SPECTROPHOTOMETRIC DETERMINATION OF CHOLESTEROL WITH DICHROMATE

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of Graduate Studies
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In Partial Fulfilment of the
Requirements for the Degree
of Master of Science
in
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by
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Dedicated to my sister W/o Bizunesh Asfaw whose affection towards me has been a lively motive for efforts of mine and my mother W/o Nedach Minuta who taught me the importance of not being out of patience in the route of any achievement.

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A C K N O L E D G M E N T S

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ABSTRACT

The oxidation of cholesterol by dichromate has been investigated for its application to quantitative determination of cholesterol in blood sera. The redox system was studied spectrophotometrically by measuring the light absorption of the reduction product, chromium(III), at 585 nm in benzene-acetic acid and pertoleum ether-acetic acid. The effects of experiemntal variables have been studied to establish the optimum conditions of the redox reaction. It has been found that consistent and reproducible results are obtained by carrying out the reaction at 80^{o}C for 1 hr. It has also been found that petroleum ether can be used as an alternative to benzene as a solvent to introduce cholesterol into the reaction system. The coloured system has been found to obey Beer's law in the concentration range 0.01-2.0mg ml^{-1} of cholesterol. Other photometric characteristics of the coloured system have also been evaluated. On the basis of these studies a simple and precise method has been developed for the determination of cholesterol. The method has been applied to the analysis of blood sera and the results obtained have been compared with clinical results. The proposed method has been found to give results which are in good agreement with those of routinely used clinical methods.

1. INTRODUCTION

Cholesterol is a monounsaturated secondary alcohol with formula $C_{27}H_{45}OH$. It is a crystalline solid and has a melting point of 149°C. Its name is derived from "Chole" (bile in Greek) as the compound is the predominant constituent of human gallstones, deposited in the bile ducts, and the generic name "Sterol" denoting "stereos" (solid in Greek) plus "Ol" (marking the alcoholic functionality). The commercial cholesterol is isolated from the spinal cords of cattle [1,2]. The structure of cholesterol is shown in Fig. 1.

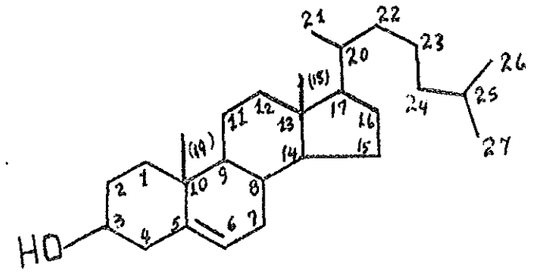


Fig. 1 Cholest-5-ene-3β-ol (cholesterol)

Most of the reactions of the molecule occur at the hydroxyl, at the double bond or at carbon 7. The hydroxyl group is equatorial and is thus freely reactive.

1.1 Source and Origin [3-6]

Cholesterol is an ubiquitous protoplasmic component in living organisms of higher animals. In the body it originates from exogenous sources, absorbed in the intestinal tract, and endogenous sources, biologically synthesized in most body tissues. Dietary cholesterol intake is largely variable but the actual amount absorbed

is small. This could be a consequence of the active enterohepatic circulation of cholesterol itself as most of the sterol is endogenous in origin and competes with the exogenous sterol. Through lymph lipoproteins, cholesterol enters the general circulation from which it is removed by the liver.

Although the range of concentrations for total serum cholesterol in healthy adults is usually stated as being approximately 140 to 250 mg per 100 ml, this range applies only to persons in younger age groups. In older age groups, serum cholesterol levels are significantly higher [5]. The approximate upper limits of normal concentration of cholesterol in various age groups are shown in Table 1. The normal range for cholesterol esters is between 70 and 75% of the total, with free cholesterol making up the remaining 25 to 30%

Table 1. Normal serum cholesterol values [4]

Age (yr)	Upper limit mg/100 ml
0-19	230
20-29	240
30-39	270
40-49	310
50-59	330

The cholesterol content of blood varies greatly with diet, age, and sex. By age 55 it averages 2.5 g/l and may be considerably higher. Females up to the age of menopause have distinctly lower level of cholesterol in blood [6].

