BIOMEDICAL ANALYTICAL CHEMISTRY

This particular aspect of analytical chemistry is the outcome of the unique amalgamation of the principles and techniques of analytical chemistry and biochemistry and was initially termed as '*clinical chemistry*' but is more recently and more descriptively known as '**biomedical analytical chemistry**'.

Presently, both serum and urine assays are being used extensively in diagnostic medicine which evidently signifies that the pharmacist of today should be fully conversant with the ever-increasingly important techniques of biomedical analytical chemistry. It is, however, necessary to make a passing reference to microbiological assays and haematological assays, also being carried out in a clinical laboratory, though it should not be treated under this topic since these methods are outside the scope of biomedical analytical chemistry.

Classical example of SGOT-PAS episodes : Patients suffering from tuberculosis (TB) when diagnosed with *para*-aminosalicylic acid (PAS) invariably showed elevated serum levels of the intracellular enzyme serum-glutamic-oxaloacetic-transaminase (SGOT) which was initially considered and treated as a drug-induced hepatic toxicity. Later, an extensive and intensive studies revealed this to be an absolutely false diagnosis. In fact, the apparent enhanced SGOT levels were actually caused on account of the interference of PAS in the SGOT assay.

In the same vein, such analytical and biochemical interferences with respect to drug interference in various biomedical assays are being profusely cited in current scientific and research journals, such as the American Journal of Hospital Pharmacy and Clinical Chemistry.

It has been established beyond any doubt that analytical interferences can only take place when a drug or its resulting metabolite happens to interfere with the analytical method adopted for the assay.

In order to have a comprehensive account on the various aspects of 'Biomedical Analytical. Chemistry', we may have to study the following *four* methods of assay with specific emphasis on their principle and applications, namely :

(*a*) Colorimetric Assays,

(*b*) Enzymatic Assays,

(*c*) Radioimmunoassays, and

(*d*) Automated Methods of Clinical Analysis.

COLORIMETRIC ASSAYS

A. Theory : In fact, *two* fundamental laws actually govern the practice of colorimeteric assays of photometry.

First Law : *Bougner's (1729) or Lambert's (1760) Law defines that—"when a beam of monochromatic light, previously rendered plane-parallel, enters an absorbing medium at right angles to the plane-parallel surfaces of the medium, the rate of decrease in radiant power with the length of light path through the absorbing*

medium `b' is directly proportional to the radiant power of the beam, i.e., the light will be diminished in geometric (not arithmetic) or exponential progression".

Alternatively, it may be explained that if a particular thickness absorbs half the light, the thickness which follows the first half and is equal to it will not absorb the entire second half, but instead only half of this half and will consequently reduce it to one-quarter. Thus, we have :

$$-\frac{\partial P}{P} = k\partial b$$

Upon integration and changing to logarithms of base 10, and substituting $P = P_0$ when $b = 0$, we may get :

$2.303 \log (P_0/P) = kb$ ... (*b*)

In other words, the radiant power of the unabsorbed light decreases exponentially as the thickness of the absorbing medium increases arithmetically,

$P = P_0 e^{-kb} = P_0 10^{-0.43\ kb}$...(*c*)

Second Law : *Bernard's (1852) or Beer's (1852) Law defines that—'the radiant power of a beam of parallel monochromatic radiation decreases in a similar manner as the concentration of the light-absorbing constituent increases".* Thus we have :

$2.303 \log (P_0/P) = k' C$ ... (*d*)

where, C = concentration of substance, and

k' = constant of proportionality.

Therefore, from Eq. (*b*) and Eq. (*d*), the two Laws may be combined and expressed with a single constant as follows :

$\log (P_0/P) = abc$ ... (*e*)

or $P = P_0 10^{-abc}$ ... (*f*)

where, *a* = absorptivity constant*.

[* **and not to be tenned as absorbancy index, extinction coeffcient or specific extinction.**]

In fact, the absorptivity constant '*a*' is dependent upon the wavelength of the radiation as well as the nature of the absorbing material, whose concentration 'C' is usually expressed in grams per litre.

Molar Absorptivity (∈) : It is the product of the molecular weight of the substance and its absorptivity and is designated by the symbol ∈.

Beer's Law (or Beer-Lambert's Law) : The combined law is invariably referred to as '**Beer's Law**', while some texts refer to this as '*Beer-Lambert's Law*'.

Eq. (*f*) is mostly expressed as shown below :

$A = abc$ ...(*g*)

where, A = absorbance,

a = absorptivity,

b = optical path length, and

c = analyte concentration.

The term $A^{1\%}_{1cm}$ designates the absorbance of a 1 cm layer of solution that essentially contains 1% by weight of absorbing solute.

It is pertinent to mention here that most of the pure pharmaceutical substances (RS) do possess a definite characteristic absorbance (*i.e.*, $A^{1\%}_{1cn}$) that forms the basis of their assay *vis-a-vis* the unknown sample.

Beer's Plot : *It is a plot of the absorbance value (along Y-axis) against a series of unknown solute concentrations in g/litre (along X-axis) thereby yielding a straight line passing through the origin.*

Therefore, the solute-concentration present in an unknown solution can be estimated conveniently from the **Beer's Plot** or sometimes referred to as the **Standard Curve**, merely by measuring the absorbance value of the solution and then finding the concentration value that corresponds to the measured absorbance value as is illustrated in the following Figure-1.

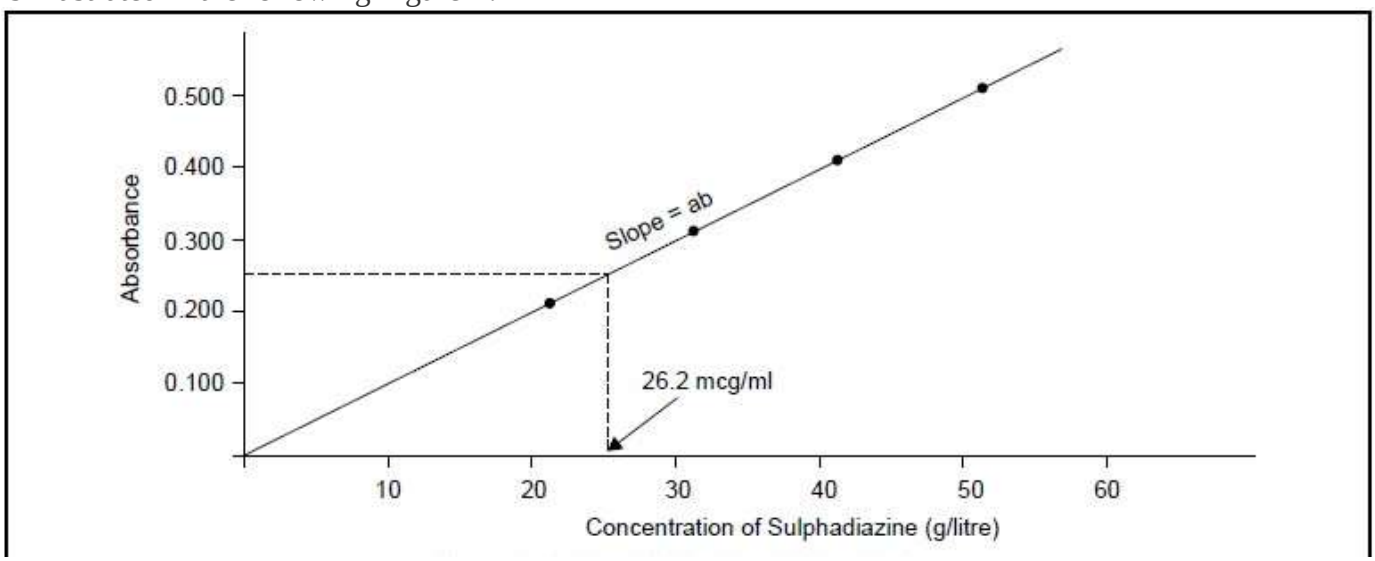
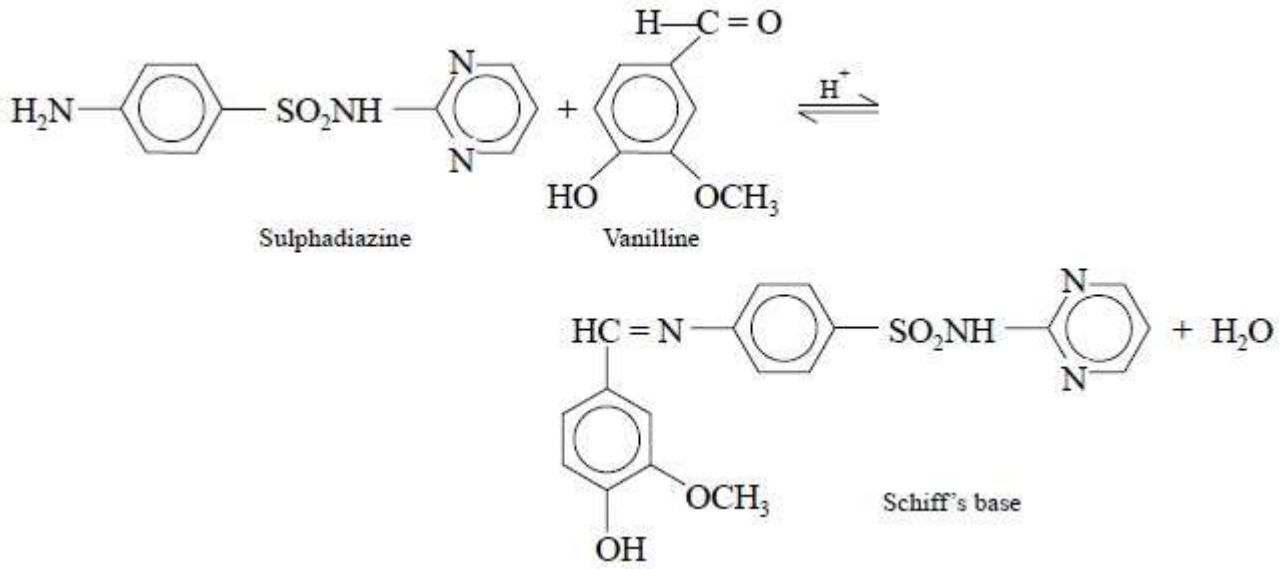


