from other acid phosphatases. The former is raised in metastatic prostatic carcinoma, and the determination is used both as an aid to diagnosis and in monitoring treatment. As long as the disease is confined to the prostate, the serum acid phosphatase remains normal, but secondary spread to other tissues causes the enzyme to leak into the plasma, giving raised values. The prostatic isoenzyme is inhibited by alcohol and L-tartrate but resistant to copper ions and formaldehyde, while red cell acid phosphatase is inhibited by alcohol, copper and formaldehyde but resistant to L-tartrate. *In vivo*, acid phosphatase is inhibited by oestrogens and promoted by testosterone.

Alkaline phosphatase

Alkaline phosphatase is present in liver, bone, placenta and intestinal tissue. In adults most is derived from the liver, but in children most is of bone origin. Partial separation of the isoenzymes is possible because of their difference in behaviour when heated at 56 °C for 15 minutes. At this temperature, bone alkaline phosphatase is more labile than the liver isoenzyme, while the placental isoenzyme is the most stable. Placental and intestinal isoenzymes are inhibited by the addition of L-phenylalanine, but bone and liver isoenzymes are resistant.

Alkaline phosphatase is excreted by the liver into the bile, and serum activity is increased in obstructive jaundice, due mainly to the damming effect of the obstruction and not to increased liver secretion. The largest increases are found in bone disease, particularly Paget's disease. Alkaline phosphatase is decreased in hypophosphatasia, a rare inborn error of metabolism.

5′-Nucleotidase is a liver isoenzyme with high specificity for the hydrolysis of adenosine monophosphate (AMP). Activity is therefore normal in bone disease and obstructive jaundice, but raised in liver disease. This enzyme does not require magnesium ions as an activator and is therefore unaffected by the addition of EDTA, a factor used in its determination.

Both acid and alkaline phosphatases may be determined by similar methods, provided the pH of the substrate has been correctly buffered. A wide variety of substrates have been used, with very different incubation times and unit values. In addition, comparison of results may be difficult because different methods may estimate different isoenzymes.

Bessey, Lowry and Brock (1946) used hydrolysis of colourless *p*-nitrophenol phosphate to form *p*-nitrophenol, which is yellow and easily measured at 400 nm. Bodansky (1933) used β-glycerophosphate as the substrate, and, after incubation for 1 hour, measured the liberated phosphate by the formation of a phosphomolybdenum blue complex. King and Armstrong (1934) measured the phenol liberated from disodiumphenyl phosphate by allowing it to react with Folin–Ciocalteau's reagent (phosphotungstic, phosphomolybdic acid reagent) in alkaline solution. This method was later modified by Kind and King (1954), who measured the phenol after reaction with 4-aminophenazone and potassium ferricyanide to form a red complex. Phenolphthalein phosphate has also been used as a substrate, with the measurement of liberated phenolphthalein in alkaline solution, which is a red indicator (Babson, 1963).

5′-Nucleotidase is determined by incubating serum with adenosine monophosphate substrate in barbiturate buffer at pH 7.5, both with and without the addition of nickel, and measuring the phosphate liberated by the formation of a phosphomolybdenum blue complex. This isoenzyme can also be determined kinetically, using the coupled reaction shown below, and measuring the resulting decrease in absorbance at 340 nm (Arkesteijn, 1976).

$$\text{Adenosine monophosphate} \xrightarrow{\text{5′-nucleotidase}} \text{adenosine} + PO_4^{3-}$$

$$\text{Adenosine} \xrightarrow{\text{adenosine deaminase}} \text{inosine} + NH_4^+$$

$$\text{2-Oxoglutaric acid} + NH_4^- + \text{NADH} \xrightarrow{\text{glutamate dehydrogenase}} \text{glutamic acid} + NAD^+$$

Reference ranges

Total serum acid phosphatase (males): less than 10 i.u./ℓ
Tartrate-labile acid phosphatase (males): less then 4 i.u./ℓ
Alkaline phosphatase: 14–80 i.u./ℓ
5′-Nucleotidase: 3–17 i.u./ℓ

Transaminases

This group of enzymes catalyse the transfer of nitrogen from an α-amino to an α-oxo group of an amino acid (*see Figure 1.16*). Those commonly determined in clinical chemistry are aspartate aminotransferase (AST) and alanine aminotransferase (ALT).

Aspartate aminotransferase

AST is widely distributed in the body, but is found particularly in heart, liver, muscle and kidney tissue. It is increased in the serum in a wide variety of conditions, including cardiac, liver and muscle diseases, due to cellular damage allowing leakage of the enzyme into the circulation. Transaminases are excreted into the bile, and are therefore also increased in the serum in obstructive jaundice, which blocks entry into the duodenum. Serum AST begins to increase about six hours following myocardial infarction, and returns to normal after about five days. At its peak, it may be raised to five times the normal level.

It may be determined colorimetrically (Reitman and Frankel, 1957) or kinetically. As shown below, addition of a colour reagent in alkaline solution to the oxaloacetate formed in the initial reaction results in the formation of a red phenylhydrazone complex. Some workers recommend converting the oxaloacetate to pyruvate before adding the colour reagent:

$$\text{Aspartate} + \alpha\text{-oxoglutarate} \xrightarrow{\text{AST}} \text{oxaloacetate} + \text{glutamate}$$

(Oxaloacetate + aniline citrate → pyruvate)

Pyruvate (or oxaloacetate) + 2,4-dinitrophenylhydrazine → red complex

The kinetic method uses the oxaloacetate formed in the reaction above to oxidize NADH in the presence of malate dehydrogenase (Karmen, 1953). The resulting decrease in absorbance is then measured at 340 nm.

$$\text{Aspartate} + \alpha\text{-oxoglutarate} \xrightarrow{\text{AST}} \text{oxaloacetate} + \text{glutamate}$$

$$\text{Oxaloacetate} + \text{NADH} \xrightarrow{\text{malate dehydrogenase}} \text{L-malate} + \text{NAD}^+$$

Alanine aminotransferase

ALT is much less widely distributed than AST, and occurs mainly in the liver. Serum activity is increased in patients with infectious

hepatitis and cirrhosis of the liver, and also in obstructive jaundice (as a result of the damming effect in the bile duct).

Its colorimetric determination is similar to that of AST. The method of Reitman and Frankel (1957) is used, but without the addition of aniline citrate as pyruvate is formed in the initial reaction:

$$\alpha\text{-Oxoglutarate} + \text{L-alanine} \xrightarrow{\text{ALT}} \text{L-glutamate} + \text{pyruvate}$$

$$\text{Pyruvate} + \text{2,4-dinitrophenylhydrazine} \rightarrow \text{red complex}$$

The kinetic method (Wroblewski and La Due, 1956) is also similar to that used for AST. Lactate dehydrogenase in the presence of NADH converts the pyruvate formed in the reaction above to lactate, while the NADH is oxidized to NAD^+. The resulting decrease in absorbance is measured at 340 nm.

$$\text{Pyruvate} + \text{NADH} \xrightarrow{\text{lactate dehydrogenase}} \text{L-lactate} + NAD^+$$

Reference range
Serum aspartate aminotransferase/alanine aminotransferase: less than 18 i.u./ℓ.

γ-Glutamyltransferase (GT)
This enzyme, which is widely distributed throughout the body but most abundant in those cells responsible for amino acid transfer, catalyses the transfer of γ-glutamyl groups from one peptide to another. Serum activity is normally very low, but increased in liver disease, particularly biliary obstruction. In acute viral hepatitis it remains elevated for longer than other serum enzymes, and is thus useful in monitoring recovery.

It is determined colorimetrically using a reaction in which *p*-nitroaniline is released by the action of the enzyme (Szasz, 1969) and the resulting yellow colour measured at 405 nm.

$$\gamma\text{-Glutamyl-}p\text{-nitroaniline} + \text{glycylglycine} \xrightarrow{\gamma\text{-GT}} p\text{-nitroaniline} + \gamma\text{-glutamylglycyl glycine}$$

Reference range
Serum γ-glutamyltransferase: Males less than 28 i.u./ℓ.
Females less than 18 i.u./ℓ.

Glucose

Glucose is constantly entering and leaving the blood. It is absorbed from the intestine and produced from the breakdown of glycogen in the liver, and leaves in order to supply the tissues with energy, or to be stored as glycogen in the liver when in excess. If the renal threshold is exceeded, it leaves the body via the kidneys. This cycle is shown in *Figure 6.6*. The blood concentration is finely controlled by the action of insulin, which increases the rate of glucose uptake by the cells.

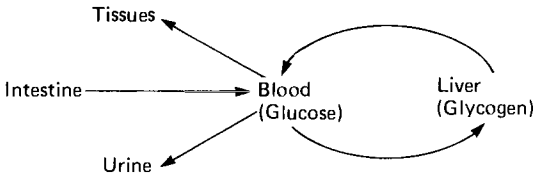


Figure 6.6 The glucose cycle

The determination of blood glucose is important in the diagnosis and treatment of diabetes mellitus, and in the investigation of insulin-secreting tumours and renal glycosuria. The glucose concentration in the cerebrospinal fluid (CSF) is slightly lower than that in blood, but in meningitis it is significantly lower, and sometimes not detected.

Older methods for determining the blood glucose actually measured reducing substances, of which glucose is the most abundant. The degree of interference from non-glucose reducing substances depended on the method of protein precipitation: with some methods glutathione remained within the cells, while with others it was released and estimated. Alkaline copper solutions, iodine and potassium ferricyanide have all been used for this determination. The former usually resulted in the formation of a phosphomolybdenum blue complex, which could be measured colorimetrically, while most iodometric methods involved volumetric analysis. The latter tended to be more specific, but also more time-consuming.

An example of the iodometric type is the method developed by Hagedorn and Jensen, in which glucose reduces a standard solution of potassium ferricyanide to potassium ferrocyanide. The excess (unused) ferricyanide is allowed to react with potassium iodide to liberate iodine, which is back-titrated with sodium thiosulphate:

$$K_3Fe(CN)_6 + \text{glucose} \rightarrow K_4Fe(CN)_6$$

$$\text{Excess } 2\,K_3Fe(CN)_6 + 2\,KI \rightarrow 2\,K_4Fe(CN)_6 + I_2$$

$$I_2 + 2\,Na_2S_2O_3 \rightarrow Na_2S_4O_6 + 2\,NaI$$

In the United Kingdom most laboratories now use a form of glucose oxidase technique.

$$\text{D-Glucose} + O_2 \xrightarrow{\text{glucose oxidase}} \text{D-gluconolactone} + H_2O_2$$

The addition of 4-aminophenol to the peroxide formed in the reaction above will, in the presence of peroxidase, produce a pink quinone complex (Trinder, 1969b).

'Dextrostix' (Ames), which work on the same principle, are plastic strips impregnated with the appropriate enzymes and a chromogenic indicator. The Beckman glucose analyser uses an oxygen-specific electrode to measure the rate of disappearance of oxygen from the equation shown above. This system, however, necessitates the use of plasma and not whole blood.

The Yellow Springs Analyser uses whole blood or plasma, and has a hydrogen peroxide sensitive electrode, the tip of which is covered by a membrane containing immobilized glucose oxidase. The current produced is proportional to the rate of production of hydrogen peroxide and thus to the glucose concentration.

Another method (Stein, Mildner and Maulbetsch, 1978) uses glucose dehydrogenase to reduce NAD^+ and then measures the rate of formation of NADH by the increase in absorbance at 340 nm.

$$\text{D-Glucose} + NAD^+ \xrightarrow{\text{glucose dehydrogenase}} \text{D-gluconolactone} + NADH$$

A similar method, using hexokinase and glucose-6-phosphate dehydrogenase (G-6PDH) in the coupled reaction shown below, and then measuring the rate of NADH production by the increase in absorbance at 340 nm was introduced by Passey *et al.* (1977).

$$\text{D-Glucose} + ATP \xrightarrow{\text{hexokinase}} \text{glucose-6-phosphate} + ADP$$

$$\text{Glucose-6-phosphate} + NAD^+ \xrightarrow{\text{G-6PDH}} \text{6-phosphogluconate} + NADH$$

Blood specimens for glucose determination should be collected into a fluoride-containing anticoagulant to prevent conversion of glucose to lactic acid in the process of glycolysis; otherwise they must be estimated immediately, or have the proteins inactivated.

CSF specimens must be collected into fluoride to prevent loss of glucose by possible bacterial action. If bacteria are present, this loss can be very rapid indeed.

Reference range
Fasting blood glucose: 3.0–5.0 mmol/ℓ

Glucose tolerance test
Diabetes can often be diagnosed by performing fasting and two-hour postprandial blood glucose determinations. The normal values are 5.0 and 10.0 mmol/ℓ respectively. If the diagnosis is in doubt a *glucose tolerance test* may be undertaken. Following the collection of fasting blood and urine specimens an oral dose of 75 g glucose is given in water. Specimens of blood are taken at 30-minute intervals for 2.5 hours, and urine specimens after 1 and 2 hours. An untreated diabetic should have a fasting blood glucose level of more than 7.0 mmol/ℓ and a maximum level during the test period of more than 10.0 mmol/ℓ. Two hours following the oral load the venous concentration should be greater than 10.0 mmol/ℓ and the capillary concentration greater than 11.0 mmol/ℓ. A lag curve, indicating delay in the production of insulin, is of no clinical significance. Normal and diabetic glucose tolerance curves are shown in *Figure 6.7*. Insulin-secreting tumours produce 'flat' curves, i.e. the blood glucose fails to rise appreciably following the oral glucose load.

Glycosylated haemoglobin

During their life span of approximately 120 days the red blood cells slowly absorb glucose, which is bound to haemoglobin. The bound (glycosylated) haemoglobin is increased in diabetes mellitus. As the bond is stable and builds up over a considerable period of time, a measure of the glycosylated haemoglobin fraction provides a better overall picture of the state of control of diabetic patients than single blood glucose determinations, which may change rapidly. On electrophoresis, the glycosylated haemoglobin fraction migrates faster than the non-glycosylated fraction, and in parallel with the fetal haemoglobin F. The three glycosylated fractions are HbA_{1A}, HbA_{1B} and HbA_{1C}. The most abundant of these is HbA_{1C}. Some methods determine the total HbA_1, while others determine the HbA_{1C} fraction. The separation is carried out by means of electrophoresis on agar gel or by ion-exchange column chromatography on the red cell haemolysate. Normally, approximately 7 per cent of the haemoglobin is glycosylated while 93 per cent remains unglycosylated. In diabetics as much as 25 per cent may be glycosylated.