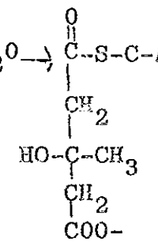
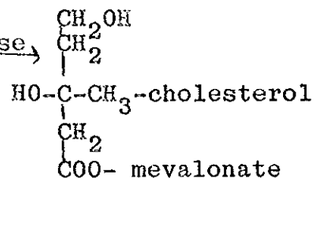
1.2 Biosynthesis of Cholesterol [7-13]

The biological synthesis of cholesterol is achieved

principally in the liver. In the work of Konrad Bloch [11] undertaken to elucidate the pathway of biosynthesis of cholesterol, acetate isotopically labeled in its carbon atoms was prepared and fed to rats. The cholesterol synthesized by these rats contained the isotopic label, which showed that acetate is a precursor of cholesterol. A series of mechanistic steps and numerous intermediates are established to occur in the route from acetylcoenzymeA(acetylCoA) to cholesterol.

The first stage in the biosynthesis of cholesterol is the formation of isopentenyl pyrophosphate from acetylCoA. This reaction sequence starts with the formation of 3-hydroxy-3-methylglutarylCoA from acetylCoA and acetylacetoCoA. 3-Hydroxy-3-methylglutarylCoA is reduced to mevalonate:

acetoacetylCoA

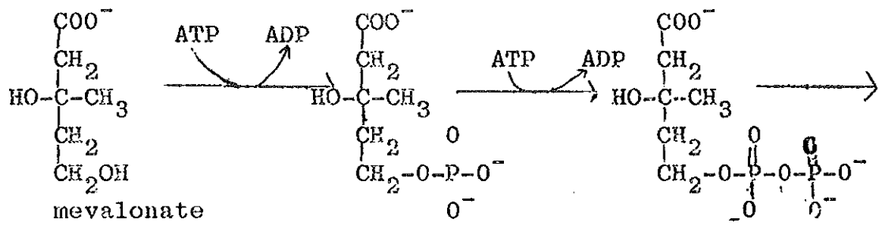
acetylCoA + H_2O →  <u>reductase</u> → 

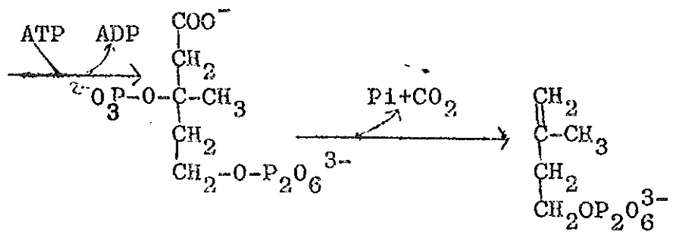
3-Hydroxy-3-methyl glutarylCoA is present both in cytosol and in the mitochondria of liver cells. The cytoplasmic pool gives rise to mevalonate for the synthesis of cholesterol. The enzyme catalyzing this irreversible step, 3-hydroxy-3-methylglutary-COA reduductase, is an important control site in the cholesterol biosynthesis shown in the following reaction:

3-hydroxy-3-methylglutarylCoA + 2 NADPH + $2H^+$ ⟶

⟶ mevalonate + 2 $NADP^+$ + CoA.