Figure-1

The colorimetric assay of sulphadiazine is based on the **acid-catalysed equilibrium reaction** that occurs between vanillin (an aldehyde) and sulphadiazine (an arylamine). The chemical species that forms as shown below is known as the **Schiff's Base** and is yellow in colour.



Transmittance. The relationship between per cent transmittance and concentration is shown in Figure-2.

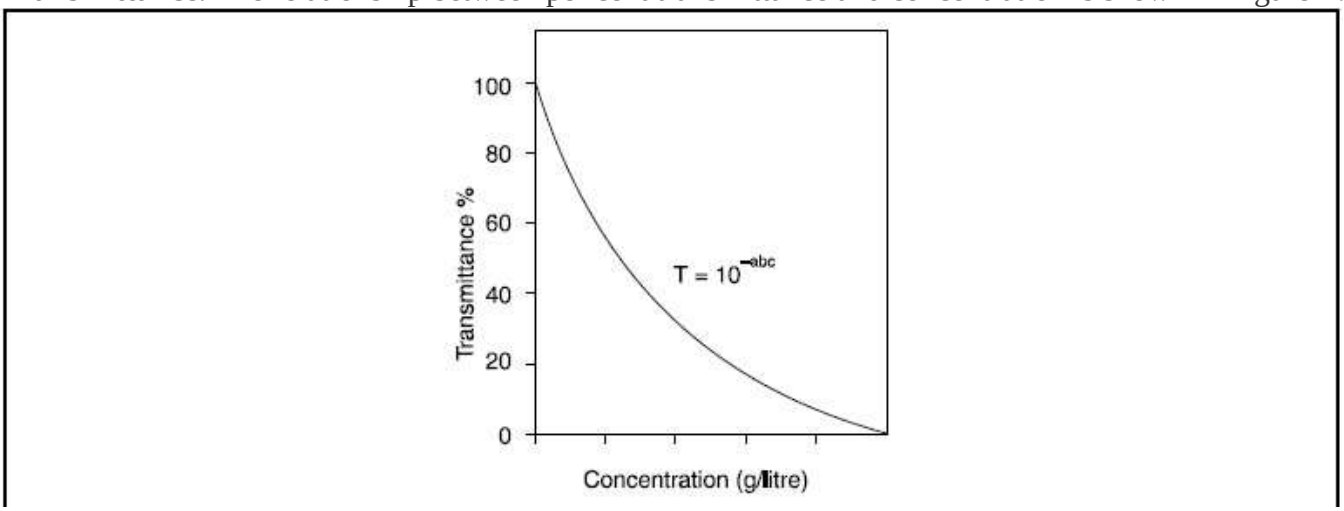


Figure-2

From Figure 2.3, it is quite evident that at lower concentrations the per cent trasmission is high and is *vice varsa* at higher concentrations.

However, a direct relationship between per cent transmittance and absorbance is illustrated in Figure-3.

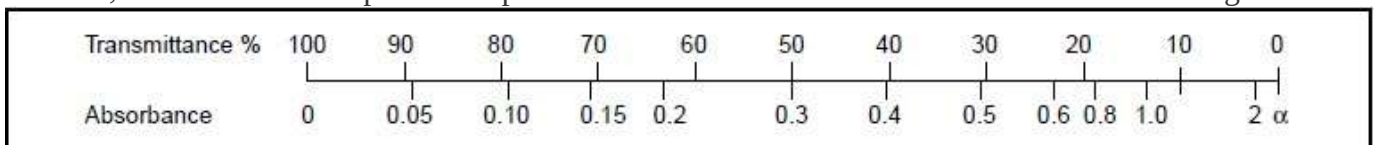


Figure-3

B. Applications in Biomedical Analytical Chemistry Colorimetric assays have a wide spectrum of applications in biomedical analytical chemistry which may be categorized under the following *four* heads, namely :

(*i*) Colorimetric Assays of Biochemicals,

(*ii*) Colorimetric Assays Involving Complexation Reactions,

(*iii*) Colorimetric Assays Involving Redox Reactions, and

(*iv*) Colorimetric Assays of Enzyme Levels.

All these four categories of colorimetric assays shall be discussed briefly with appropriate examples, wherever necessary, to have an indepth knowledge and better understanding of the practical aspects.

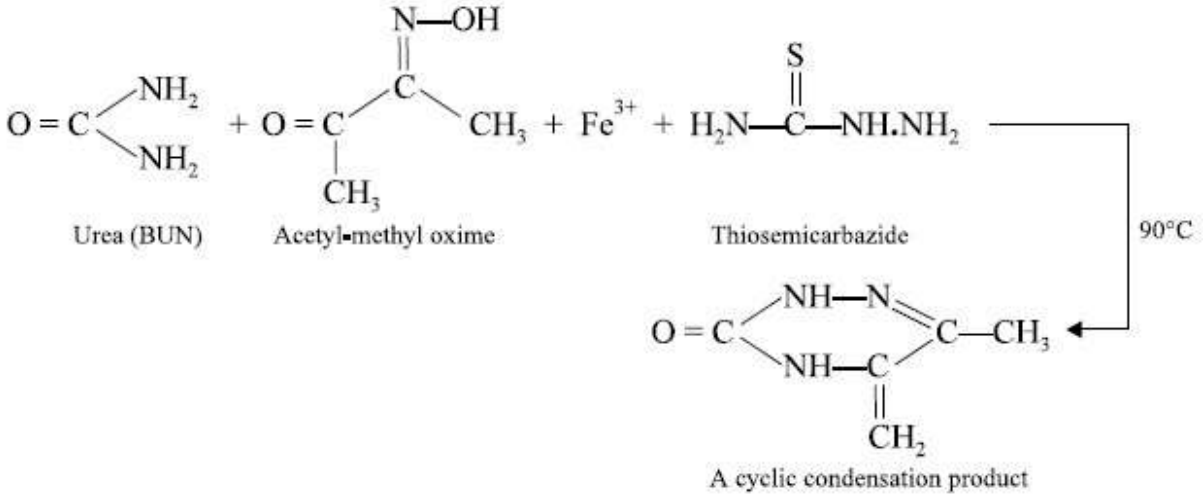
1. Colorimetric Assays of Biochemicals

In this context, the discussion shall be restricted to the colorimetric assays of urea (BUN), bilirubin and cholesterol. However, the clinical significance of these substances and the extent to which they are present in biological fluids; besides the various drugs that usually interfere with their assay are also described adequately in the following pages :