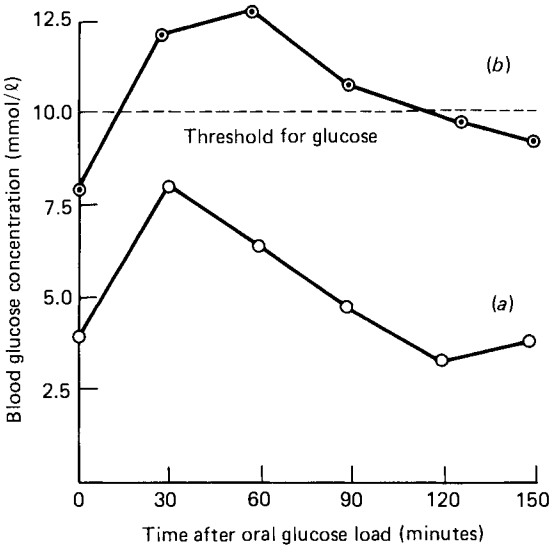


Figure 6.7 Glucose tolerance test. (*a*) Normal and (*b*) diabetic response to an oral dose of 75 g glucose

Urinary glucose

Urine does not normally contain sufficient glucose for detection by routine methods. The presence of glycosuria therefore requires further investigation, although it may not necessarily be of pathological significance. 'Labstix' (Ames), which incorporate a glucose oxidase system similar to that described for blood determination, may be used for this purpose. Benedect's reagent provides a non-specific test for urinary reducing substances by reduction of the alkaline copper reagent to a green or yellow complex depending on the amount of reducing substance present.

Hormones

Until the advent of radioimmunoassay (RIA) techniques, which allow the detection and determination of minute quantities of hormones, direct hormone assay was rarely possible in routine clinical laboratories.

Generally, the laboratory investigation of endocrine function involves one or more of the following:

1. Measurement of the hormone metabolite(s) in urine, e.g. the determination of vanilylmandelic acid as a measure of adrenal medullary function.
2. Measurement of a parameter affected by the action of the hormone, e.g. plasma calcium concentration as a measure of parathyroid function.
3. Direct determination of the hormone by radioimmunoassay.

Obviously direct determination of a specific hormone is preferable to indirect measurements. Direct measurement is becoming increasingly available, but where it is not, it will still be necessary to use indirect methods.

Stimulation and/or suppression tests may also provide valuable diagnostic information. Since the production of many hormones is stimulated by tropic hormones produced by another organ (e.g. cortisol from the adrenal cortex in response to ACTH from the anterior pituitary), measurement of hormone (or metabolite) levels before and after stimulation or suppression may help to identify the source of the dysfunction.

Steroid hormones

All steroids possess the basic cyclopentanoperhydrophenanthrene ring structure. The rings are lettered, and the carbon atoms numbered according to the convention shown in *Figure 6.8*. They are classified according to the substituents at C-17. Steroid hormones do not have long side chains at C-17 as do other classes of steroids. They have a total of 18 to 21 carbon atoms, and most active hormones have a Δ^4-3-oxo grouping, although this is reduced during metabolism. They also have angular methyl groups at C-10 and C-13.

Oestrogens (the female sex hormones) are derived from the parent substance oestrane and have phenolic A rings, thereby eliminating the possibility of having an angular methyl group at C-10. The principal oestrogen is 17β-oestradiol (*Figure 6.8*). Determination is by radioimmunoassay (RIA). In normal subjects, oestrogen production is increased following the administration of clomiphene, which stimulates the anterior pituitary to secrete LH and FSH, thus stimulating ovulation. It also increases in response to human gonadotropic hormone, which stimulates the ovaries to produce oestrogens.

Androgens (the male sex hormones) are derived from the parent substance androstane, and have 19 carbon atoms. Dehydroepiandrosterone (DHA) is secreted by the adrenal cortex in response to

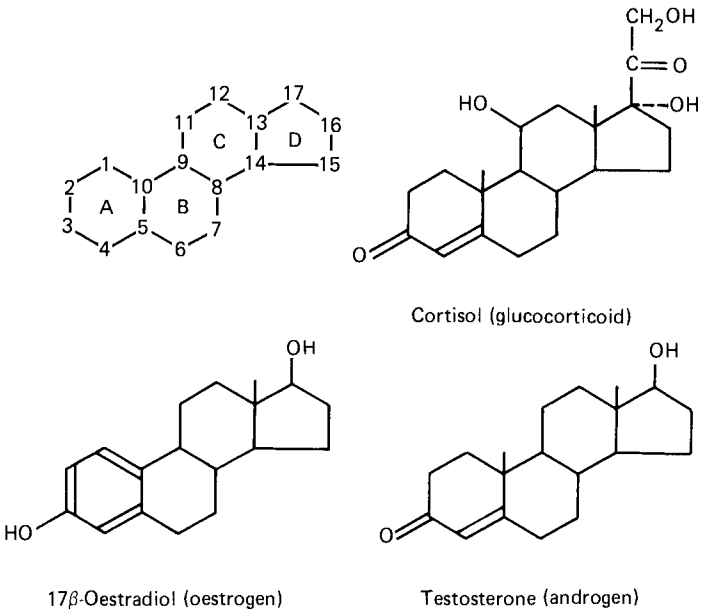


Figure 6.8 The basic cyclopentanoperhydrophenanthrene ring structure of steroid hormones, showing conventional lettering of rings and numbering of carbon atoms

ACTH stimulation, and is therefore present in both males and females. Testosterone (*Figure 6.8*) is the principal androgen secreted by the testes in response to LH and FSH stimulation. Both DHA and testosterone are excreted as the 17-oxosteroid metabolites aetiocholanolone and androsterone, respectively (*Figure 6.9*).

Androgenic function is assessed mainly by radioimmunoassay of the hormone in blood or by the Zimmerman reaction on the urinary 17-oxosteroid metabolites. In this method the sulphate conjugates are freed by acid hydrolysis, and the oestrogen 17-oxosteroids are removed by washing with alkali. The free 17-oxosteroids are then extracted with solvent, evaporated to dryness and allowed to react with alkaline *m*-dinitrobenzene (*Figure 6.9*) to give a purple complex with maximum absorbance at 520 nm. Interfering chromogens are removed by ether extraction. The reaction is given by all substances with the CH_2—CO—CH_2 grouping, so that it is important to ensure that none of the apparatus used for the test is contaminated with acetone (CH_3—CO—CH_3).

The principal glucocorticoid secreted by the zona fasciculata of

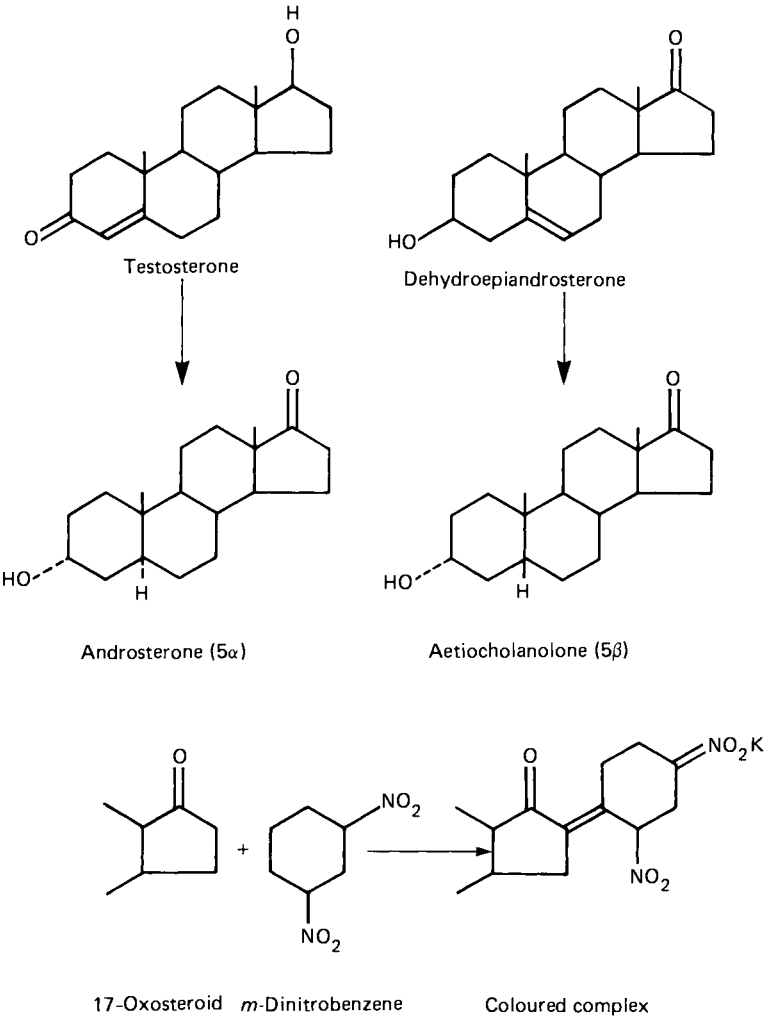


Figure 6.9 The metabolism of androgens and the reaction of their 17-oxosteroid metabolites with alkaline *m*-dinitrobenzene (Zimmerman reaction) to assess androgenic activity

the adrenal cortex is cortisol. This has 21 carbon atoms and a substituted 17-hydroxyl group. It is metabolized and excreted as the tetrahydro derivative, and is conjugated with glucuronic acid.

Cortisol is determined by RIA, using a competitive binding technique in which an excess of ^{3}H-labelled cortisol is added to the

unlabelled cortisol from the patient and allowed to compete for the binding sites of a constant amount of cortisol-binding globulin. The excess free cortisol is separated from that which is bound by absorption onto charcoal, and the activity is then measured. The activity of the free tritiated [^{3}H-] cortisol is inversely proportional to the concentration of cortisol in the test sample. The technique of Mattingly (1962) extracts the cortisol with dichloromethane and, after removal of the solvent, the fluorescence is measured at 530 nm in sulphuric acid. The urinary 17-hydroxycorticosteroid metabolites of cortisol may be measured by enzyme hydrolysis of the conjugates, followed by oxidation of the freed steroids to 17-oxosteroids and their subsequent determination by the Zimmerman reaction as above.

Adrenocortical function is commonly assessed by tests designed to stimulate or suppress the secretion of steroid hormones either by direct action on the adrenal cortex or by an effect on the governing feedback mechanism.

The synthesis and secretion of cortisol is controlled by the level of circulating ACTH. The functional capacity of the adrenal cortex can be assessed by measuring cortisol in the blood or its metabolites in the urine, after an exogenous dose of ACTH. In anterior pituitary hypofunction there will be a slow rise in cortisol while in adrenal cortical disease there will be no appreciable rise in either plasma cortisol or its urinary metabolites. Exogenous ACTH can produce unpleasant allergic reactions, which can be avoided by using Synacthen (Ciba), a synthetic steroid containing 24 of the amino acids of ACTH but deficient in the antigenic part of the molecule.

The pituitary–ACTH–adrenal axis may be investigated by use of the metyrapone (Metapirone) test. This substance inhibits the 11β-hydroxylation of 11-deoxycortisol, which is necessary to give cortisol the essential 11-OH group. In normal subjects the hypothalamus responds to a decrease in plasma cortisol levels by secreting corticotropin releasing factor (CRF), which stimulates the anterior pituitary to release ACTH. Normally, therefore, an initial fall in the plasma cortisol level is followed by an increase due to an excess ACTH production sufficient to overcome the 11β-hydroxylation inhibition.

In hypopituitarism the anterior pituitary fails to produce ACTH and no subsequent increase in plasma cortisol will be observed. Similarly, the urinary 17-oxogenic steroids should normally rise following ACTH stimulation.

Dexamethasone is a powerful synthetic glucocorticoid which, given in an oral dose, markedly reduces cortisol secretion by inhibiting the secretion of ACTH from the anterior pituitary and

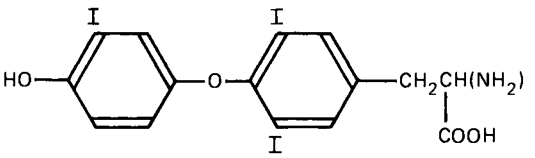
thus suppressing the production of adrenal cortical hormones.

Overactivity of the adrenal cortex may be due to primary aldosteronism, which causes increased reabsorption of sodium and water from the distal renal tubules, and thus oedema. It may also be due to Cushing's syndrome, where increased production of cortisol increases gluconeogenesis and thus causes muscle wasting. Increased androgen production gives rise to the adrenogenital syndrome, with precocious secondary sexual development in children, and hirsutism in female adults.

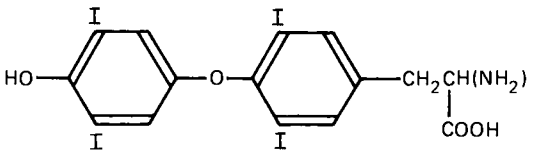
The opposite effects are observed with hypoactivity. In Addison's disease there is inadequate renal tubular reabsorption of sodium and water, causing severe plasma electrolyte disturbance, with decreased sodium and increased potassium levels, and decreased gluconeogenesis, thus lowering blood glucose levels.

Thyroid hormones

Most of the circulating thyroid hormones thyroxine (T_4) and triiodothyronine (T_3) are bound to thyroid-binding globulin (TBG), in which form they are not active. Only the very small free fraction is biologically active. The structure of T_3 and T_4 is shown in *Figure 6.10*.