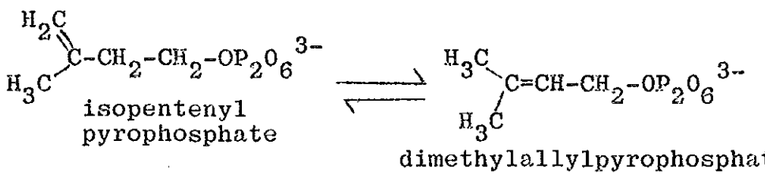
Mevalonate is converted into 3-phospho-5-pyrophosphomevalonate by three consecutive phosphorylations. This labile intermediate loses CO_2 and inorganic phosphate, pi, which yields 3-isopentenyl-pyrophosphate:

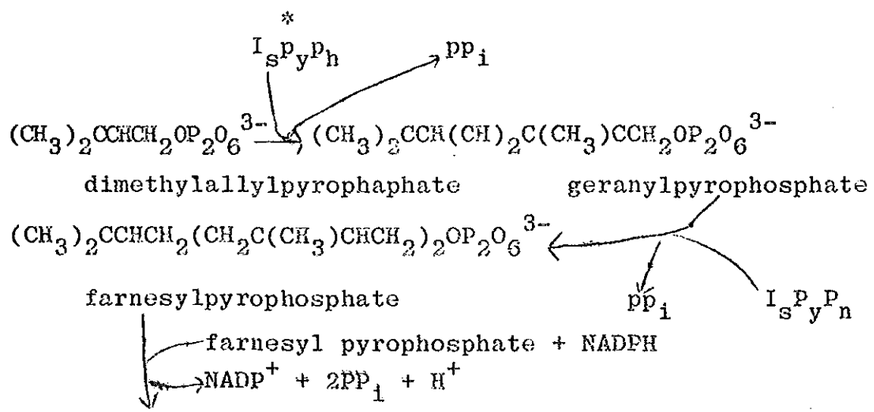




3-phospho-5-pyrophosphomevalonate isopentenylpyrophosphate

The isopentenylpyrophosphate so obtained is used for squalene synthesis. This stage in the synthesis of cholesterol starts with the isomerization of isopentenylpyrophosphate to dimethylallylpyrophosphate:





$(CH_3)_2CCHCH_2(CH_2C(CH_3)CHCH_2)_2-(CH_2C(CH_3)CHCH_2)_2CH_2CHCH(CH_3)_2$

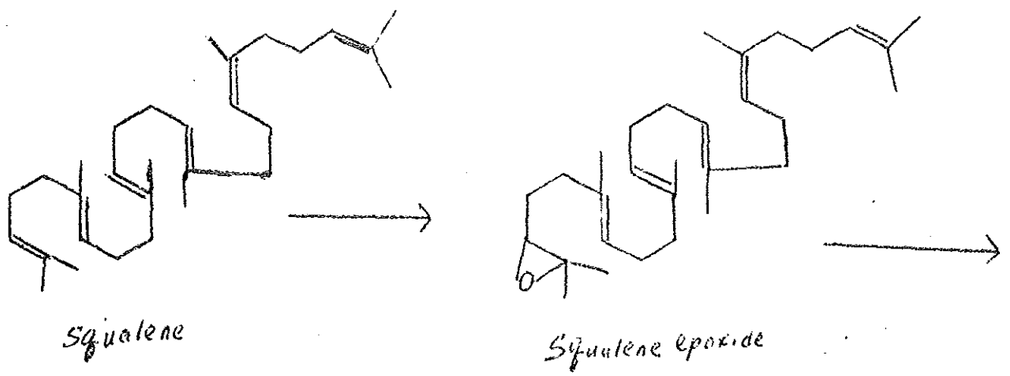
squalene

(* $I_2P_yP_h$: isopentenylpyrophosphate)

The two C_5 units condense to form geranylpyrophosphate (C_{10}) which condenses with another molecule of isopentenylpyrophosphate to form farnesylpyrophosphate(C_{15}). The last step in the synthesis of squalene is a reductive condensation of two molecules of farnesylpyrophosphate:

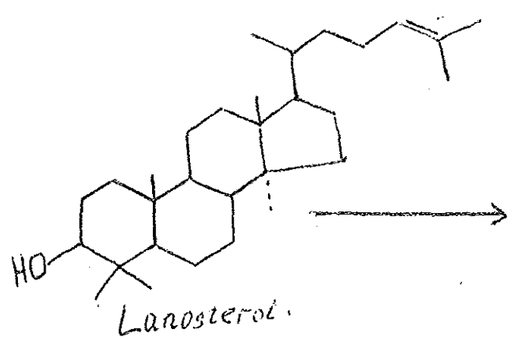
2 farnesylpyrophosphate + NADPH → squalene (C_{30}) + $2PP_i$ (C_3) + $NADP^+$ + H+