1.1. *Urea (BUN)*

The extent of urea (BUN) present in biological fluids is normally determined in many Auto Analyzers by the following method :

The quantity of substance having an unknown structure is determined at 520 nm spectrophotometrically, while the normal BUN level is determined by averaging the BUN levels of a number of normal subjects.



Profile of BUN-levels

- normal BUN level is 10-15 mg per 100 ml,

- Enhanced BUN levels clearly signify a renal dysfunction, for instance urinary tract obstruction and nephritis *i.e.*, inflammation of the kidney.

- Increased incidence of BUN is also found in subjects suffering from diabetes, hepatic disorders and gastrointestinal disturbances,

- Decreased BUN level is usually indicative of acute hepatic dysfunction and excessive dehydration,

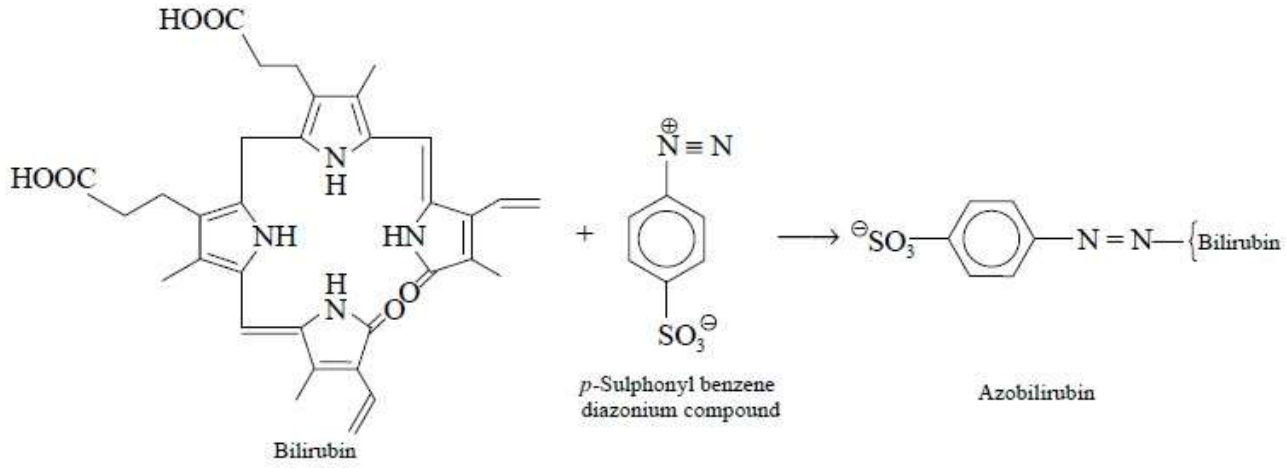
- A few important drugs, namely : thiazide diuretics (*e.g.*, chlorothiazide, hydroflumethiazide, bendroflumethiazide, benzthiazide, cyclothiazide etc.), neomycin, tetracyclines, methyldopa etc., help in enhancing the BUN levels perhaps due to interference with normal renal function,

- Phenothiazines (*e.g.*, promethazine, chlorpromazine, ethopropazine etc.) on the contrary causes a significant decrease in BUN levels due to lowering of urea production from the liver, and

- Substances that are inherently present in the serum and absorb at 520 nm shall interfere with these measurements, and therefore, necessary corrections for these materials have got to be made adequately.

1.2. *Bilirubin*

Bilirubin is diazotized with *para*-sulphonyl benzene diazonium compound and the absorbance of the resulting azobilirubin is measured at 600 nm to determine bilirubin level in the biological fluid *e.g.*, blood serum. In usual practice, a serum blank is run simultaneously by reacting the serum with caffeine, sulphanilic acid and tartaric acid, and the absorbance of the blank is measured at 600 nm which is subsequently subtracted from the azobilirubin absorbance initially obtained before the bilirubin level is finally determined.



Profile of Bilirubin Levels

- Normal bilirubin level ranges between 0-1.5 mg per 100 ml,

- Enhanced bilirubin level may suggest drug toxicity, bile-tract obstruction, hepatitis and hepatic dysfunction,

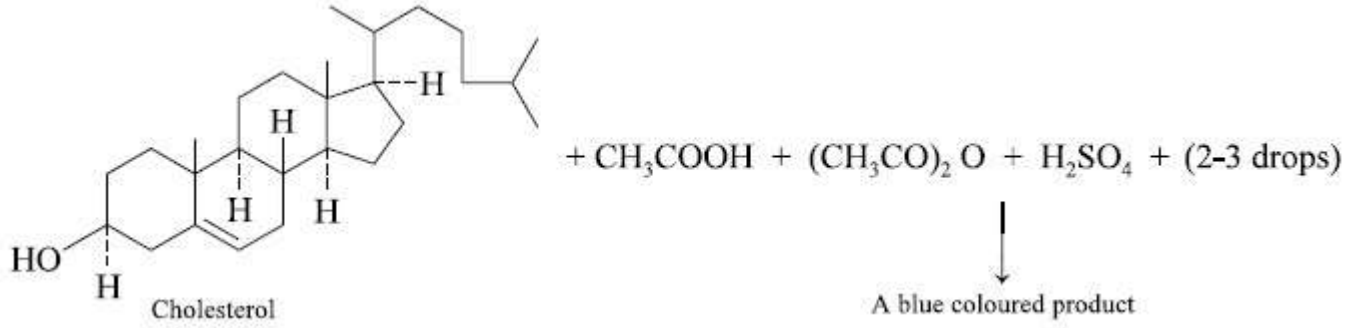
- As normal bilirubin level commences from zero, hence conditions responsible for its decrease are practically non-existent,

- Increased bilirubin levels are caused due to the intake of large doses of such drugs as : chloroquine, vitamin K, sulpha-drugs, tetracyclines, paracetamol, nicotinic acid and monoamine oxidase inhibi-tors (*e.g.*, iproniazid RP 1.0 ; nialamide RP 1.8 ; isocarboxazid RP 3.1 ; phenelzine RP 18 ; pheniprazine RP3l ; and tranylcypromine RP 45), where RP designates the 'Relative Potency' based on the tryptamine potentiation test. The elevated levels are due to hepatic injury, and

- Drugs that interfere with the assay are, namely : (*a*) phenylazopyridine hydrochloride—a coloured drug, (*b*) azo-compound forming medicinals, and (*c*) degradation product of novobiocin.

1.3. *Cholesterol*

Cholesterol interacts with glacial acetic acid and acetic anhydride to result into the formation of a coloured product whose absorption is measured at 630 nm and this is found to be directly proportional to the level of cholesterol present in the serum. The reaction may be expressed as follows :



The above reactions is also referred to as the Libermann's Reaction.