Tri-iodothyronine (T_3)



Thyroxine (T_4)

Figure 6.10 The structure of thyroid hormones T_3 and T_4

One of the principal effects of these hormones is to stimulate the uptake of oxygen by nerve tissue, and one of the earliest methods of assessing thyroid function was to measure the oxygen consumption by allowing the patient to breathe oxygen from a closed-circuit apparatus, the expired carbon dioxide being absorbed by soda lime, and the amount of oxygen consumed compared to the reference value found in controls matched for age, sex and body surface area. A major problem was how to keep anxious patients at mental and physical rest, so that considerable technical (and psychological) skill was required to achieve good results. Furthermore, since oxygen consumption is also increased in fever, cardiac failure and pulmonary disease, and decreased in malnutrition, Addison's disease and nephrosis, this test was not specific.

The determination of the protein-bound iodine (PBI) gave a much better indication of thyroid function, but was technically difficult to perform and suffered from interference from drugs, pregnancy and hormone therapy, so that results did not always correlate well with the clinical findings. Almost all (99.6 per cent of the total T_3 and 99.96 per cent of the total T_4) of the circulating thyroid hormone is bound to protein and this must be precipitated and washed to remove trapped free iodine, and then oxidized to liberate the iodine from its bound state. The liberated iodine is measured by reaction with ceric sulphate, which is reduced to the cerous state.

Radioimmunoassay is now used to determine the uptake of T_3 (T_3U) by resin, and double antibody RIA to determine the total T_4. An index of the amount of free thyroxine (FTI) can be obtained from the ratio of the total T_4 to the uptake of T_3 expressed as a percentage. This, however, requires two separate determinations. Direct radioimmunoassay of free T_4 and T_3 in plasma is now possible. In the free T_4 test, a mixture of ^{125}I-labelled T_4 and test plasma containing free T_4 is allowed to compete for a limited number of binding sites on a specific T_4-antibody. The antibody-bound fraction is separated by centrifugation, and the labelled iodine assayed in the supernatant.

A similar technique is used for the determination of thyroid stimulating hormone (TSH) in order to assess dysfunction of the hypothalamus–pituitary–thyroid axis. In primary hypopituitarism the TSH is raised, as there is no suppression by the normal feedback mechanism.

Catecholamines

The principal biochemical interest in catecholamines is in the investigation of catecholamine-producing tumours. In adults, these

are chromaffin cell tumours, usually of the adrenal medulla, which produce an excess of adrenaline and noradrenaline, the main metabolite of which is 3-methoxy-4-hydroxymandelic acid (VMA; *Figure 6.11*) In children, they are neuroblastomas, which secrete an excess of dopamine, which is metabolized to homovanillic acid (HVA; *Figure 6.12*).

Gas chromatographic separation is probably the best method of assay, as all the metabolites are identified and quantified. It is not, however, suitable for routine analysis, and most laboratories con-

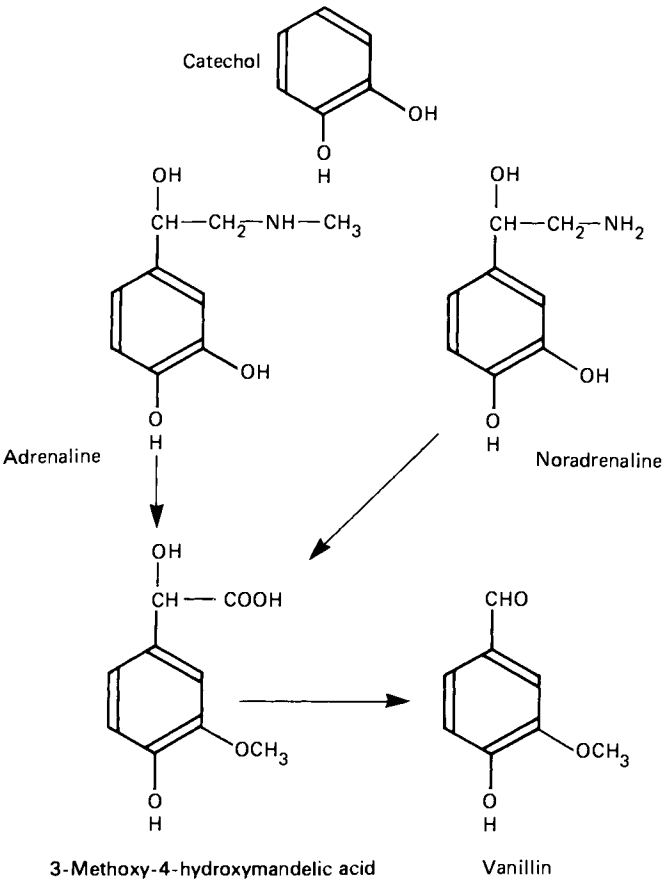
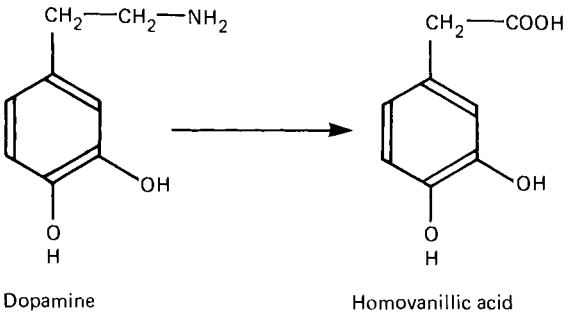


Figure 6.11 Catecholamines and their main urinary metabolite 3-methoxy-4-hydroxymandelic acid. This can be determined by extraction into ethylacetate and oxidation to vanillin, which absorbs at 360 nm



Dopamine Homovanillic acid

Figure 6.12 The formation of homovanillic acid

centrate on the determination of the major urinary metabolite, VMA.

The method of Pisano, Crout and Abraham (1962) is based on the extraction of VMA into ethylacetate and its subsequent oxidation with periodate to vanillin, which absorbs at 360 nm. Sandler and Ruthven (1961) use a similar technique but separate the VMA by absorption chromatography on alumina.

Plasma catecholamines can be determined fluorimetrically by conversion to fluorescent adrenolutins with potassium ferricyanide, following absorption chromatography.

Chromatographic separation of phenolic acids and their location with *p*-nitroaniline has also been used as a screening test for both VMA and HVA.

Reference range
Urinary VMA: less than 35 μmol/24 hours (Pisano)

Phosphate

The concentration of phosphate in the blood is controlled by parathormone, which increases the reabsorption of phosphate from bone, and by the kidneys, which secrete phosphate in response to parathormone. The concentration of phosphate within the cells is much greater than in the plasma, as it is an essential component of nucleic acids. It is therefore important in the determination of plasma phosphate to ensure that there is no leakage due to haemolysis or increase due to the activity of phosphatases after collection of the specimen. For this reason, plasma should be separated as soon as possible after collection.

There has been no significant change in the technique recommended by Fiske and Subbarow in 1925. Phosphate ions are allowed to react with acid molybdate to give phosphomolybdic acid, which is reduced to a phosphomolybdenum blue complex. The reducing agent is usually 1,2,4-aminonaphthol sulphonic acid, but other agents such as stannous chloride and hydroquinone may also be used. The original method requires protein precipitation, but the use of sodium laurylsulphate allows the proteins to remain in solution without causing interference.

Reference range
Plasma phosphate: 0.6–1.3 mmol/ℓ.

Proteins

Most plasma proteins are produced in the liver, the exception being the immunoglobulins, which are produced in the reticuloendothelial system. They are a large and extremely varied group of substances, but all have the common property of consisting of chains of amino acids linked by peptide (CO—NH) bonds. Because of their great diversity and the fact that different protein fractions do not all behave in the same way, it has been difficult to standardize methods of determining total protein. The only accurate determinations, therefore, are those for specific proteins. In all other cases an assumption has been made which may well not be correct, especially if the normal protein fractions are present in altered proportions, or if abnormal proteins are present.

Some diseases, however, are associated with characteristic changes in the serum protein pattern. Multiple myelomatosis is characterized by a high total protein concentration and an abnormal monoclonal γ- or β-globulin band on electrophoresis. In the nephrotic syndrome the total serum protein and albumin are lowered and the α_2-globulin fraction is increased.

The determination of total serum proteins by the method of Hock and Marrack (1945) is based on the premise that 16 per cent of the total protein is nitrogen. The procedure, however, is time-consuming and technically difficult, and thus unsuitable for a busy routine laboratory. The proteins in the first sample are precipitated, and the protein-free supernatant is then used to determine the non-protein nitrogen. A second sample, without protein precipitation, is used to determine the total nitrogen content. The difference (protein nitrogen) represents 16 per cent of the total protein content.

$$\text{Serum total protein} = \text{protein nitrogen} \times \frac{100}{16}$$
$$= \text{protein nitrogen} \times 6.25 \text{ g}/\ell$$

In this technique, the large protein molecules are broken down by the action of sulphuric acid in the presence of a suitable catalyst (e.g. selenium dioxide, potassium permanganate, hydrogen peroxide) to form ammonium sulphate. The ammonia is either determined by nesslerization or distilled in a Kjeldahl apparatus into standard acid, the excess of which is then determined by volumetric analysis.

The most commonly used technique (using Biuret reagent) assumes that the number of peptide linkages in the different protein fractions is constant. Chelation with Biuret reagent (alkaline copper tartrate) forms a deep blue complex, which is read at 555 nm. It is not read at the maximum absorbance as the coloured reagent interferes at this wavelength (Reinhold, 1953).

Folin–Ciocalteau's reagent reacts with the phenolic amino acids to give a phosphomolybdenum blue complex in alkaline solution (Greenberg, 1929). The method assumes a constant amount of tyrosine in the total proteins.

The 'field' method of Moore and Van Slyke (1930) is based on the formation of a copper proteinate when protein is added to a copper sulphate solution. By using a series of copper solutions of differing specific gravities and observing the behaviour of the copper proteinate drop which is formed (i.e. whether it rises, falls or remains stationary before finally sinking to the bottom of the container) the protein concentration can be calculated from the observed specific gravity of the copper proteinate. This method is used to determine the adequacy of the haemoglobin level in blood donors.

Total serum/plasma protein can also be determined by measuring the specific absorbance at 215 nm (which assumes a constant proportion of peptide bonds) and at 280 nm (which assumes a constant proportion of tyrosine). The method, however, is technically unsuitable for routine use.

Refractive index (RI) measurements have also been used. The refractive index of water is increased in direct proportion to the concentration of the solutes dissolved in it. As the solute in by far the greatest concentration in plasma is protein, this would seem to be a very simple and accurate means of determining the total protein concentration. Unfortunately the method requires a clear, colourless solution for accurate measurements, which makes it unsuitable for many specimens.

Albumin

Albumin may be measured by the same methods as total protein if prior fractionation is carried out. This is done with ammonium or sodium sulphate and sulphite solutions and is known as 'salting out'. The globulins are precipitated with varying efficiency, depending on the salt concentration, temperature and extraction procedure. The albumin is then assayed in the supernatant.

Binding with bromocresol green (BCG) buffered at pH 3.6 (Northam and Widdowson, 1967) or with bromocresol purple (BCP), (Pinnell and Northam, 1978) is reported to be very specific, and is a very simple procedure. Following electrophoretic separation, albumin and other protein fractions can be quantitatively determined by binding with dyes and measuring the intensity of the areas stained. Unfortunately, the different protein fractions do not take up the dye to the same extent, which renders the method unreliable.

Specific protein determination

By using pure specific protein standards and the immunological techniques described earlier, it is now possible to detect and determine minute quantities of specific proteins. The single radial diffusion technique (Mancini, Carbonara and Heremans, 1965) requires virtually no additional laboratory equipment, and is easily preformed.

Reference ranges
Total serum protein: 61–77 g/ℓ.
Serum albumin: 41–51 g/ℓ.

Renal calculi

Most renal calculi are inorganic, and the majority are mixtures of calcium phosphate and calcium oxalate. Phosphate stones, which may be ammonium magnesium or calcium phosphate, are fairly common, but pure calcium oxalate stones are rare. Mixtures of uric acid and phosphate are not uncommon, but stones consisting purely of uric acid or cystine are rare. Calculi are easy to identify chemically if a simple pattern of analysis is followed.

The first step is to heat a small portion of the powdered calculus over a flame on a piece of foil or crucible. If the stone dissappears it is organic, while any remaining ash is inorganic.

Inorganic analysis

1. Dissolve a portion of the original stone in dilute (2M) hydrochloric acid. Effervescence indicates the presence of carbonate. Make the solution just alkaline with dilute ammonia. A precipitate indicates the presence of oxalate or phosphate (or both). Render the solution just acid with dilute acetic acid. Oxalates will not redissolve but phosphates will redissolve in the acid solution.
2. Take a little of the dilute acid solution originally prepared and make this alkaline with ammonium oxalate. Calcium will precipitate as calcium oxalate.
3. If phosphate is found but not calcium, the stone contains ammonium magnesium phosphate. Test for magnesium by adding a drop of Magneson II reagent (0.5 per cent *p*-nitrobenzene-azo-α-naphthol in 0.25 M-sodium hydroxide) and make alkaline with ammonia. A blue precipitate is formed in the presence of magnesium.

Organic analysis

1. Test for uric acid by dissolving a little of the original stone in concentrated nitric acid and very slowly evaporating to dryness in an evaporating basin. Cool, and add a few drops of ammonia solution. In the presence of uric acid this will produce a purple complex (murexide, or ammonium purpurate).
2. Test for cystine by adding 5 per cent sodium cyanide solution (POISON) followed after a few minutes by a few drops of saturated sodium nitroprusside solution. Cystine gives a magenta colour. Hexagonal cystine crystals may also be prepared from the evaporation of the acid solution originally prepared.

Triglycerides

The determination of plasma triglycerides is important in the differential diagnosis of hyperlipidaemic states. The triglyceride level is raised in most of the hyperlipidaemias, but particularly in Fredrickson's type I (*see* Chapter 1), which is caused by a deficiency of lipoprotein lipase. As triglycerides are present in the plasma mainly as chylomicrons, a very high concentration gives the plasma a milky appearance. Specimens should be taken in the fasting state to avoid estimating the chylomicrons transporting the most recent meal. The triglyceride level is lowered in a-β-lipoproteinaemia and hypo-β-lipoproteinaemia, and raised in Tangier disease.