The final stage of cholesterol biosynthesis starts with the cyclization of squalene. The transformation of squalene to cholesterol requires molecular oxygen and is initiated by a microsomal enzyme system. The system utilizes O_2 and NADPH to form squalene-2,3-oxide. The reaction can be visualized [8-10] as proceeding through a carbonium ion created by attack of a proton on the oxygen atom of the epoxide ring. Squalene epoxide is then cyclized to lanosterol by a cyclase. There is a concerted movement of electrons through four double bonds, a migration of two methyl groups in this remarkable cyclization [11]. Finally, lanosterol is converted into cholesterol by the removal of three methyl groups, the reduction of one double bond by NADPH, and the migration of the other double bond [12,13].

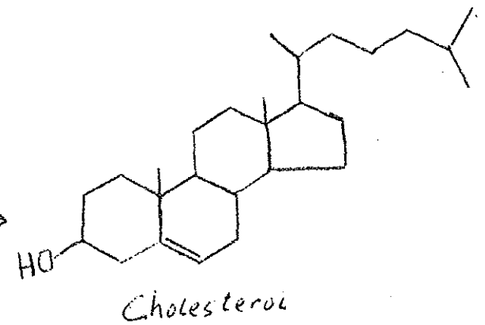


Squalene

Squalene epoxide



Lanosterol



Cholesterol

The elucidation of the mechanism of biological synthesis of cholesterol makes it possible to seek ways of inhibitng the synthesis in cases of hypercholesterolaemia. The most suitable medicines for use against hypercholesterolaemia in human beings are those which inhibit cholesterol fromation at the point between 3-hydroxy-3-methylglutaryl CoA and mevalonate, where physiological regulation of cholesterol biosynthesis takes place[3].

1.3 Transport and Metabolism of Cholestero [2,14-16]

For the safe transport of cholesterol in blood, its concentration and its tendency to escape from the blood

stream to cell membranes via passive exchange must be controlled. Multicellular organisms solve this problem of cholesterol transport by esterifying the sterol with long-chain fatty acids and packaging these esters within the hydrophobic cores of plasma lipoproteins. Cholesterol and its esters are among the water-insoluble group of fatty compounds present in blood, lipids. Because of their hydrophobic property lipids are transported in blood not in the free form but in association with the soluble proteins. This combination is known as lipoprotein. The lipoproteins in blood exist in varying density namely, chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL), and high density lipoproteins (HDL) [15].

Most serum cholesterol is carried by LDL which delivers the cholesteryl ester directly to cells that need cholesterol. But these esters are too hydrophobic to pass through cell membranes. Consequently one may expect a problem of distribution to the inner part of the cell. However the lipoprotein receptors solve the problem by binding the LDL and carrying it into the cell through receptor-mediated endocytosis [16]. The internalized lipoprotein is delivered to lysosomal enzymes which hydrolyze the cholesteryl esters to liberate free cholesterol.

The released cholesterol is used by the cell for synthesis of plasma membranes, bile acids, and steroid hormones, or stored in the form of cytoplasmic cholesteryl ester droplets. The unwanted proportion of the substance is eliminated from the body by chemically converting it to water-soluble derivatives, bile acids, and by physically

excreting it as such in the mixed micelle present in bile.

The metabolic scheme is given in Fig. 2.