Profile of Cholesterol Levels

- Normal total cholesterol level is 200 mg per 100 ml,
- Increased cholesterol levels in serum are found in patients suffering from chronic hepatitis, atherosclerosis (deposit of fat in arteries of heart) and hypothyroidism,
- Decreased cholesterol levels in serum is indicative of liver ailment and hyperthyroidism,
- Corticosteroids (*i.e.*, steroidal compounds) found in urine that possess biological properties resembling those of adrenal cortical extract, either in the increase or decrease of cholesterols levels,
- Oestrogens, for instance : estrone, estriol, estradiol etc., are found to lower the cholesterol levels,
- The broad-spectrum antibiotic chlorotetracycline and the aminoglycoside antibiotic kanamycin are observed to lower the cholesterol levels by forming salts with bile acids (*e.g.*, cholic acid, deoxycholic acid and chenodeoxycholic acid) in the intestinal canal,
- Likewise, the antoconvulsant phenytoin sodium and neomycin—an aminoglycoside antibiotic also decrease the cholesterol levels, and
- Interestingly, penicillamine—a degradation product of penicillin and phenothiazines—the histamine H_1—receptor antagonists, such as : promethazine teoclate, methadilazine hydrochloride, trimeprazine tartrate are found to increase the cholesterol levels.

ENZYMATIC ASSAYS

A. Theory :

All colorimetric enzymatic assays essentially involve the measurement of the activity of an ezyme under the following *two* circumstances, namely :

(*a*) When substrate is in large excess, and

(*b*) When enzyme concentration is in large excess.

A.1. Substrate Present in Large Excess :

In reality, an enzyme reaction is nothing but a special kind of generalized reaction that may best be expressed as follows :

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} P + E \qquad \text{.....(a)}$$

Where, E = Enzyme,
S = Substrate
ES = Enzyme-substrate complex, and
P = Product.

From Eq. (*a*), we have,

Rate of Product Formation = V_{max} [S]/K_m + [S] ...(*b*)

Where, $K_m = (k_2 + k_3) / k_1$,

V_{max} = Max. rate of reaction

Assuming, [S] to be in large excess [S] >> K_m,

From Eq. (*b*) we have :

Rate of Reaction = V_{max} [S]/[S]

or Rate of Reaction = V_{max} ...(*c*)

Example : In order to measure the activity of an enzyme E, such as creatine phosphokinase (CPK), the concentration of the substrate S, for instance creatine, should be in large excesses so that the products measured shall be in the linear portion of the curve (Part 'A') in Figure-4.

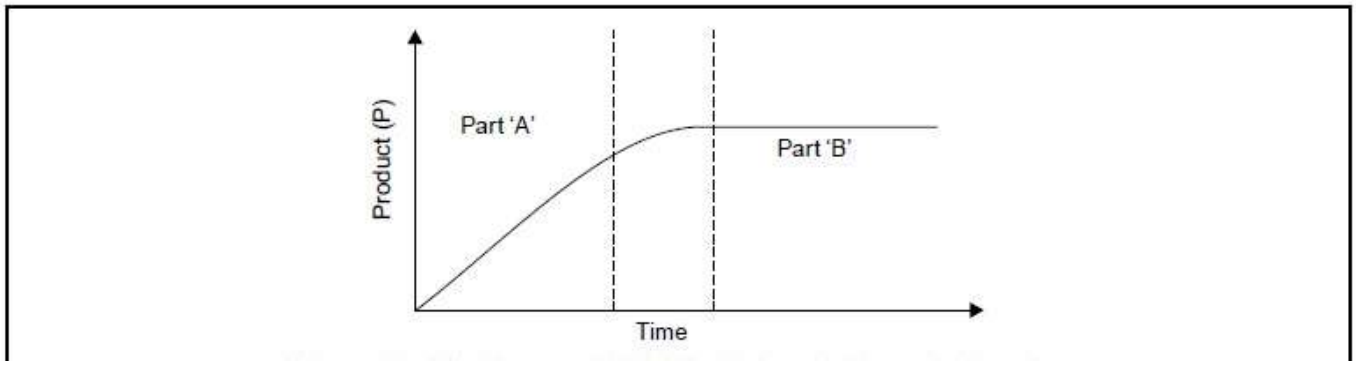


Figure-4

Therefore, with a view to obtaining the best results, the two experimental parameters, namely : the temperature (constant-temperature-water-bath) and the time (phaser) should always be kept constant in order that the rate

of reaction, as determined by the amount of product formed, specially designates the activity of the enzyme under assay, and devoid of the influence of any other variables on the reaction rate.

A.2. Enzyme Concentration in Large Excess

In order to analyze the quantity of substrate (S) present in a biological sample glucose oxidase is added in excess of the actual amount needed for the complete conversion of all the substrate to product ; and to achieve this object the reaction is allowed to run for a fairly long duration (*i.e.*, to complete the reaction). It can be seen evidently in Part 'B' of Figure 2.5, wherein the sepecific reaction time the substrate (S) has been consumed completely and consequently, the concentration of the product achieves a maximum value.

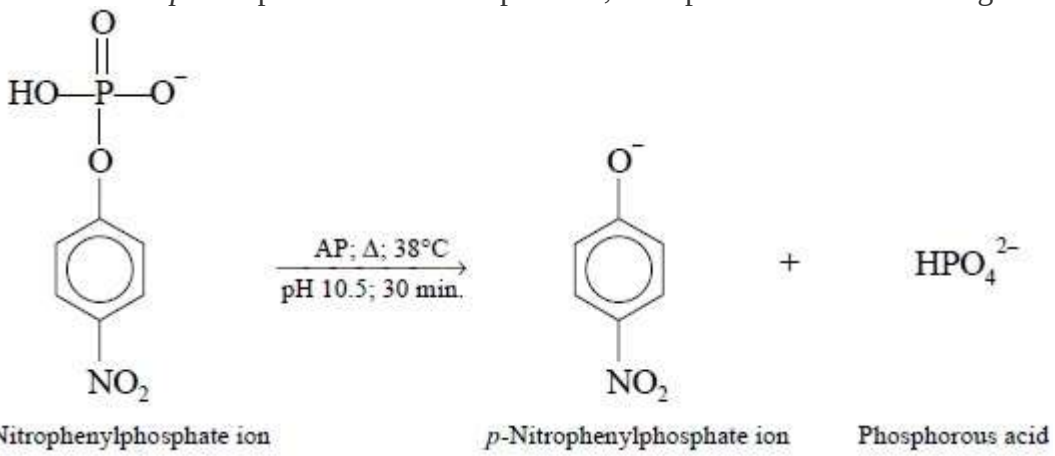
1. Assay Methods

A few typical examples of colorimetric assay of enzyme levels will be discussed briefly hereunder :

1.1. *Alkaline Phosphatase (AP)*

Theory :

Alkaline phosphatase is responsible for the cleavage of O-P bonds. It is found to be relatively non-specific and this characteristic permits the AP level to be assayed based on the fact that *p*-nitrophenylphosphate ion gets converted to *p*-nitrophenolate anion at pH 10.5; as expressed in the following reaction.



In actual practice, *p*-nitrophenylphosphate is present in large excesses, and the reaction is carried out at 38°C for 30 minutes. The resulting amount of *p*-nitrophenolate ion is estimated by the help of an usual standard curve employing known concentrations of *p*-nitrophenolate prepared from *p*-nitrophenol.

Bessey-Lowry Activity :

One unit of activity may be defined as the amount of enzyme present in 1 millilitre of serum that liberates 1 μ mol of *p*-nitrophenol (0.1391 mg)* per hour at pH 10.5 after 30 minutes at 38°C.

Elimination of Interference due to Coloured Drugs

p-Nitrophenol is colourless, whereas the phenolate ion under basic conditions is yellow in appeanace. Therefore, the elimination of interference due to coloured drugs present in the serum is accomplished effectively by *first*, measuring the absorbance of the serum under basic conditions, and *secondly*, under acidic conditions. Thus we have :

$A_{p\text{-nitrophenolate}} = A_{basic} - A_{acidic}$

Profile of AP-levels

- Normal AP-levels in adults range between 0.8 to 2.3 Bessey-Lowry units and in children between 2.8 to 6.7,

- Increased AP-levels are observed in patients suffering from liver diseases, hyperparathyroidism and in rickets,

- Decreased AP-levels could be seen in patients suffering from hypoparathyroidism and pernicious anaemia (*i.e.*, an anaemia tending to be a fatal issue).