Older methods of analysis depend on the colorimetric or fluorimetric determination of glycerol formed by previous saponification. Recently, fully enzymatic procedures have become available which have increased both the sensitivity and the specificity of the determination (Bucolo and Davis, 1973). In the method illustrated below, lipase hydrolyses triglyceride to glycerol and fatty acids. Glycerol reacts with ATP in the presence of glycerol kinase to form ADP, which liberates pyruvate from phosphoenol pyruvate by the action of pyruvate kinase. Lactate dehydrogenase converts the pyruvate to lactate in the presence of NADH. The rate of production of NAD^+ can then be measured by the decrease in absorbance at 340 nm.

$$\text{Triglyceride} \xrightarrow{\text{lipase}} \text{glycerol} + \text{fatty acid}$$

$$\text{Glycerol} + \text{ATP} \xrightleftharpoons{\text{glycerol kinase}} \text{glycerol phosphate} + \text{ADP}$$

$$\text{ADP} + \text{phosphoenolpyruvate} \xrightleftharpoons{\text{pyruvate kinase}} \text{ATP} + \text{pyruvate}$$

$$\text{Pyruvate} + \text{NADH} \xrightleftharpoons{\text{lactate dehydrogenase}} \text{lactate} + \text{NAD}^+$$

The recommended standards for triglyceride determinations are triolein or tripalmitin.

Reference ranges
Fasting plasma triglycerides:
Males: 0.7–2.1 mmol/ℓ.
Females: 0.6–1.5 mmol/ℓ.

Urea

Urea is formed from the ammonia liberated by the interconversion of amino acids in the liver, and its concentration in the blood is raised in many disease states. In renal disease it is used as a measure of the total waste products which have accumulated in the body because they cannot be excreted, and it is thought that it is the phenolic acid waste products which cause the coma in uraemia, rather than the urea itself. It is raised whenever there is increased metabolism of proteins or amino acids. In hepatic failure it is lowered, as the cells responsible for the production of urea are severely damaged.

Urea is determined colorimetrically. Two common methods are

based on the production of ammonium carbonate by the action of the enzyme urease.

$$CO(NH_2)_2 + 2\,H_2O \xrightarrow{\text{urease}} (NH_4)_2CO_3$$

In the Nessler reaction the ammonia is coupled with mercuric potassium iodide to produce yellow ammonium mercuric iodide ($NH_2Hg_2I_2$). This is a very sensitive reaction and any excess ammonia produces a brown precipitate. It is necessary to remove proteins. This is usually done by the addition of sodium tungstate and sulphuric acid (forming tungstic acid) after incubation with urease and before the addition of Nessler's reagent.

The Berthelot reaction, first described in 1895, is also extremely sensitive but results in a more stable complex by coupling with phenol hypochlorite in the presence of sodium nitroprusside, after urease incubation. The resulting deep blue complex is not normally read at the peak absorbance, but at 560 nm.

Many laboratories use a direct reaction with urea. The Technicon, autoanalyser, for example, uses a system in which urea is coupled with diacetylmonoxime to form a yellow complex.

As urea is rapidly broken down by bacterial action, urine specimens should always contain a preservative such as thiomersal, and blood specimens should be refrigerated if not processed on the day of collection. Whole bood, serum or plasma are all suitable for the estimation, but if whole blood is used the proteins must first be removed. Blood collected into fluoride oxalate anticoagulant should not be used for urease methods as fluoride inhibits the action of urease.

Reference range
Plasma urea: 3.2–7.1 mmol/ℓ.
Urine urea: 250–600 mmol/24 hours.

Blood urea nitrogen

Confusion sometimes arises in the United States where it is customary to report urea as blood urea nitrogen (BUN), and the values are given in milli- (or micro-) grams. Urea has a molecular weight of 60 and contains two atoms of nitrogen, as shown by the molecular formula, which is $CO(NH_2)_2$. Thus, 60 milli-(or micro-) grams of urea is equivalent to 28 milli- (or micro-) grams of BUN. When the results are expressed in millimoles there is no numerical difference between urea and BUN values.

Uric acid

Uric acid is the end point of purine metabolism in man, and is formed from the breakdown of nucleic acids (*see Figure 1.1.*) It is increased in the blood in gout, and deposited as urate crystals in and around the joints. Increased concentrations in the urine may lead to the formation of renal urate calculi. These are detected by the murexide test (*see* page 131).

Most of the older chemical tests use phosphomolybdic acid for serum determinations. After tungstic acid protein precipitation, phosphomolybdic acid is added in the presence of alkali to produce a phosphomolybdenum blue complex (Brown, 1945). Both sodium carbonate and sodium cyanide have been used to raise the pH; cyanide is the more sensitive but has the disadvantage of being extremely toxic. Recently introduced methods involve the use of uricase, which oxidizes uric acid to allantoin.

Uric acid has a maximum absorbance at 292 nm, while allantoin does not absorb in the ultraviolet range. The difference in absorbance at 292 nm before and after incubation with uricase therefore gives a measure of the uric acid concentration (Liddle, Seegmiller and Lester, 1959). Morgenstern and Klein (1966) base their determination on the reduction of copper by uric acid, measuring the formation of a neocuproin complex before and after the incubation with uricase.

Reference range
Plasma or serum urate:
Males: 0.25–0.51 mmol/ℓ.
Females: 0.18–0.39 mmol/ℓ.

Chapter 7

Management in clinical chemistry

Clinical chemists need to be aware of the importance of their managerial role in addition to being able to understand and perform their technical functions, as only good management can ensure that laboratory investigations are efficiently performed and controlled. Of the many aspects of management, only those which directly affect the performance of the technical work will be discussed, as the remainder are beyond the scope of this book.

The maintenance of equipment and supplies

Much equipment is installed by the manufacturer, but if this has not been done it is essential that the installation instructions are properly carried out and that the principles of operation are fully understood by each operator using it. Many suppliers of complex equipment offer staff training courses prior to installation and full use should be made of this facility. It is imperative to select the trainee with great care. This should normally be the person(s) who will subsequently be responsible for its use and maintenance, and who will be required to train others.

The maxim that 'cleanliness is next to godliness' applies equally to machines and men. Quite apart from the aesthetic appearance, much harm may be caused by equipment which has been allowed to become dirty. Corrosion due to chemical spills, dirty or worn bearings, and many other forms of neglect all contribute to shorten the useful life of laboratory apparatus, as well as making it less efficient. Check lists for routine operation and maintenance are a simple and efficient means of ensuring that essential functions are properly carried out. The manager needs to know which parts are most likely to be required as spares and keep adequate supplies in stock, with due regard to cost, shelf-life and ease of availability. If the instrument is vital to the running of the laboratory, an adequate back-up service must be available. If not, it is important to decide how much 'down time' can be permitted and where help can be

obtained in order to restore normal service. Often telephone assistance is available, and simple tests to check the instrument may be carried out while 'on line' to the service engineer. This may enable faults to be corrected quickly and easily, and without the expense of a service visit.

Consumables such as reagents and glassware should be kept in reasonable constant supply, with due regard to lead (delivery) time, shelf-life, available storage space, quantity discounts etc., and efforts should be made neither to stockpile nor to under-order, and to avoid continual 'crisis' management.

Ergonomics

The arrangement and amount of working space has a marked bearing on the comfort, safety and efficiency with which the laboratory worker operates. Ideally he should be seated comfortably, with sufficient knee space under the bench, and all items required for use should be arranged in an arc in front of him so that they may be reached without bending or stretching, or the need to constantly move away from the working area during a given task. Instruments should be purchased with the operator in mind as well as the chemical merit. Equipment which is difficult or tiring to use and maintain will inevitably result in less efficient output than well-planned, well-sited apparatus.

Safety

That there are dangers in chemical laboratories is universally accepted, but that there are special dangers in the clinical chemistry laboratory has often been overlooked. Most of the specimens received for analysis are blood, urine or cerebrospinal fluid, which are all normally sterile. They may, however, be infected due to a variety of causes. The risk of infection to the laboratory worker lies not so much with the known 'high risk' samples, but with those which are unexpectedly found to be infectious. All specimens should therefore be handled with caution and care.

In order to achieve maximum safety for the laboratory worker with minimum disruption to the work flow, a balance must be sought between precautions which are essential and the freedom which may be enjoyed. Some of the more common hazards, and the means of avoiding them, are discussed below.

Concentrated acid solutions should be stored in a cool but not

cold (acetic acid will freeze at 16.3 °C) well-ventilated area, away from contact with other chemicals and well away from the working area. They should always be stored at floor level, never on a shelf. The storage area should be enclosed by a low wall lined with an acid-resistant material so that any spillage following an accident can be contained. A quantity of solid sodium bicarbonate should always be within easy reach, as should a cold water tap and hose, and a quantity of absorbent material, to be used in the event of an accident. Any spillage should be mopped up immediately after diluting well with water (CARE) and neutralizing with sodium bicarbonate. Goggles or an eye shield should be worn to protect the face and eyes, and thick rubber gloves to protect the hands. Concentrated acetic and nitric acids should be stored well apart from each other in order to prevent the formation of nitrates. Acids should not be stockpiled, and only small amounts required for immediate use should be kept in the working area. They must always be transported in safety carriers or containers, and not hugged or carried by the neck of the bottle. Acid burns should be washed immediately with copious amounts of running water and neutralized with sodium bicarbonate, except for the eyes, which should only be washed with water or physiological (0.85 per cent) solutions of sodium chloride.

In general, concentrated alkalis are less dangerous than their acidic counterparts, but they must still be treated with respect. They should be stored away from direct contact with acids, preferably at floor level, and with the same facilities for diluting and absorbing spillages. Burns should be treated by copious irrigation with cold running water.

Solid chemicals should be stored in such a way as not to allow contact between highly reactive chemicals. Picric acid should only be stored in small quantities, and must be covered with water at all times. Obsolete and surplus chemicals should not be retained in the store but should be removed, preferably by a qualified contractor.

Flammable liquids should be stored away from the general chemical store, and away from the main laboratory, preferably in a separate outside building. This should be clearly labelled with approved signs, and strict 'no smoking' rules must be obeyed in its vicinity. The amount of flammable liquid in the store should be strictly limited and only the quantities required for immediate use should be kept in the laboratory area. The store should be cool and well ventilated. Flammable liquids should not be allowed to flow into the main drainage system, except for small quantities of well-diluted water-miscible solvents washed down with copious volumes of cold running water. In all other cases the solvent waste should be

retained and collected into separate containers, one for chlorinated and the other for non-chlorinated hydrocarbons. These should be removed regularly by a qualified contractor.

Special precautions are required when working with isotopes. Highly active standards, etc. must be diluted behind lead shielding in a special fume cupboard or safety cabinet, set apart for this purpose, and bearing approved 'radioactive' hazard warning signs. The fume cupboard outlet should be above the rest of the building and should not have a deflector to direct the exhaust downwards. Filters should be easy to remove and exchange when necessary. The radioactivity should be monitored before and after each use with a Geiger counter and the air flow measured regularly with an anemometer. If there is excess radiation and/or defective air flow the fume cupboard must be declared unsafe and not used until the fault is corrected. The floor and benches should have smooth, non-porous, easily cleaned surfaces with welded seams. Sinks should have an integral, not separate drainer. There should be at least 12 air changes per hour in any room used for isotope work.

Isotopes should be stored in a lockable, shielded, labelled cupboard bearing the approved radiation signs on the outside. It is useful to line work surfaces with a disposable plastic surface such as 'Benchkote' which can be easily removed and replaced when contaminated. Users should wear rubber gloves which are disposed of after use and radiation film badges which are checked and replaced regularly. The room should be monitored regularly with a Geiger counter for excessive radiation and if necessary put out of action until safe. For both β- and γ-emitting substances the background should not be allowed to exceed 20 counts per second. Work surfaces should be decontaminated by washing with 10 per cent surfactant in order to achieve this level.

The disposal of radioactive substances must be very carefully controlled. Small quantities of weak, well-diluted, aqueous isotopes may be discharged into the general sewage system. Solid waste should be incinerated, and fluids used for scintillation should be collected into labelled containers and disposed of by a qualified contractor.

All workers must be properly instructed in the safe handling of isotopes, and great care must be taken to maintain the equipment at very low radiation levels by means of good housekeeping techniques.

The ever-increasing use of electronic equipment in the laboratory requires added precautions in order to prevent accidents. Equipment must be properly wired and fused, and care taken not to overload the circuit. The use of multiple adaptors should be discouraged. A

qualified electrician's advice and help should always be sought with electrical problems and for the installation of new or replaced electronic equipment. The wiring should be checked regularly and replaced if necessary. Refrigerators used for the storage of reagents should be spark-proof and the siting of electronic equipment should be well planned (e.g. well away from water sources).

In chemical pathology the hazard from microbiological agents lies in the unexpected. All specimens should therefore be considered as potentially hazardous. Special care is needed with specimens known or suspected to be infected, and those from high risk groups of patients such as drug addicts and those receiving renal dialysis. The use of screw-capped specimen containers minimizes the release of airborne contaminants, and initial sample handling in a Class I open-fronted safety cabinet further reduces the risk. The cabinet should contain at least one HEPA (high efficiency particulate air) filter and be positioned away from draughts and cross-currents and the general stream of laboratory traffic. The air intake should be at least 0.75 linear metres per second, and the room should have at least six air changes per hour. The filtered air should not be recirculated within the building. Regular servicing is essential, and this should include changing the primary filter. Before this is done, however, the cabinet must be disinfected by placing in it a 500 ml beaker containing 35 ml formalin and carefully adding 10.0 g solid potassium permanganate. This quantity of potassium permanganate *must* not be exceeded, in order to avoid the risk of explosion. The cabinet front should then be sealed off and left overnight. Any residual formalin is removed the following day by opening the front a little and activating the fan for a few minutes. The filters may then be safely inspected and changed. They should be handled with disposable rubber gloves and placed immediately in a plastic bag which is then sealed and incinerated.