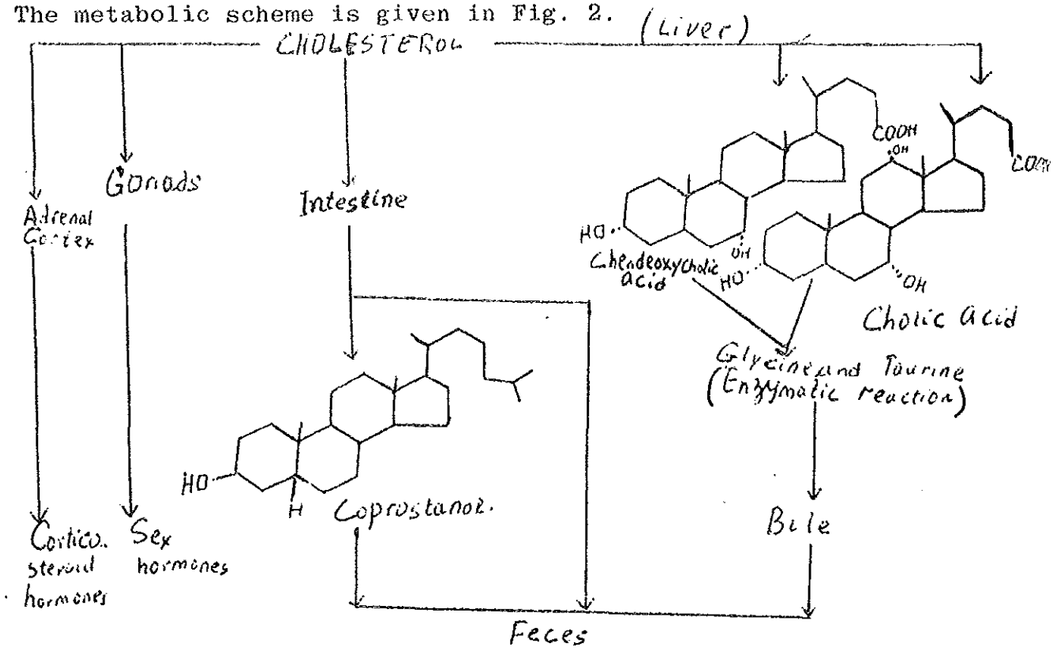


Fig.2 Metabolic fate of cholesterol

1.4 Function and Pathogenic Effect [17-22]

Cholesterol is an essential constituent of all types of cell membranes in higher organisms; it moderates the fluidity of the membranes. As mentioned above it is also the precursor of bile acids and steroid hormones of the adrenal cortex and sexual glands [15].

Bile acids are the powerful emulsifiers for dietary fats and they flow from the liver into bile duct and small intestine. Later a large fraction is reabsorbed in the duodenum and is returned to the liver for reuse. Bile acids also serve as activators of lipolytic enzymes in the intestinal tract.

Steroid hormones are very essential to life. Some of them are responsible for the development of female and

male sex organs and secondary sex characteristics, whereas some others promote glyconeogenesis and accumulation of glycogen in liver. The rest of the steroid hormones account for the regulation of the Na and K levels of the body [18].

On the other hand, cholesterol is a pathogen lethal to a great number of people. Over 10% of the adult population suffers from the formation of gallstones [19]. The present treatment of the disease is either by surgical removal, with the accompanying risks and expenses, or by oral therapy with drugs designed to dissolve stones in situ. Two types of gallstones can be distinguished on the basis of their approximate compositions; cholesterol and pigment stones [20]. The major constituent of cholesterol stones is cholesterol. Dissolution therapy is used for these stones but can be ineffective for the pigment type.

Elevated concentrations of serum total cholesterol, i.e., the sum of free and esterified cholesterol, are major factors in the development of atherosclerosis, an extremely common human disease which thickens the arterial walls leading to decreased blood flow.