Interference due to Bilirubin

Bilirubin is eliminated by dializing the incubated *p*-nitrophenolate ion (at pH 10.5, and maintaining at 38°C for 30 minutes) into 2-amino-2-methyl-1-propanol, without carrying out the blank determination stated earlier.

There are a few medicinals that cause increased bilirubin levels which ultimately enhances AP-levels ; unless and until a corrective measure is taken in the respective procedure one may be left with false AP-level enhancement. Some typical examples are, namely : amitriptyline, chloropropamide, erythromycin, phenylbutazone, sulpha-drugs and tetracyclines.

Materials Required :

0.01 M *p*-Nitrophenol (dissolve 140 mg of *p*-nitrophenol in 100 ml of DW) : 1.0 ml ; 0.02 N NaOH (dissolve 160 mg in 200 ml DW) : 200 ml ; 5 ml of alkaline-buffered substrate (l M *p*-nitrophenylphosphate) (dissolve 7.5 g glycine, 0.095 g anhydrous $MgCl_2$ and 85 ml of 1 N NaOH to 1 litre with DW ; and mixing with an equal volume of a solution prepared by dissolving 0. l0 g of *p*-nitro-phenylphosphate in 25 ml of water) ; temperature bath previously set at 38°C ; alkaline phosphatase for unknowns (commercial source) ; working standard [dilute 0.50 ml of a solution of *p*-nitrophenol (10.0 mol/ litre, 0.139 g/100 ml) to 100 ml with 0.02 N NaOH].

Procedure :

Table-1

Cuvet	Working Standard (ml)	NaOH 0.02 N (ml)	Alkaline Phosphatase Units/ml	A_{410}
1	1.0	10.1	1.0	-
2	4.0	7.1	4.0	-
3	8.0	3.1	8.0	-
4	10.0	1.1	10.0	-
5	10.5	0.6	10.5	-

(1) First of all prepare a standard calibration curve as per Table-1.

(2) Plot a graph of absorbance A *Vs* units of alkaline phosphatase per millilitre.

(3) Proceed for the assay of AP in the serum sample sequentially as follows :

(*i*) Pipette 1.0 ml of alkaline—buffered substrate into each of two test tubes and keep in a water-bath preset at 38°C,

(*ii*) When both the test tubes have attained the temperature equlibrium, add 0.10 ml of serum and water to these tubes separately. The one with water serves as a reagent blank and is always needed per set of unknowns. Now, put the two tubes for incubation for exactly 30 minutes period,

(*iii*) Enzyme activity is arrested by adding 10.0 ml of 0.02 N NaOH to each tube. Remove them from the water-bath and mix the contents thoroughly,

(*iv*) Read out the absorbance of the unknown tube at 410 nm against the 'reagent blank' tube,

(*v*) Transfer the contents from the cuvets to the respective test-tubes and add 0.1 ml of HCl (-~ 11.5 N to each tube and mix the contents carefully. This operation removes the colour developed due to *p*-nitrophenol,

(*vi*) Again read out the absorbance of the serum sample against the reagent blank tube at 410 nm. This gives the colour due to the serum itself,

(*vii*) Now, the corrected reading is achieved by subtracting the reading obtained in step (*vi*) from the reading in step (*v*). The alkaline-phosphatase activity of the serum as Bessey-Lowery units is obtained from the calibration-curve step (*i*). Under these experimental parameters, we have :

1 Bessey-Lowry Unit = 5×10^{-8} mol of *p*-Nitrophenolate anion.

Thus, one unit of phosphatase activity liberated 1 μ mol of *p*-nitrophenol (l μ mol = 0.1391 mg) per hour per millilitre of serum under specified conditions.

Note : In case, a value more than 10 Bessey-Lowry Units is obtained, it is always advisable to repeat the process either with a smaller volume of serum or a shorter incubation period, and then finally adjust the calculations accordingly.

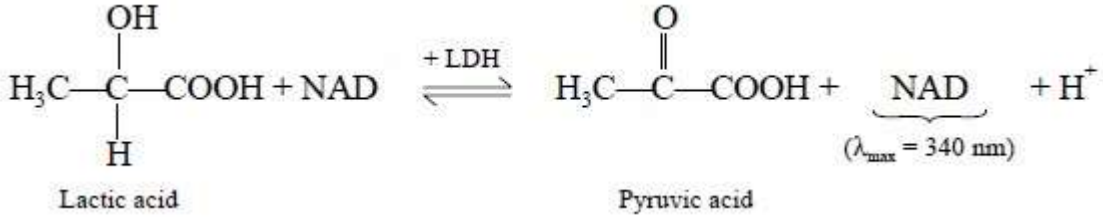
(4) Report the concentration of AP in units per millilitre.

1.2. *Lactate Dehydrogenase (LDH)*

Theory :

The method of LDH assay is based on kinetic analysis. In a kinetic enzymatic assay a unit of enzyme activity is defined as '*the quantity of enzyme that brings about a certain absorbance increase in 30 seconds or 1 minute at a fixed temperature (for instance 25 ± 0.2°C)* '.

The kinetic assay of LDH is based on the conversion of lactic acid to pyruvic acid, in the presence of nicotinamide adenine dinucleotide (NAD), and is closely monitored at intervals of 30 seconds or 1 minute by measuring the increase in absorbance at 340 nm. In this particular instance lactic acid available in an excess to ensure that the increase in pyruvic acid is linear with time, *i.e.*, directly proportional to time. The reaction involved may be expressed as follows :



The liberated nicotinamide-adenine-dinucleotide hydrogenase (NADH) has an absorption maxima at 340 nm, whereas lactic acid. NAD^+ and pyruvic acid do not absorb at all at this wavelenath.

Temperature Correction Factor :

The rate of the above reaction is temperature dependent. Hence, if the temperature (experimental) is higher or lower than that used to define a unit of activity, a definite correction factor should be applied as per Table-2.

Table-2

S. No.	T (°C)	Tf (25°C)	S. No.	T (°C)	Tf (25°C)
1	20	1.45	11	30	0.69
2	21	1.35	12	31	0.64
3	22	1.24	13	32	0.59
4	23	1.15	14	33	0.55
5	24	1.07	15	34	0.51
6	25	1.00	16	35	0.47
7	26	0.92	17	36	0.44
8	27	0.85	18	37	0.41
9	28	0.80	19	38	0.38
10	29	0.74	20	39	0.35
			21	40	0.33

From Table-2 it may be observed that :

(*a*) At a temperature beyond 25°C (Tf = 1.0), the absorbance increases at a faster rate than at 25°C due to enhanced rate of reaction and enhanced formation of NADH, thereby lowering the correction factor from 1.0 *e.g.*, 0.80 at 28°C,

(*b*) At a temperature lower than 25°C the rate of reaction is slower than at 25°C, thereby increasing the correction factor from 1.0 *e.g.*, 1.24 at 24°C, and

(*c*) Rule of thumb suggests that for each 10°C rise in temperature the reaction rate is almost doubled and the correction factor is halved, for example : at 35°C the correction factor is 0.47 (or 1.0/2 −~ 0.47).

Profile of LDH-levels :

1) Normal LDH levels are as follows : Absorbance Units per ml : 42 to 130, International Units per ml : 0.20 to 0.063

2) LDH level in serum is found to be increased in 8 to 10 hours after a myocardial infarction (*i.e.*, development or presence of an infarct in the heart) ; obviously the heart muscle is destroyed and consequently the enzymes leak into the serum,

3) Increased LDH levels are found in patients suffering from diseases related to liver and renal func-tions, cancer and pulmonary infarction,

4) Drugs like codeine and morphine help in enhancing LDH levels.