Specimens known or suspected to contain category B_1 or B_2 organisms must be labelled 'Danger of Infection' and handled separately and with additional caution, using disposable rubber gloves.

Spillages of specimens must be cleaned up promptly with a suitable disinfectant. For most purposes 10 per cent sodium hypochlorite is suitable but this should never be used on metals. Centrifuges and other metallic equipment requiring decontamination should be cleaned with 2.5 per cent gluteraldehyde in 0.3 per cent bicarbonate buffer at pH 7.5. Non-metallic work surfaces may be cleaned with 1 per cent sodium hypochlorite solution.

Urine specimens should be discarded carefully into the sewage system having first been decontaminated overnight with 10 per cent

hypochlorite solution. Blood and faecal specimens should be placed in a sealed container and incinerated, as should disposable needles and syringes used to collect specimens.

Confidence in chemical analysis

The workload of many laboratories has increased to such a degree that it has become difficult or impossible to handle without the aid of mechanization or automation. There is, however, no point in carrying out vast numbers of determinations either manually or mechanically if the validity of the results cannot be assured. This requires confidence regarding several major issues:

1. The patient sample and identity
2. The accuracy of the test result
3. The precision obtainable with similar tests
4. The elimination of non-analytical errors.

The following is confined to a discussion of accuracy and precision in the analytical test, although errors may of course be due to other factors (*see above*).

Accuracy

By this, we mean the closeness of the value obtained to the true result. The more specific a test is for a constituent, the less likelihood there is for interference from other substances. For example, enzymatic determination of blood glucose gives considerably lower values than most other methods, since the latter estimate other non-reducing substances in addition to glucose. Enzymatic methods tend to be more specific than general chemical techniques, and also more accurate. This somewhat sweeping statement requires qualification for each determination, as it is by no means universally applicable. Methods should be selected for their specificity for the test substance and efforts taken to avoid techniques known to be influenced by other substances.

Standard solutions containing known concentrations of the test substance should be analysed in parallel with the tests, as should reagent and sample blanks in order to correct for interference from either the reagents themselves or other substances present in the sample. Where possible, the standards should be *primary standards*, with weighed-in constituents; if this is not possible, they should be

standardized against a primary standard before use so that there is absolutely no doubt concerning their true concentration.

In spectrophotometric analysis the test sensitivity should be adjusted to read only that part in which the Beer and Lambert laws are obeyed. Gross inaccuracies can arise when the test concentration exceeds the capacity of the reagents, producing a solution of very intense colour. In such cases it is necessary to repeat the analysis with less, or a more dilute sample. (Simple dilution of the coloured solution will not give an accurate result.)

Recovery experiments are a very useful guide to accuracy. A known amount of the analyte is added to one of two identical test solutions, both of which are analysed. The difference in concentration can be used to calculate the percentage of added analyte recovered.

The accuracy of a test is influenced by whether tests and standards can be treated in an identical manner. For example, since plasma has a very high protein content (60–80 g/ℓ) and therefore a very much higher viscosity than aqueous standard solutions, their aspiration rates during flame emission spectrophotometry will be different, which requires correction. Similarly, in continuous flow systems involving the removal of interfering proteins, the rate of dialysis of the test sample may be retarded by the presence of protein, while the standards are unaffected. In contrast, some determinations are made more specific by dialysis. For example, in the determination of creatinine by continuous flow methodology using dialysis and the Jaffe reaction, larger non-creatinine interfering chromogens are removed because they cannot pass across the dialysis membrane. This method, therefore, is more specific than its manual counterpart.

Precision

This is the ability to produce the same result on repeated analysis, either within the same analytical batch or between different batches of analyses. In order to be of real clinical value, chemical results must be both accurate and precise. An awareness of the many factors which can cause errors is vital in order to ensure this.

Human errors are an extremely important factor. Poor pipetting technique and inadequate mixing of solutions, inaccurate timing of incubation periods, inaccurate reading or setting of instruments and inaccurate calculations all contribute to the errors which *all* staff can make, however experienced or careful they are. Fatigue is another factor which tends to produce poor results, particularly with large batches of manual tests. For these, automated equipment will prove invaluable, as it is designed specifically for continuous analysis and

often gives greater precision than is possible with the best technical staff performing manual tests. This benefit, however, is lost if equipment is not properly maintained. Faulty or broken pipettes and heating baths, cracked filters and incorrectly adjusted spectrophotometers all contribute to laboratory errors – and to its reputation.

Specimens accepted for analysis must be of the correct type, properly labelled and receive the correct treatment before analysis. For some determinations fresh specimens are required. For analytes showing diurnal variations in concentration the time of collection must be known. Specimens for enzyme determinations may have to be frozen if analysis is not to be carried out immediately, since some are labile and likely to deteriorate. For some tests, blood may have to be separated immediately after collection to prevent diffusion of substances or ions across the cellular membrane, or enzyme activity from producing changes by the addition of an enzyme poison.

The first step in quality assurance is an awareness of inadequacies in technique and in the treatment of specimens. Every laboratory worker should be willing to act on his quality control findings and to reject suspect batches of results. Only by such positive action will the laboratory staff and clinicians gain true confidence in the analytical results. Some of the simple statistical methods which may be used to increase confidence are discussed below.

Statistical methods of evaluation

Standard deviation

If we were to plot the results of a single specimen of blood analysed 100 times for the glucose concentration we might well obtain a histogram similar to that shown in *Figure 7.1*, where the most frequently occurring value (mode) is the most probable true value, with the other values evenly scattered about the mode. In this example the mode is the same as the mean value. In this simple illustration, 65 of the results fall between 5.8 and 6.2 mmol/ℓ; 95 fall between 5.6 and 6.4 mmol/ℓ; and almost (but not quite) all between 5.4 and 6.6 mmol/ℓ.

Replotting this histogram as a frequency distribution curve (*Figure 7.2*) reveals a symmetrical or *normal*, type of distribution. The standard deviation (σ) is the mean difference from the overall mean, and is expressed by the general formula

$$\sigma = \sqrt{\frac{\Sigma(x-\bar{x})^2}{n}}$$

where x = an observed vlaue, $\bar{x}$ = the mean value of x (i.e. $\bar{x}/n$),

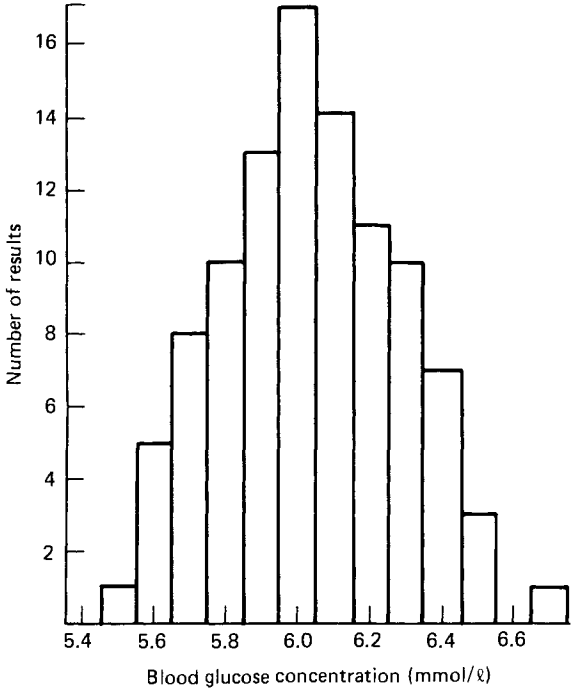


Figure 7.1 Histogram showing the results of 100 blood glucose determinations performed on a single specimen

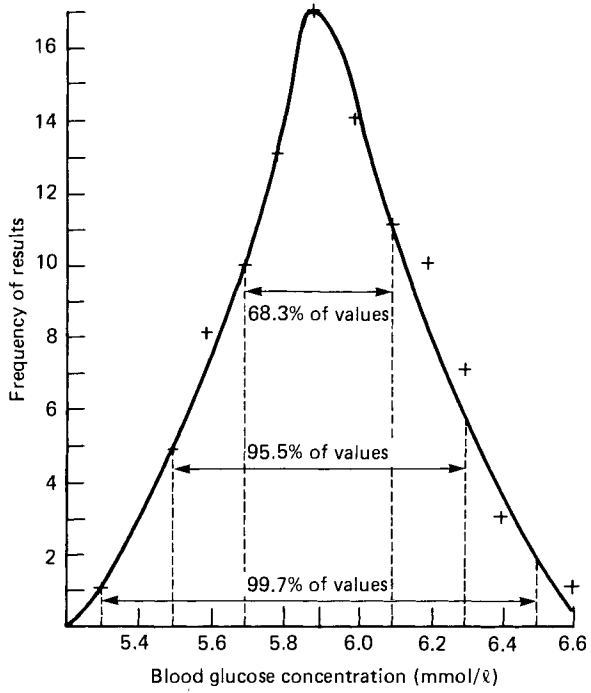


Figure 7.2 The same results as in *Figure 7.1* plotted as a distribution curve

n = the number of determinations, Σ = the sum of (observed samples), and σ = standard deviation.

If the number of determinations is less than 35, the above formula is biased and a small correction is necessary to allow for this. This is done by substituting $n-1$ for n in the above formula. Therefore when $n<35$

$$\sigma = \sqrt{\frac{\Sigma(x-\bar{x})^2}{n-1}}$$

In our glucose example the standard deviation is 0.2 mmol/ℓ. Statistically it can be shown that

68.3 per cent of the results will fall within $\pm 1\,\sigma$ of the mean;
95.5 per cent of the results will fall within $\pm 2\,\sigma$ of the mean; and
99.7 per cent of the results will fall within $\pm 3\,\sigma$ of the mean.

Which of the 100 results, then, from our sample are acceptable? The most frequently used criterion is that $2\,\sigma$ on either side of the mean value would be acceptable. These are termed the 95 per cent confidence limit. In other words, we could be 95 per cent sure that our result is correct. The accepted 95 per cent confidence limit for these determinations in the sample above would be 6.0 ± 0.4 mmol/ℓ. It has been suggested that limits of $2\,\sigma$ be called *warning limits* and that $3\,\sigma$ be taken as *action limits*, beyond which, if controls fail to achieve these targets, positive action must be taken at once to keep methods in control.

When results are the same or similar, comparison using the standard deviation is easy, but it is often necessary to compare the precision obtained over a wide range of values. One method of allowing for the difference in magnitude of the values is to use the *coefficient of variation* (c). This is expressed as

$$c = \frac{\sigma \times 100}{\bar{x}}$$

A standard deviation of 0.2 has a much greater significance at a concentration of 3.0 mmol/ℓ than at 30.0 mmol/ℓ. For example, the coefficient of variation at 3.0 mmol/ℓ, when $\sigma = 0.2$, is

$$c = \frac{0.2 \times 100}{3.0} = 6.7$$

whereas at a concentration of 30.0 mmol/ℓ, when $\sigma = 0.2$,

$$c = \frac{0.2 \times 100}{30.0} = 0.67$$

The lower the value of c the more precise is the determination.

It has been suggested that in order to compensate for the wide variation in range between different determinations the allowable error should be taken as one-tenth of the normal range for that determination. It is more important, however, that each laboratory establishes its own allowable margin of error, which should neither be so narrow that its achievement is virtually impossible, nor so wide that it allows slackness.

Trends

It is often useful to follow the daily statistical values for individual laboratory tests, as the observation may reveal changing trends and allow action to be taken early enough to prevent an analysis going out of control. It has been shown that if sufficient daily data are available, the daily mean value for a particular test should be the same, provided that the population samples are comparable. A large clinic sending an unusually large batch of samples, for example, may bias the results for selected determinations. Such bias must be taken into consideration. For this reason it is useful to set *truncation limits* and to exclude results above and below these values from the calculation of the daily mean.

When the daily mean values are plotted against time (Levy–Jennings curve), a method which is well controlled will show a variation about the expected mean from day to day, but with no continued trend in any single direction (*Figure 7.3*). A continuing tendency to rise or fall may mean that the reagents, standards or technique are changing, or that the equipment is not functioning correctly, and that action needs to be taken to prevent the method from becoming uncontrolled. By plotting the cumulative sum of the difference in the daily mean value (cusum), the trend is magnified and thus more apparent to the observer. *Figure 7.3* shows the daily mean values for plasma urea determination for which the expected mean was 6.0 mmol/ℓ. Observation of the graph of the daily mean values shows that the general pattern is for the results to fall within 1 s.d. of the expected mean, with only occasional results outside these limits. However, the same values replotted as a cusum reveal a definite trend upwards for the centre part of the observed period, which was found to be caused by a faulty spectrophotometer bulb. The cusum shows that after replacing the bulb the trend was corrected during the latter part of the test period.

Non-analytical errors

It has been shown that a considerable number of non-analytical errors occur even in well regulated laboratories (McSwiney and Woodrow, 1969). These can only be minimized by attention to detail

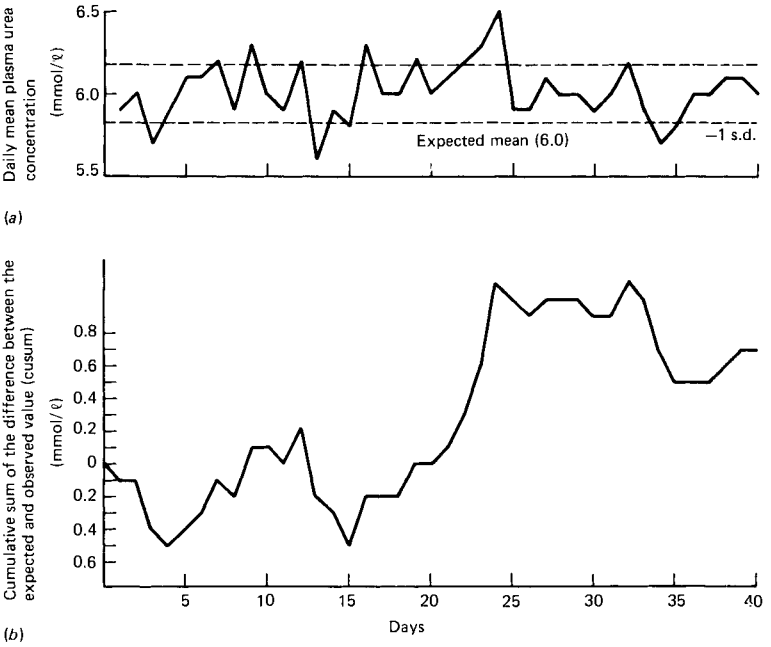


Figure 7.3 The assessment of trends in the statistical values produced by a laboratory. (*a*) Levy–Jennings curve of the daily mean values of plasma sodium concentration. (*b*) Cumulative sum (cusum) of the differences between the observed and expected daily mean values

and careful cross-checking. The commonest serious non-analytical errors are clerical, caused through misreading or transposing figures. It is important to remember that this type of error, if undetected, can have more serious consequences for the patient than an analytical error, as with modern methods of analysis and control these are seldom gross. On-line methods of computerized reporting may do much to help, but information still has to be put into the computer by a human operator at some stage, and computer programmes themselves are devised by man.