The very property that makes cholesterol useful in cell membrane is its very low solubility in water, the property which also makes it lethal. Because when the substance accumulates in wrong place, for example within the wall of an artery, it can not be readily mobilized, and its presence eventually leads to the development of an atherosclerotic plaque. The potential for errant cho-

lesterol deposition is aggravated by its dangerous tendency to exchange passively between blood lipoproteins and cell membranes. The LDL receptor possesses two important properties: its high affinity for LDL and its ability to cycle multiple times in and out of the cell. It allows a large amount of cholesterol to be delivered to body tissues, while at the same time keeping the concentration of LDL in blood low enough to avoid the building up of atherosclerotic plaques. When the LDL receptor function is inappropriately diminished as a result of genetic defects or in response to regulatory signals, the protective mechanism is lost, cholesterol builds up and atherosclerosis ensues [21].

From the foregoing discussion it is evident that knowl - edge of the cholesterol content both in gallstone and blood is an essential prerequisite for medical measures to be taken against diseases [11,22].

In view of high mortality rate from the cardiovascular diseases and th preponderance of gallstones in the adult population the determination of cholesterol in common matrices appears to be of great importance.

2. THE EXISTING METHODS OF CHOLESTEROL DETERMINATION

A literature survey reveals that there has been quite a large number of methods developed for the quantitative determination of cholesterol though no universally accepted reference method is available[23] except the very expensive isotope dilution/mass spectrometry technique which has been considered a candidate reference method[15]. In this section is presented a brief discussion of the major methods classified, for convenience, into early and modern methods.

2.1 Early Methods of Cholesterol Determination

Several early methods are briefly mentioned below primarily because of historic interest[24].

(i) Gravimetry. Cholesterol is precipitated as the diditonide after saponification in blood samples and the digitonide weighed[25].

(ii) Nephelometry. Cholesterol digitonide can be suspended and quantified by nephelometric measurements[26].

(iii) Turbidimetry. The turbidity produced upon addition of sodium alcoholate is measured[34]. A NaOH solution in ethanol is added to serum and precipitate appeared in the solution. The light transmitted through this turbid solution was measured at 550 nm and compared with the incident light.

(iv) Gas chromatography[27-29]. Cholesterol in serum sample is extracted with acetone-ethanol(1:1,v/v) solvent system and then analyzed by gas chromatography.

(v) Thin layer chromatography. Free and esterified cholesterol are first separated by thin layer chromatography and are then determined separately with the ferric ammonium chloride method[30]. The method is based on the Zak reaction[46]. Ferric chloride dissolved in glacial acetic acid was frozen. Cold($4^{o}C$) concentrated sulphuric acid is added to the ferric solution with gentle swirling. This mixture is added to the cholesterol samples and is shaken vigorously, the colour system is measured at a fixed time at 560 nm. In the reaction ferric ion is reduced to ferrous ion and the cholesterol is oxidized to polyene product[44]. The esterified cholesterol is hydrolyzed before subjected to the reaction.

(vi) Fluorimetry. These methods are based on Liebermann-Burchard reaction[32] or the Tschugaeff reaction[33] and the resultant fluorescene is measured. Under the action of the Liebermann-Burchard reagent, cholesterol displays a fluorescence with an emission maximum located at 570-590 nm. A fluorimetric method was developed based on this principle which alllowed measurement of cholesterol in serum. In the procedure serum is treated with acetic acid and acetic anhydride in chloroform, then with concentrated sulphuric acid; the mixture is centrifuged after 40 min and the fluoresence of the supernatant is read.