Materials Required :

Dermatube LDH provided by Worthington Biochemical, USA.

Procedure :

The following steps need to be followed in a sequential manner :

1) Dissolve the contents of Dermatube LDH (containing NADH and lactic acid) with 2.8 ml of DW,

2) Put this solution in a cuvette and then insert it in a colorimeter previously warmed up to 25°C. Set the wavelength at 340 nm. Carefully adjust the absorbance of this solution to 0.1 by making use of the proper variable control as explained earlier,

3) Remove the cuvette and add to it 0.2 ml of serum. Mix the contents of the cuvette and replace it quickly in position. Carefully record the absorbance exactly at intervals of 30 seconds for 2 to 3 minutes. In case, the absorbance happens to rise very rapidly, repeat step 3 by diluting 0.1 ml of the serum to 0.2 ml with DW,

4) From the foregoing measurement of absorbances calculate an average A/min,

5) Note the temperature at which the reaction is carried out accurately and then find out Tf from Table 2.10.

6) Report the LDH concentration as follows :

$$\text{Absorbancy Units of LDH per ml} = \frac{(\Delta A/\text{min}) \times (100) \times \text{Tf}}{\text{ml of serum used}}$$

$$\text{International Units* of LDH per ml} = \frac{(\Delta A/\text{min}) \times (100) \times \text{Tf}}{(6.2 \times 10^3) \times (\text{ml of serum used})}$$

RADIOIMMUNOASSAYS (RIAS)

An assay method based on immunological antibody-hapten (Ab-Ha) reaction that makes use of a radioactive tracer is usually known as radioimmunoassay. A hapten (or haptene) is a small molecule that represents the portion of an antigenic molecule or complex which determines its immunologic specificity, for instance : cortisol ; whereas an antibody is a relatively large protein that is specific for certain haptens. An antibody is generated by binding the hapten to a protein, resulting into the formation of an antigen that specifically stimulates the immune system to produce antibodies specific for the hapten.

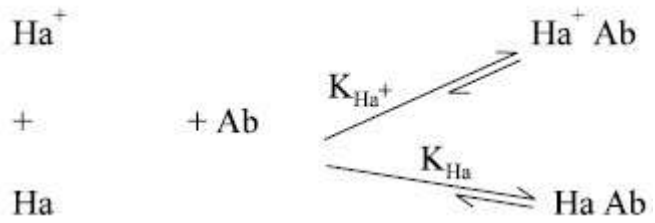
The assays that utilize protein instead of antibody are normally termed as **competitive protein bind-ing assays**. As an antibody is also a protein, therefore, a radioimmunoassay may be looked upon as a type of competitive protein binding assay.

Theory :

Generally, a radioimmunoassay makes use of a radioactive hapten and subsequently the percent of radioactivity bound to the antibody is measured. The radioactivity is determined by the help of a Geiger-Müller Counter or Geiger-Counter or G-M Tube and sometime by a Scintillation Counter.

First of all, a '*Standard Curve*' or a '*Calibration Curve*' is plotted between the reciprocal value (*i.e.*, $1 \times \%^{-1}$ radioactivity bound to the antibody) versus the amount of standard for a series of unknowns. Thus, the amount of hapten present in the unknown sample is measured from the plotted calibration curve conveniently.

The radioimmunoassay is based on the evolved competition between the combination of radioactive (Ha^+) and nonradioactive (Ha) hapten to the antibody as represented below :



Let us assume that the binding constants for Ha^+ and Ha are equal ; now, for a fixed quantity of Ha^+ but an increased concentration of Ha. The ultimate impact would be that lesser Ha^+ shall be bound. In actual practice, however, the use of Tritium (H^3) or Carbon-14 (C^{14}), which helps to render the Ha radioactive, ulti-mately maintains the equality of these binding constants, namely : K_{Ha^+} and K_{Ha} . It also confirms that the chemical properties of both radioactive (Ha^+) and nonradioactive (Ha) entities are more or less the same as far as the antibody is concerned.

Salient Features of Radioimmunoassays

- They belong to a class of extremely sensitive methods of analysis,
- Sample required for assay is usually very small *e.g.*, 1 ml of serum,
- Concentrations upto the nanogram range *i.e.*, 10^{-9} g can be measured accurately,
- A large number of hormones and drugs which find their abundant usage in a bad way, namely :
- cortisol (17-hydroxycorticosterone or hydrocortisone), insulin, morphine, barbiturates (sedatives), vitamin B_{12}, digoxin and human growth hormones, such as : somatotropin (elaborated in the placenta),
- Incidence of interferences observed in the radioimmunoassays are fairly insignificant by virtue of the highly specific hapten-antibody complexation reaction, and
- Exceptions do occur when two 5-substituted barbiturates present together cannot be assayed by this method, obviously due to interference.

1. Cortisol (In Plasma)

Theory :

Cortisol (or hydrocortisone) was introduced in the year 1951, for the treatment of rheumatoid arthritis. It has a significant effect on protein metabolism. It also exerts widespread effects on carbohydrates, lipid and protein synthesis (or anabolism). The cardinal side effects such as excessive potassium excretion and sodium retention, enhanced gastric acidity, oedema, psychosis and negative nitogen balance are some of the exaggerated manifestations of the normal metabolite functions of cortisol.

Most importantly, the determination of cortisol levels is considered useful in the diagnosis and treatment of various ailments, namely : Addison's Disease *i.e.*, pernicious anaemia—a condition whereby the maturation of the red cells may not proceed beyond the stage of megaloblasts; Cushing's Syndrome.

Adrenal Tumours :

The assay-method is entirely based on the Schwartz-Mann Kit. According to this method, cortisol is first extracted from the plasma using CH_2Cl_2 (methylene chloride). In the actual radioimmunoassay the cortisol present in the extract competes with Cortisol-H^3 (*i.e.*, the radioactive tracer) for the common binding sites on transcortin, which is incidently not an antibody but a cortisol-binding protein. Now, the free cortisol is quantitatively removed by adsorption on dextran-coated charcoal from the one bound to the transcortin. Finally, the bound radioactivity (due to Cortisol-H^3) is measured which is then employed to calculate exactly the amount of cortisol present in the sample by the help of a Standard Curve (or Calibration Curve).

Materials Required :

Schwartz-Mann-H^3 Cortisol RIA-Kit ; liquid scintillation counter, centrifuge.

Procedure :

The various steps to be followed sequentially for the assay of cortisol in plasma are as follows :

- The cortisol is usually extracted from the samples drawn from the patients, as follows :

Place 100 µ l of plasma in each of two tubes and add 2.5 ml of methylene chloride. Shake the contents of the tube vigorously for 10 minutes and transfer 0.5 ml of clear extract (*i.e.*, the lower layer) to another tube. Evaporate the methylene chloride either at 35°C in an oven or in a stream of N_2. The extract thus obtained is employed in the following step.

- The following steps *viz.*, Step 1 to Step 15, related to the procedure for the assay and the calibration curves must be performed simultaneously :

Sequence	Preparation of Calibration Curve	Clinical Determination
Step-1	Consecutively number 18 glass tubes,	Use tubes containing the dried extract,
Step-2	Pipette phosphate buffer (0.04 M having buffer pH 7.4) into tubes as follows :	Pipette 800 µl phosphate buffer into each tube,

Tube	Phosphate Buffer (µl)
1, 2	1300
3, 4	900
5, 6	800
7, g	800
9, 10	700
11, 12	700
13, 14	800
15, 16	700
17, 18	700

Step-3 Add cortisol standard solution (A or B from Schwartz-Mann Kit*) as follows : Add no standard to patient sample tubes,

Tube	Cortisol Standard	Cortisol ng/Tube
5, 6	NONE	0
7,8	25 µl A	0.5
9, 10	50 µl A	1.0
11,12	100 µl A	2.0
13, 14	25 µl B	4.0
15, 16	50 µl B	8.0
17, 18	100 µl B	16.0

*Manufactured By : Mountain Ave., Orangeburg N.Y.