Quite misleading results can be produced by analysing specimens which have been incorrectly collected or stored. The reception, storage and preparation of specimens for analysis is far from being the most exciting aspect of clinical chemistry, but it is certainly the most important. Without a check on the identity, time of collection and type of the sample, and its subsequent correct treatment and

storage, there is little to be gained, and often much to be lost, from even the most sophisticated analytical procedure.

Work simplification and mechanization

The large increase in demand for laboratory investigations has led to increasing mechanization and automation of routine analytical techniques.

Despite their apparent complexity, many of the instruments are basically fairly simple, and use the same chemical reactions as manual techniques. The mechanical and electronic design, however, is often extremely complex and beyond the scope of this book. Apart from speeding up the work, modern instruments usually allow better quality control than manual methods because of their improved precision in pipetting etc. Well-maintained machines do not tire, but continue to produce consistent results throughout the day and night. It is well known that human efficiency fluctuates throughout the day, reaching a particularly low ebb during the latter part of the afternoon.

Many instruments for spectrophotometric analysis simply dilute samples and reagents, and compare the resulting absorbances at predetermined time intervals against similarly treated standards. Often all the relevant data can be fed into a microprocessor, obviating the need for further calculation and avoiding possible operator error. As the basic concept of analysis is the same as in manual determinations, these instruments are not described further. Those described below have been selected to illustrate different concepts in analytical technique. They are not necessarily the most modern and up-to-date versions available, but the basic principle illustrated still applies to their more sophisticated successors.

The Technicon 'Autoanalyser' system

This is a continuous flow system of analysis, and in its simplest form consists of a series of linked modules through which samples, standards and reagents flow in a continuous stream. The reactions are not necessarily taken to completion, but the time taken for both standards and test solutions to complete the circuit is identical, and a direct comparison of the results of standards and test samples is therefore permissible. Most chemical methods employed in this system are designed to produce a coloured complex for spectropho-

tometric assay, but flame emission spectrophotometry, ion-selective electrodes and fluorimetry have all been used successfully.

The sample consists of a circular plate with small plastic cups holding the samples and standards. This rotates past a post holding a sample probe which dips alternately into the passing samples or standards and a wash solution. The length of time the probe spends in each solution is controlled by a cam which allows a variation in the sample:wash ratio.

The master unit of the system is the proportioning pump. This consists of manifold pump tubes stretched over a series of rollers and held in position by plastic end blocks. The tubes are of different known internal diameters, allowing variation of the flow rate between 0.05 and 3.90 ml per minute. A heavy platten is clamped over the tubes, against which they are pressed by the action of the rollers, thus pushing forward their contents by peristaltic action.

A special feature of this system is air segmentation. As the diluted samples pass along the tube, a small amount of liquid tends to adhere to the inner surfaces. If allowed to continue, this would contaminate subsequent samples. By the introduction of a small air bubble at regular intervals this layer of liquid is pushed along the tube, which is effectively 'scoured' by the air bubble. Having introduced air into the stream, however, it is essential to remove it before the stream enters the colorimeter flow cell to be read. This is done by careful adjustment of the volume allowed into the flow cell and by placing a 'debubbler' immediately before entry of the stream into the flow cell. All the bubbles and some of the liquid pass upwards through the debubbler, while the remainder of the stream is drawn through the flow cell.

Mixing of specimens, diluent and reagents is done in glass coils whose alternately ascending and descending limbs mix their contents as they rise and fall throughout the length of the coil. Protein interferes with many analyses, and in this system it is removed by dialysis across a cuprophane membrane, the donor and recipient streams flowing in concurrent directions (*Figure 7.4*). The emergent donor stream containing the protein is either allowed to run to waste or may be used in a second determination in which it does not interfere. The recipient stream passes on to the next stage of the analysis. Further reagents, if required, may be introduced into the main stream at this point. If heat or delay is necessary for the reaction the stream is passed through a very long glass coil which may, if necessary, be heated to the required temperature.

The colorimeter has both test and reference photocells to counteract fluctuations, and interference filters to select light of a very narrow band pass width. The differences in electrical output between

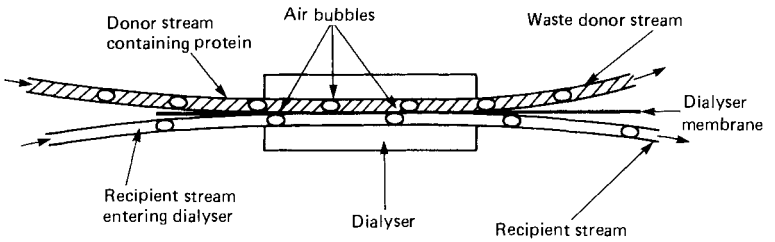


Figure 7.4 The principle of dialysis to remove protein, as used in the autoanalyser (Technicon). The recipient stream leaving the dialyser contains protein-free dialysis residue and dialysed small molecular weight substances for analysis

the test and reference photocells are fed into a recorder which charts them as a series of peaks and troughs (*Figure 7.5*). A direct comparison between those produced by the standard and those produced by the specimen gives the quantitative results.

This was the first large-scale automated system introduced into clinical chemistry and its advent brought about a marked change in

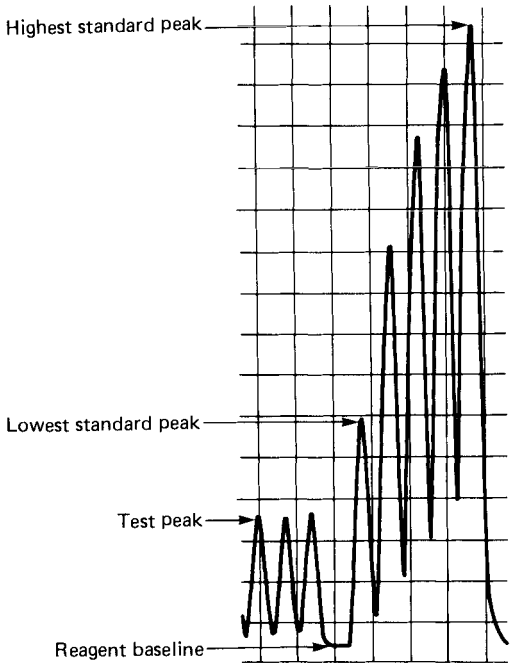


Figure 7.5 Section of the recorder chart produced by the Autoanalyser (Technicon) from blood glucose determinations (*see text for details*)

the pattern of work by allowing increased throughput with improved accuracy and precision. To many laboratories even this now seems slow, but the Autoanalyser can now cope with multiple analyses at much faster rates than the original system.

The sequential multiple analyser (SMA) was developed from the above system. In the early models up to 12 channels were linked together, each with its own analytical cartridge fixed to the main console of the machine. The sample is split by plastic stream splitters, allowing each fraction to enter a different cartridge, or, if only very small samples are required, allowing a fraction of a prediluted sample to be taken. Air segmentation is critical and is controlled by an air bar fitted to the proportioning pumps. The resultant final solutions are fed into the colorimeters, which are designed to convert the readings into signals directly proportional to the concentration of the analyte. The charts are calibrated in units ready for reporting, and only a small portion of the plateau peak at the steady state is printed (*Figure 7.6*). The larger instruments have an oscilloscope which shows all the peaks being formed on all channels. The timing is altered by changing a series of phasing coils, which have known delay times. Careful selection of the appropriate coil ensures that the solution reaches the colorimeter only when the steady state has been reached. The SMAC member of the team (*Figure 7.7*) is computer controlled and does not require manual phasing, uses much smaller sample volumes, thus allowing more determinations to be performed simultaneously at a faster rate and does not require the removal of the air bubbles before the solution enters the colorimeter.

The Technicon RA 1000 system is a discrete analyser using a very small sample volume and eliminates carry-over problems. This is made possible by coating the internal surfaces of both reagent and sample probes with a fluorohydrocarbon 'random access fluid' which is immiscible with sample and reagents, and is inert. After inspiration of the sample into the probe, it is ejected into the sample cup by an air bubble pushing the sample out of the probe encapsulated in a film of random access fluid, which falls to the bottom of the sample cup. In a similar manner the reagents are dispensed, and neither sample nor reagents come into direct contact with the walls of the probes, thus completely eliminating carry-over and cross contamination.

The Vickers Multichannel 300 system

In contrast to the continuous flow system of the Technicon autoana-

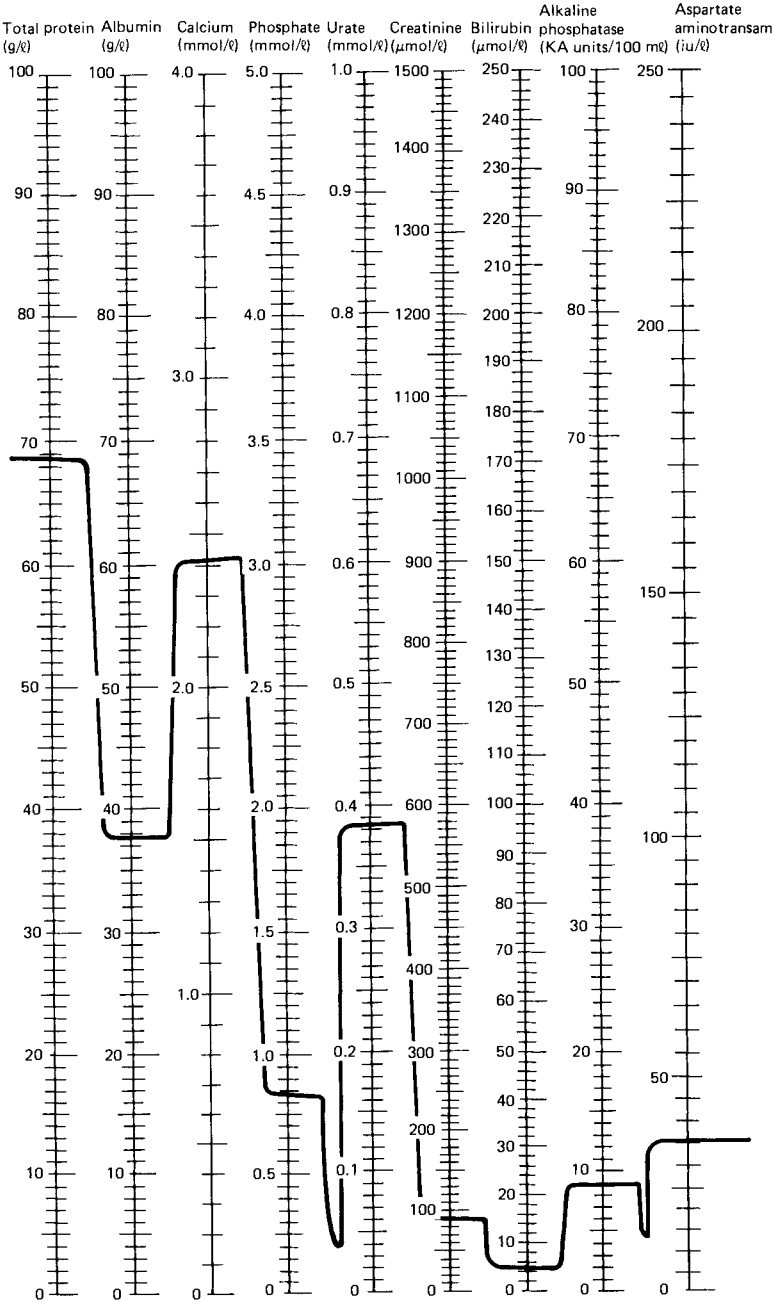


Figure 7.6 Section of a sequential multiple analyser (SMA 12/60) chart

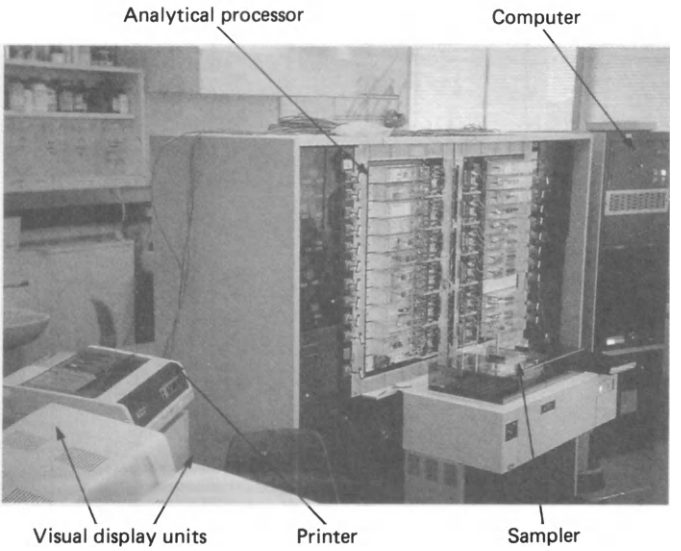


Figure 7.7 The SMAC (Technicon) – a computer controlled sequential multiple analyser

lyser this is a discrete analytical system for the simultaneous determination of multiple analyses.