Step-4	Add 100 µl transcortin solution from SM-Kit to tubes 1, 2 and 5 through 18 and mix gently,	Add 100 µl transcortin solution to each tube,
Step-5	Add 200 µl cortisol (– H^3) to tubes 1 through 18 mix for 3 seconds on a vortex mixer. Set tubes 1 and 2 aside until Step-13,	Add 100 µl cortisol (– H^3) to each tube and mix,

Step-6 From this point onwards the various tubes are treated as follows :

Step-7 Incubate tubes 3 through 18 and all patient sample tubes in a pre-set constant temperature water-bath at 45°C for exactly 5 minutes,

Step-8 Immediately after Step-7 incubate tubes 3 through 18 and all patient tubes in an ice-water bath (0 to 4°C) for 30 minutes. Shake the rack several times at short-intervals to ensure that the tubes attain 0-4°C rapidly,

Step-9 Quickly add 0.5 ml of cold dextran-coated charcoal suspension to tubes 3 through 18 and to all patient sample tubes so as to get rid of free cortisol. Do not add to tubes 1 and 2,

Step-10 Keep tubes 3 to 18 and all patient sample tubes in an ice-water bath for 10 minutes,

Step-11	Centrifuge all tubes either at 1240 × g for 10 minutes at 4°C or centrifuge for less time at higher speeds,
Setp-12	Consecutively number a set of scintillation vials,
Step-13	Pipette out 1.0 ml of solution from tubes 1 and 2 into correspondingly numbered scintillation vials. These vials will give the total count per assay. Also pipette 1.0 ml of each clear supernatant into a correspondingly numbered scintillation vial,
Step-14	Add 10.0 ml of UNOGEL to each vial. Shake each vial to solubilize the contents: An emulsion should form, and
Step-15	Count the radioactivity in the vials in sequence for 1 to 10 minutes. The count time should be long enough to accumulate 10,000 to 15,000 counts.

- **Results :** Average the counts per minute in vials 3 and 4. This is the blank value. Now, subtract the blank from all other counts per minute to obtain the actual counts per minute and average the counts per minute for vials 1 to 2 to find the total count per minute. The percent bound may be calculated using the following expression :

$$\% \text{ Bound} = \frac{\text{Counts per minute}}{\text{Total counts per minute}} \times 100$$

Finally, plot the percent bound *Vs* nanograms (ng) per tube of cortisol standard either on linear or on semilog paper and make use of this Standard Curve to calculate the amount of cortisol present in the unkown samples.

AUTOMATED METHODS OF CLINICAL ANALYSIS

Theory : An '*Autoanalyzer*' serves as the most versatile and important instrument in a well-equipped '*clinical laboratory*' that caters for the rapid screening of serum levels for upto forty (40) important chemical substances in the field of diagnostic medicine. These autoanalyzers may be either 'Single Channel' *i.e.*, performing only one determination on each sample or Multichannel' *i.e.*, carrying out several different determinations on each sample.

A few important substances that are routinely analyzed in a clinical laboratory with the aid of an 'Autoanalyzer' are, namely : serum-glutamic-oxaloacetic transaminase (SGOT) ; creatine-phophokinase (CPK); alkaline-phosphatase (AP) belonging to the class of enzymes ; and a host of biochemical substances, for instance : bilirubin, serum albumin, blood urea nitrogen (BUN), uric acid, creatinine, total protein, glucose, cholesterol, besides a few common inorganic ions, such as : Cl^-, Ca^{2+}, K^+, Na^+.

The basic principles underlying both automated and unautomated methods of analysis are more or less the same. Out of the broad-spectrum of biological samples blood analysis is the most common one. There exists a number of parameters which may be assayed, and spectrophotometry is ideally suited for nearly all of them, a few typical examples are cited in Table-3.

Table-3

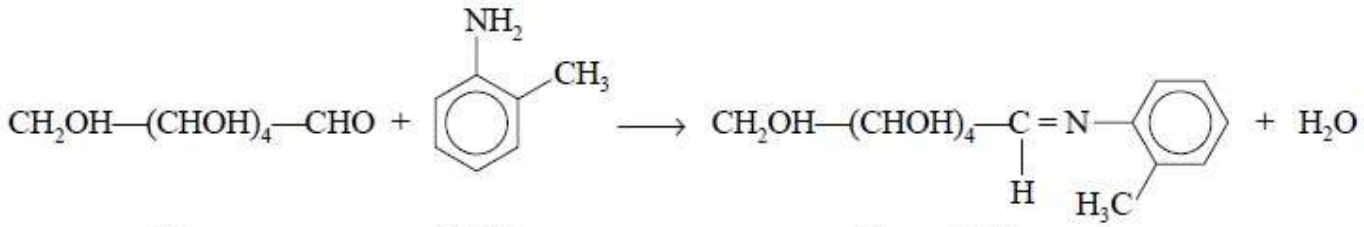
S. No.	Analyte	Reagents(s) and/or Procedure(s)	Wavelength λ (nm)
1.	Bilirubin	Diazotized sulphanilic acid (Ehrlich's Reagent)	540
2.	Cholesterol	Acetic-anhydride (Liebermann-Burchard Reagent)	625
3.	Glucose	Glucose reduces Cu^{2+} to Cu^{+} ; & Cu^{+} reduces phosphomolybdic acid (Folin-Wu)	420
4.	Glucose	*o*-Toluidine	635
5.	Phosphate (Inorganic)	Na_2MoO_4, *p*-Methylaminophenol sulphate	700
6.	Urea (Nitrogen)	Urease, Na_2WO_4, Nessler's Reagent	490

Explanation : Glucose (having an aldehyde functional moiety) reduces Cu^{2+} to Cu_2O (*i.e.*, Cu^{+}) as per the following reaction :

$$C_6H_{12}O_6 + 2Cu^{2+} + 6H_2O \rightleftharpoons Cu_2O + C_6H_{12}O_7 + 4H_3O^{+}$$

As some other sugars are also present in blood sample, and besides the above reaction not being abso-lutely stoichiometric, it has become necessary in actual practice to establish an emperical calibration curve using known concentrations of glucose. The above reaction is allowed to proceed for exactly 8 minutes at 100°C. To the resulting solution phosphomolybdic acid is added, which is subsequently reduced by Cu_2O to give rise to an intensely coloured 'molybdenum blue' that is measured at 420 nm accurately.

Alternatively, glucose forms a specific complex with *o*-toluidine according to the following reaction that forms the basis of the colorimetric assay :



Glucose *o*-Toluidine Glucose-toluidine complex

The diagnostic green colour is usually developed for exactly 10 minutes at 100°C and measured subse-quently at 635 nm.

1. Instrumentation

The schematic diagram of an Auto Analyser is shown in Figure-5. The major component parts com-prise of the various important sections namely : the preparation section, the reaction section and the analysis section which will be discussed briefly here.