The instrument (*Figure 7.8*) consists of four main units:

1. The main console, responsible for accepting, diluting and identifying specimens.
2. The reaction rotor units, each responsible for the determination of one or more tests.
3. The distributor, which delivers the primary specimen dilution to each reaction console and also houses the laundry which cleans and dries the primary dilution cups.
4. The process controller, which collates the information from the photometers and address reader, and monitors the entire system.

The major stages of the analytical cycle are shown in *Figure 7.9*. Specimens are collected and centrifuged in special bar-coded vials which identify each specimen. These are loaded into magazines from which they enter the analytical system at a sample rate of 300 per hour. A primary dilution is made into a cup on the distributor returning from the laundry unit. This cup then passes behind each reaction console in turn, at which a secondary transfer dilutor takes

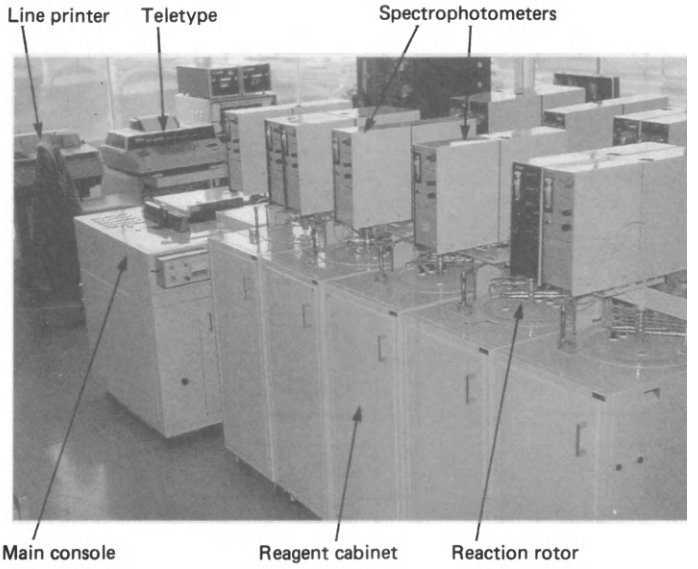


Figure 7.8 The MC 300 (Vickers) multichannel analyser

an aliquot from the cup into a small sample cup recessed into the temperature-controlled head of the reaction console. Appropriate reagents are then added at predetermined intervals and the reaction allowed to proceed. The final stage is the transfer of the reaction mixture from the reaction cup into the photometer. The arrival of the reaction mixture in the photometer is timed to coincide with that of the specimen vial in the address reader on the main console. The computer correlates the address and test results, which are then printed out by a line printer.

The entire system is controlled by a computer which is also able to perform statistical analysis of the results for management purposes. The instrument is standardized by means of comparative readings from control 'standards' introduced into the system at regular intervals and by calibration of the photometers at the beginning of each day. The instrument cannot be standardized by the use of primary standards.

The system is discrete, extremely fast, and ideal for large batches of analyses. It is not suitable for small-batch analysis, nor is it possible to select a particular analytical programme. A high degree of technical skill is required for its operation as it has a large number of mechanical parts which require fastidious attention.

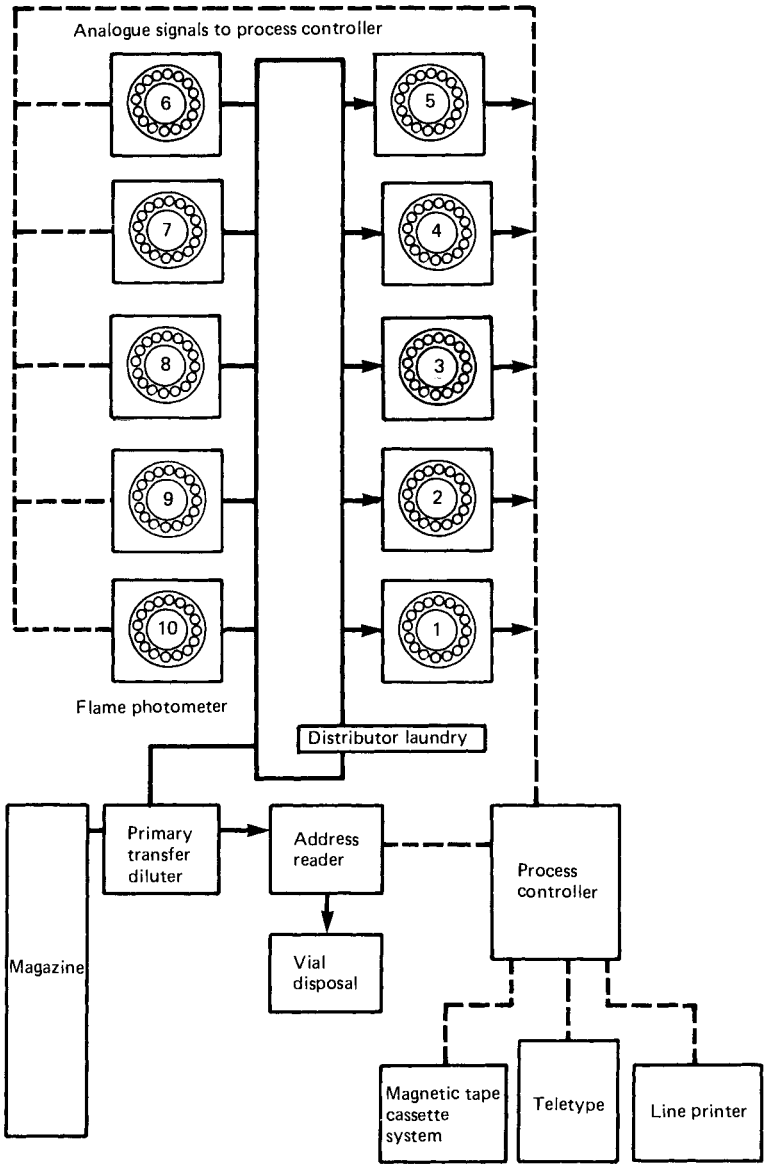


Figure 7.9 The MC 300 analyser: flow diagram showing the major stages in the analytical cycle

Rotary centrifugal analysis

This form of analysis provides a rapid, discrete means of analysing medium-sized batches of a single constituent. It is simple to operate and easily changed from one determination to another in a few seconds. The instrument consists of three main components:

1. A pipettor unit
2. An analytical unit
3. A computer unit

The system described here is the Centrifichem 400 (Union Carbide), but the same principle applies to other systems of rotary centrifugal analysis. The pipettor unit is shown in *Figure 7.10*. Standard solutions are placed in predetermined positions on the sample tray and the specimens in the remainder. A transfer disc (*Figure 7.11*) is placed in the centre of the sample tray, and the unit then delivers sample (or standard) to the outer wells of the disc and reagent to the inner wells. As the wells are separated there is no mixing of sample and reagent at this stage. The loaded transfer disc is placed on the rotor (*Figure 7.12*) of the analytical unit and locked and sealed in position. The rotor is ringed by optical cuvettes which are in close contact with the transfer passage on the transfer disc so

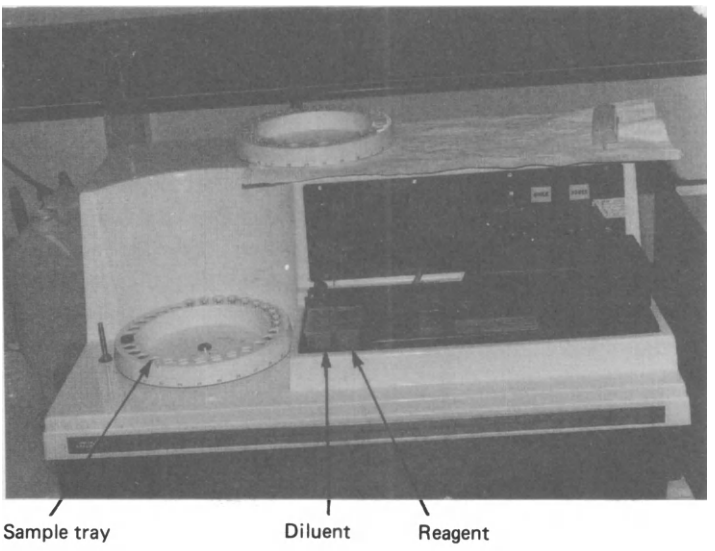


Figure 7.10 The Centrifichem 400 (Union Carbide) rotary centrifugal analyser: pipettor unit

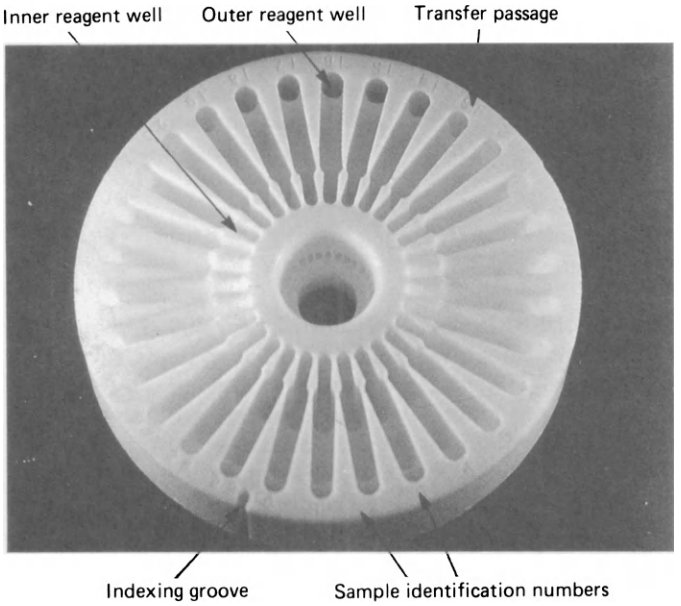


Figure 7.11 Centrifichem 400: transfer disc (*see text for details*)

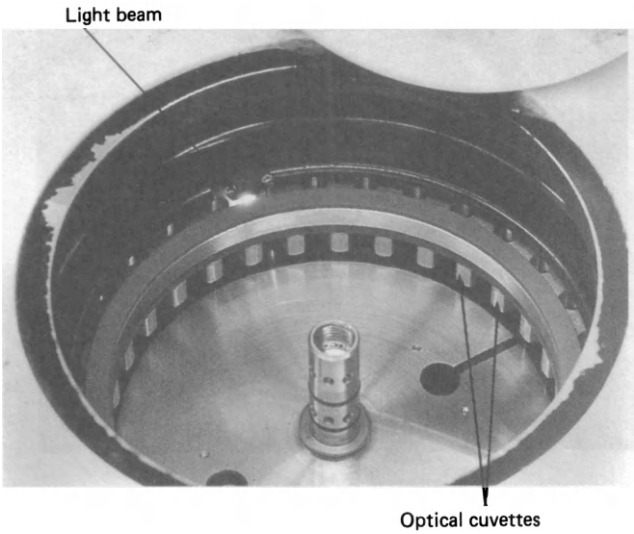


Figure 7.12 Centrifichem 400: analytical unit rotor

that when the centrifuge is operated both reagent and sample, from the inner and outer wells respectively, pass through the transfer passage into the cuvettes lining the rotor, where they are mixed. Rapid and complete mixing is achieved by giving a short burst of air into the cuvette during the first few seconds of centrifugation. The cuvettes have optical surfaces on both the top and bottom. As they rotate, they are monitored by a light beam which passes through them to a selected interference filter and then to a photomultiplier tube. In this way an absorbance is obtained from all cuvettes on each revolution.

For a predetermined time interval a mean of eight readings is taken and compared to those from the standards occupying preset positions on the transfer disc (whose concentrations have been entered into the computer unit), and the results are then printed.

For kinetic enzyme determinations readings are taken at predetermined time intervals, and the rate of increase or decrease in absorbance per unit time is measured and converted by the computer into the appropriate units of enzyme activity.

The Automatic Clinical Analyser (Du Pont)

This instrument provides a discrete system for single or multiple analysis of single or small batches of specimens by the simple addition of a test pack appropriate for the required determination. It is not designed, nor is it suitable, for very large numbers of specimens, for which it would be both too slow and too costly. It is useful in large laboratories for paediatric and emergency work which may not be able to be processed with the main batches of work, and in small laboratories where the total work load may be small but the repertoire wide. Its virtue is that only one analytical instrument is required, that it requires no initial 'warm up' period, and that the operational procedure is the same for all tests. In addition, it requires only a single operator. Apart from the standardization, the system is entirely out of the operator's control. Reagents cannot be prepared in the testing laboratory, as they are sealed into the test packs.

The specimen is placed in a small plastic cup from which the patient identity card containing any information required by the laboratory is suspended (*Figure 7.13*). This is supported by the rails of the input tray. Behind this are suspended (*Figure 7.14*) the appropriate analytical test packs which are optically clear plastic envelopes containing pockets for up to seven different reagents which may be required for the test. The test packs have an integral coded perspex header containing up to two sample filling apertures.

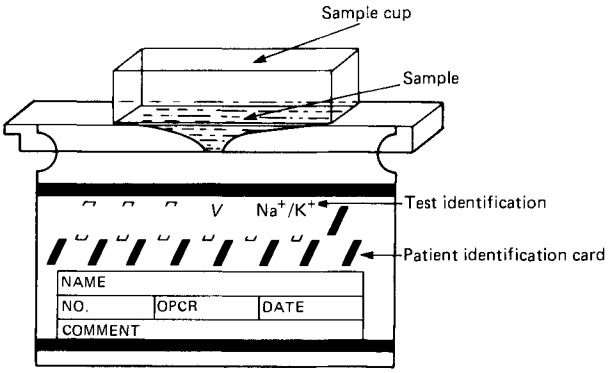


Figure 7.13 Automatic Clinical Analyser (ACA; Du Pont): sample kit

The header may also contain a chromatographic column to remove interfering substances. Protein is removed by passing the specimen through a column of porous glass beads coated with protein-precipitating reagents. Gel filtration and ion-exchange columns are also used. Following the last test pack an empty sample kit signals to the microprocessor that no further tests are required for that specimen.

On activating the instrument, the sample kit is pushed into the shuttle, and then to the left. Ultraviolet lamps transfer the information from the patient identification card to photosensitive paper.

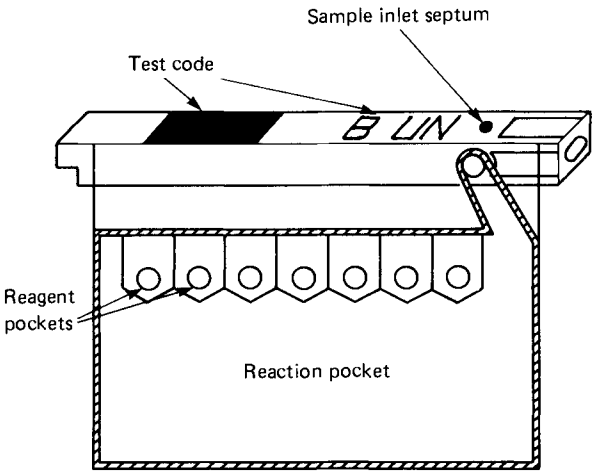


Figure 7.14 The ACA: analytical test pack

The following analytical test pack then enters the shuttle and the controller decodes the information on the header and allows the correct volumes of specimen and buffered diluent to be delivered by the filling station needle through the apertures. The test kit is then attached to a supporting pin on the transport chain and passes to the right into the temperature chamber, where a preheater raises the temperature of the pack to 37 °C. Any reagents contained in the first four pockets are then mixed with the diluted specimen in the plastic envelope by the first breaker–mixer. A series of in-built delay stations allows incubation of the reagent mixture should this be required for the reaction to proceed. A second breaker–mixer then permits the addition of any reagents from the last three pockets in the test pack. The pack finally passes into the photometer, where the resulting absorbance is read. The microprocessor compares this to the standard information previously fed into the instrument, and converts the reading into appropriate units which are printed below the patient identification details transferred earlier to light sensitive paper. After the last test result has been printed the paper is cut and delivered via the exit tray. The operational diagram for the system is shown in *Figure 7.15*.

The Kodak Ektacheme 400 system

This system, which can handle up to 500 tests per hour, is based on a dry slide technique of analysis. The slides are sandwiched, as shown

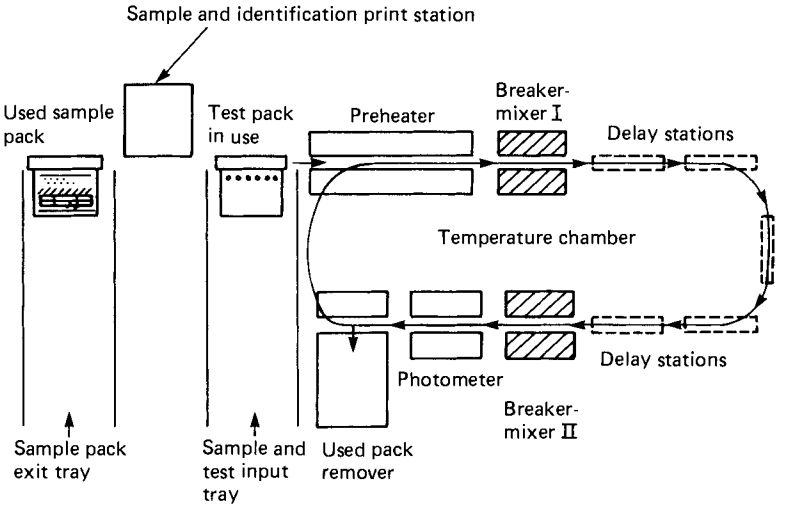


Figure 7.15 The ACA: operational diagram

in *Figure 7.16* for urea analysis, and consist of a 'spreading' layer, which evenly distributes the sample across the surface, and reagent layers, which contain the necessary reagents for the analysis. Interfering protein is removed by the introduction of a semipermeable membrane between the reagent layers to prevent the passage of large molecular weight substances from one layer to the next. Titanium dioxide is added to the spreading layer to enhance the reflectance obtained in colour reactions. The final colour produced is measured by reflectance densitometry. The outer layer of the sandwich is a clear plastic support.

The system can be used for spectrophotometric and electrometric determinations. Bar-coded cartridges containing the slides are housed in carousels until required. They are selected by the monitor and incubated after the addition of a 10 µl sample. The incubation period is five minutes for colorimetric methods and three minutes for electrometric methods. The slides are then fed into the appropriate measuring unit, and the results printed automatically.

The introduction of new methods

The clinical value of the results being sent out from the laboratory needs to be regularly reassessed. As new instruments become

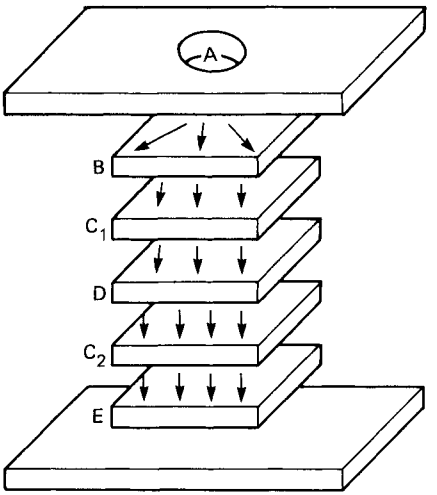


Figure 7.16 Kodak Ektacheme system: dry slide for urea analysis. A = aperture; B = spreading layer; C = reagent layers (C_1 = urease; C_2 = indicator); D = semipermeable membrane; E = plastic support

available and medical and surgical techniques advance, the repertoire of the laboratory may have to be adjusted by either adding new tests or discarding those no longer required. The introduction or removal of a method, however, should only be undertaken after careful consideration of the following points:

1. Clinical validity.
2. Technical merit.
3. Accuracy and precision.
4. Cost effectiveness.
5. Social acceptability and safety.

Clinical validity

The clinical value of performing a particular test depends on the ability to interpret the test result, and on its bearing on the diagnosis or treatment of the patient.

Technical merit

The following questions need to be answered before introducing a new technique into the routine of a laboratory:

1. Is the test easy to perform?
2. Is it time-consuming compared to other methods available?
3. Is additional equipment required?
4. Is special expertise required to perform the test?
5. Are the specimen requirements advantageous?
6. What substances interfere with the determination?
7. Are the reagents stable and easy to prepare?
8. Does the method give good correlation with the reference method?
9. Can the method be easily and accurately standardized?

Accuracy and precision

It is important to know the variation both within a given batch and between different batches of the same determination. This can be done either by replicate analysis or by retaining specimens from one analytical batch and their subsequent repeat analysis on a later batch.

The accuracy must be assessed by comparison with a reference method. Particularly with extraction techniques, recovery experiments should be performed in order to assess the effectiveness of each stage in the procedure.

Cost effectiveness

The following questions need to be considered:

1. Is the new method cheaper when compared to other methods?
2. Could initial installation costs be offset by using the equipment for any other tests?
3. Could the method save on labour costs?

Social acceptability and safety

Is the method safe for both patient and laboratory worker? Does it create any unnecessary hazard(s) e.g. pollution, toxic fumes, radiation?

The establishment of a reference range

If the reference range for the new test is not known, this must be established before the test can be introduced for routine use. Results may differ, for example, with age, sex, diet, diurnal variation, race, occupation, environment and even altitude. The greater the number of subjects tested to obtain the reference range(s), the more likely they are to be reliable. The generally accepted 'normal' or reference range is the 95 per cent confidence limit, or the mean ± 2 s.d. In order to calculate this, *all* values obtained should be included in the initial calculations. Those deviating more than ± 3 s.d. from the mean are then excluded (a process known as truncation) and the remainder used to recalculate both the mean and 2 s.d.

References

ALBEP, J. J., WARWICK, G. R., WREBE, D. *et al.* (1978) *Clinical Chemistry*, **24,** 853

ARKESTERJN, C. L. M. (1976) *Journal of Clinical Chemistry and Biochemistry*, **15,** 155

BABSON, A. L. (1963) *Clinical Chemistry*, **11,** 789

BESSEY, O. A., LOWRY, O. H. and BROCK, M. J. (1946) *Journal of Biological Chemistry*, **164,** 321

BODANSKI, A. (1933) *Journal of Biological Chemistry*, **101,** 93

BROUGHTON, P. M. G. (1956) *Biochemical Journal*, **63,** 207

BROWN, H. (1945) *Journal of Biological Chemistry*, **158,** 601

BUCOLO, G. and DAVID, H. (1973) *Clinical Chemistry*, **19,** 476

CALI, J. P., MANDEL, J., MOORE, L. and YOUNG, D. S. (1972) *National Bureau of Standards Special Publication*, 260

DIEHL, H. and ELLINGSHOE, J. (1956) *Analytical Chemistry*, **28,** 822

ENNOR, A. H. and ROSENBERG, H. (1954) *Biochemical Journal*, **57,** 203

FISKE, C. H. and SUBBAROW, Y. (1925) *Journal of Biological Chemistry*, **66,** 375

GINDLER, E. M. and KING, J. D. (1972) *American Journal of Clinical Pathology*, **58,** 376

GITELMAN, H. J. (1967) *Analytical Biochemistry*, **18,** 521

GLYNN, J. P. and KENDAL, S. E. (1975) *Lancet*, **1,** 1147

GREENBERG, D. M. (1929) *Journal of Biological Chemistry*, **82,** 545

HOCK, H. and MARRACK, J. (1945) *British Medical Journal*, **2,** 151

JENDRASSIK, L. and GROF, P. (1938) *Biochemische Zeitschrift*, **297,** 81

KARMEN, A. (1953) *Journal of Clinical Investigation*, **34,** 131

KERR, F., PATEL, A. R., SCOTT, P. D. R. and TOMPSETT, S. L. (1968) *British Medical Journal*, **3,** 291

KIND, P. R. N. and KING, E. J. (1954) *Journal of Clinical Pathology*, **7,** 322

KING, E. J. and ARMSTRONG, A. R. (1934) *Canadian Medical Association Journal*, **31,** 376

KLEIN, B., FOREMAN, J. A. and SEARCY, R. L. (1970) *Clinical Chemistry*, **16,** 32

LEVINE, J. B. and ZAK, B. (1964) *Clinica Chimica Acta*, **10,** 381

LIDDLE, L., SEEGMILLER, J. E. and LESTER, L. (1959) *Journal of Laboratory and Clinical Medicine*, **54,** 903

LIM, F. (1974) *Clinical Chemistry*, **20,** 871 (Abstract 088)

LIN, T. Z. and OKA, K. H. (1980) *Clinical Chemistry*, **26,** 69

LUBRAN, M. A. (1961) *Clinica Chimica Acta*, **6,** 594

MALLOY, H. T. and EVELYN, K. A. (1937) *Journal of Biological Chemistry*, **119,** 481

MANCINI, G. A., CARBONARA, A. O. and HEREMANS, J. F. (1965) *Immunochemistry*, **2,** 235

MCSWINEY, R. R. and WOODROW, D. A. (1968) *Journal of Medical Laboratory Technology*, **26,** 340

MOORE, N. S. and VAN SLYKE, D. D. (1930) *Journal of Clinical Investigation*, **8,** 337

MORGENSTERN, S. and KLEIN, B. (1966) *Clinical Chemistry*, **12,** 748

MOSS, G. A., BONDAR, R. J. L. and BUZZELLI, D. M. (1974) *Clinical Chemistry*, **20,** 871 (Abstract 089)

NORTHAM, B. E. and WIDDOWSON, G. M. (1967) *Technical Bulletin No. 11.* Association of Clinical Biochemists

O'DONNELL, M. D., FITZGERALD, O. and MCGEENEY, K. F. (1977) *Clinical Chemistry*, **23,** 560

PASSEY, R. B., GILLUM, R. L., FULLER, J. B., URRY, F. M. and GILES, M. L. (1977) *Clinical Chemistry*, **23,** 131

PINNELL, A. E. and NORTHAM, B. E. (1978) *Clinical Chemistry*, **24,** 80

PISANO, J. J., GROUT, J. R. and ABRAHAM, D. (1962) *Clinica Chimica Acta*, **7,** 285

PRICE, C. P., HAMMOND, P. M. and SCAWEN, M. D. (1983) *Clinical Chemistry*, **29,** 358

REINHOLD, J. G. (1953) *Standard Methods of Clinical Chemistry*, edited by M. Reiner, p. 188. New York: Academic Press

REITMAN, S. and FRANKEL, S. (1957) *American Journal of Clinical Pathology*, **28,** 56

ROSALSKI, S. B. (1967) *Journal of Laboratory and Clinical Medicine*, **69,** 696

ROSALSKI, S. B. and WILKINSON, J. H. (1964) *Journal of the American Medical Association*, **189,** 61

SANDLER, M. and RUTHVEN, C. R. J. (1961) *Biochemical Journal*, **80,** 78

SAX, S. M. and MOORE, J. J. (1967) *Clinical Chemistry*, **13,** 175

SCHMIDT, F. J. (1961) *Klinische Wochenschrift*, **40,** 585

SIBLEY, J. A. and FLEISHER, G. A. (1954) *Mayo Clinic Proceedings*, **29,** 591

STEIN, W., MILDNER, I. and MAULBETSCH, R. J. (1978) *Journal of Clinical Chemistry and Biochemistry*, **16,** 225

SZASZ, G. A. (1969) *Clinical Chemistry*, **15,** 124

TRINDER, P. (1954) *Biochemical Journal*, **57,** 301

TRINDER, P. (1969a) *Annals of Clinical Biochemistry*, **6,** 24

TRINDER, P. (1969b) *Journal of Clinical Pathology*, **22,** 246

WARBURG, O. and CHRISTIAN, W. (1943) *Biochemische Zeitshrift*, **314,** 149

WATSON, D. (1960) *Clinica Chimica Acta*, **5,** 637

WROBLEWSKI, F. and LA DUE, J. S. (1955) *Proceedings of the Society for Experimental Biology and Medicine*, **90,** 210

WROBLEWSKI, F. and LA DUE, J. S. (1956) *Proceedings of the Society for Experimental Biology and Medicine*, **91,** 569

Index