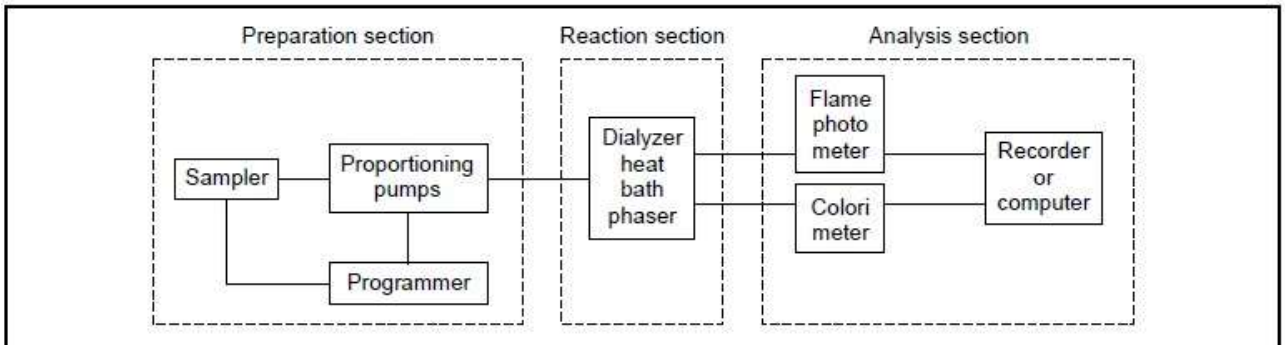


Figure-5

1.1. *Preparation Section*

This particular section of the Auto Analyzer consists mainly of the sampler, proportioning pumps, and programmer. First, the sampler introduces a fixed quantity of serum sample into the 'analysis train', which varies from one instrument to another instrument supplied by different manufacturers. For instance, the SMA-12 Survey Auto Analyzer possesses 12 analysis trains or streams as illustrated in Figure-6.

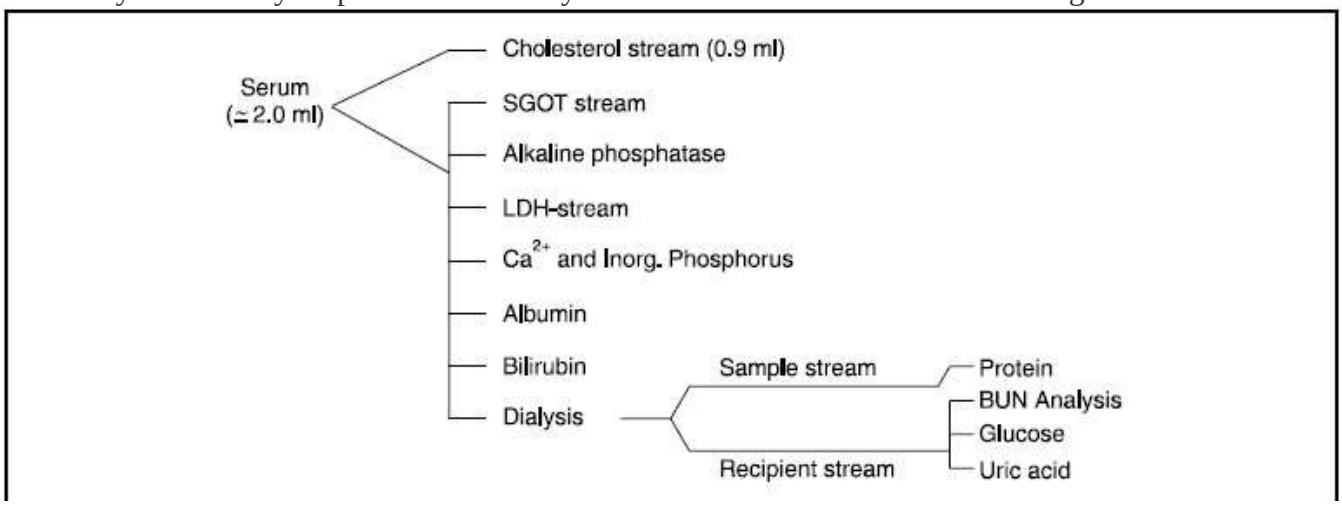


Figure-6

The proportioning pump controls the rate of advancement, *viz* 10 inch/minute, of each sample through the analysis stream. Hence, a fixed length of tubing is equivalent to a fixed amount of time. Each analysis stream is made of transparent plastic flexible tubing, and each patient-sample is separated from one another by an air-bubble.

1.2. *Reaction Section*

The reaction section essentially comprises of the dialyzer, heat bath and phaser, and obviously the reaction takes place in this zone. Let us consider the following generalized reaction :

$$aA + bB \underset{k_2}{\overset{k_1}{\rightleftharpoons}} cC + dD \qquad ...(a)$$

$$K = \frac{[C]^c[D]^d}{[A]^a[B]^b} \qquad ...(b)$$

Where , $[C]^c$ = Molar concentration of substance C raised to the *c*th power,

A = Component in serum (*e.g.*, cholesterol), and

B = Reactant that reacts with A to give a coloured product.

Evidently, B is added always in excess to ensure :

(a) rapid reaction, and

(*b*) complete reaction by forcing the reaction to the right in accordance to the *Le Chatelier's principle*.

Now, the rate of forward reaction = $k\,[A]^a\,[B]^b$

Hence, the rate constant may be expressed as follows :

$k = Ae^{-Ea/RT}$ .....(c)

where , R = Gas constant (1.99 cal/K-mol),

T = Temperature, and

E_a = Activation energy of the reaction as depicted in Figure-7.

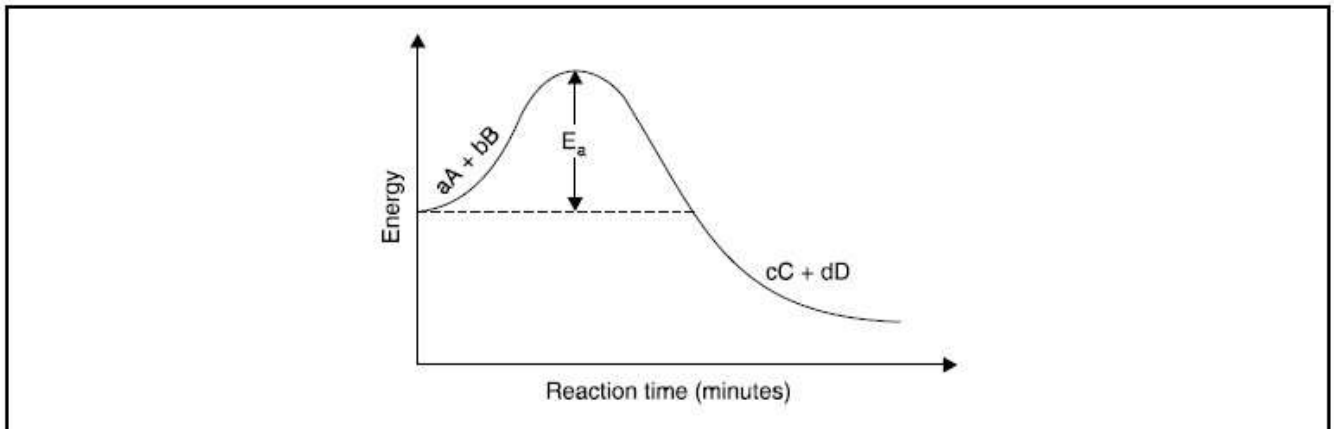


Figure-7

From Eq. (*c*) it may observed that as the temperature T is enhanced then the rate of reaction also enhances simultaneously because a higher value of T offers a smaller negative exponent of *e* or a larger number. Therefore, in actual experimental operations temperature is increased by the aid of a heat-bath so as to accelerate the reaction which in turn allows the reaction to attain equilibrium state as rapidly as possible.

Naturally at a very high temperature there is every possibility for decomposition of either the products or the reactants.

1.3. *Analysis Section*

The recent advancement in the field of *computer technology* and *anlytical instrumentation* it has become very easy and convenient to have the analyical data from a series of biological samples processed at high speed as digital readouts or on computerized recorders. Many hospitals round the globe make extensive use of advanced computer softwares for data processing as stated beiow :

- Uptodate listing of various laboratory tests,
- Listing of drugs and metabolites that cause interference both biochemically and analytically,
- Storing of levels of biologically important compounds for various disease states, and
- A tentative diagnosis for a patient based on his serum sample under investiation together with the drugs and dosages being administered and the levels of biologically important compounds.

Caution : Nevertheless, the concerned physician or pharmacist must exercise his or her own expertise and knowledge while prescribing drug(s) to a patient along with these computerized data informations.