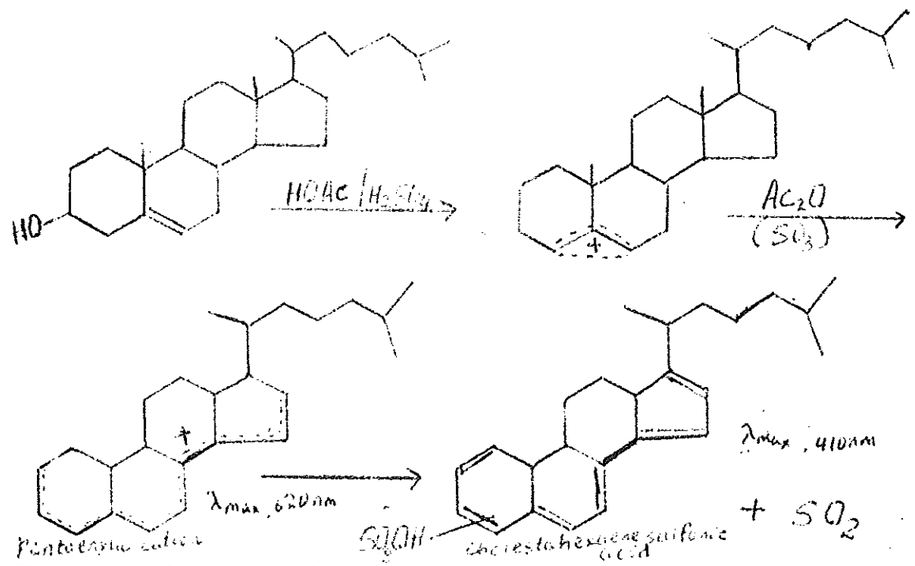
In the Tschugaeff reaction, on the other hand,

a solution of cholesterol in glacial acetic acid is treated with acetyl chloride and zinc chloride. A green fluorescence is developed and is applied for the determination of total cholesterol in samples of blood.

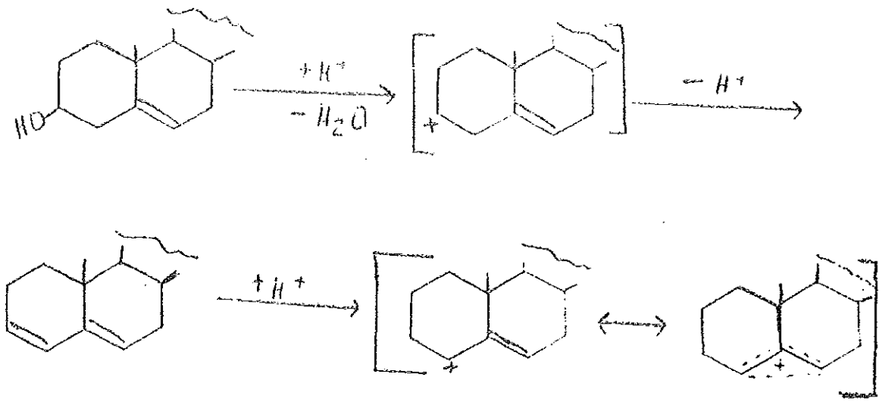
(vii) Photometric methods . The photometric methods are, in fact, the ones most frequently used and, hence, discussed below in more detail. Most of the photometric methods are based on the Liebermann-Burchard reaction.

Liebermann in 1885 described the reaction of cholesterol with concentrated H_2SO_4, in which colours are produced changing from red through violet to blue-green. Burchard[35] applied this reaction to cholesterol in $CHCl_3$. Numerous techniques employing the Liebermann-Burchard colour reaction have been introduced since then. The techniques most widely used are as follows: that of Bloor[36] and its modification[37], of Schoenheimer and Sperry[38] and modifications[39,40], of Carr and Dekter[41], and that of Abell et al[42] and modifications[43].

The Liebermann-Burchard(L-B) colour reaction is complex and the detailed, mechanism is recently proposed [44-46]. The colour is attributed to the polyenyl carbocation carying a delocalized positive charge.



Cholesterol is oxidized, in steps, by SO_3 and the reduction product is SO_2. Burke and coworkers[44] reported spectroscopic and kinetic evidences for the mechanism given above. They followed the reaction by measuring visible light absorption and observed bands at 620 and 410 nm corresponding to the pentaenylic cation and cholestahexaene sulfonic acid, respectively. According to the proposed mechanism the reaction starts with protonation of the -OH group in cholesterol and subsequent loss of water giving the carbonium ion of 3,5-cholestadiene. The oxidation of this allylic carbonium ion by SO_3 yields the cholestapolyene carbonium ion shown, together with equivalent amount of SO_2. Notably, no cationic species are given in the above scheme of reaction mechanism in going from the allylic to the pentaenylic cation, because none were observed spectrally. The precursor to colour formation is the initial formation of the carbonium ion of 3,5-cholestadiene as shown below:



The nonclassical resonance structure involving the bridge-head C-5 carbon contributes significantly to the stability of this ion. The overall reaction can be summerized as in the following balanced chemical equation:

$$\text{Cholesterol} + 5H_2SO_4 \longrightarrow \underset{\text{sulfonic acid}}{\text{Cholestahexaene}} + 4SO_2 + 10H_2O$$

The L-B reaction is affected by many variables including the concentration of H_2SO_4 and water, the acetic acid content of the acetic anydride, the solvent employed, the time of the reaction, effects of light, and temperature[40]. It is believed that the acetic anhydride serves only as a diluent for the H_2SO_4 since it can be replaced by acetic acid, ethyl acetate or butanol. The absorption peaks of the coloured product are at 410 nm and 620 nm. The yellow component of the green L-B colour is more stable than the blue component but is affected by light[47]. The L-B reaction carried out at room temperature in chloroform solution yields 10-30 % more colour with cholesterol esters than with cholesterol and therefore gives incorrect values of total cholesterol if a saponification step is not included[48]. In the method of Schoenheimer

and Sperry [38] and its modifications, the esters are saponified prior to colour development and the inaccuracy caused by differences in colour equivalence is eliminated.

Methods in which the L-B colour reaction is carried our directly on serum without prior extraction have become very popular[49]. Tonks[47] reported in his critical review of the methods that these direct methods give spuriously high results and that inaccuracies occur if bilirubin levels are elevated above normal values.

Though the Liebermann-Burchard colour reaction has been the most popular for detemination of cholesterol, it suffers interference from steroids normally present in common matrices of cholesterol[15].

Other colour reactions useful for determination of cholesterol are also known including the Tschugaeff[33] and the $FeCl_3$-H_2SO_4[37] reactions. The Tschugaeff reaction employs acetyl chloride and zinc chloride in glacial acetic acid.

2.2 Modern Methods of Cholesterol Determination

The basic principles and remarkable steps in procedures of main methods of cholesterol determination developed after 1970s are discussed in brief.

2.2.1 Gas Liquid Chromatographic(GC) Methods

The references to the gas liquid determination of cholesterol in blood sera and extracts from common samples are too numerous to cite. However, only a few of them appear to have analytical merit[50-53], one of these is Driscol's method. Driscol[50] has developed a sensitive gas chromatographic method for quantitative analysis of

free and esterified cholesterol in serum. When the concentration of total cholesterol is of interest, the serum samples are saponified with alcoholic potassium hydroxide to liberate cholesterol. The extract is then subjected to GC analysis. The internal standard addition technique is used and quantification is based on the measurement of peak height ratios.

Although the method provides a good accuracy and involves less manual labour, compared with chemical methods, its sensitivity is susciptible to minor changes in experimental conditions such as changing a septum or cylinder of carrier gas. It also involves prior extraction of cholesterol and hydrolysis of the cholesteryl esters, in the case of the determination of total cholesterol.

2.2.2 High Performance Liquid Chromatographic (HPLC) Methods.

Liquid chromatography(LC) has been used in the analysis of lipids[54], in general. Its application in cholesterol analysis has, however, been limited because cholesterol absorbs very little ultraviolet(UV) radiation in the wavelength range in which most UV detectors used in LC operate. With recent advances in LC-detector technology, high-performance spectrophotometers with low-volume flow cells are now commercially available that allow chromatographic eluates to be monitored at wavelengths as low as 200 nm. By coupling a high-performance reverse-phase chromatographic column and such a detector Duncan and coworkers [55] have been able to measure cholesterol photometrically at 200 nm as it is eluted from the chromatographic column.

The technique offers measurement of free, total, and esterified cholesterol in blood serum samples.

The esters of cholesterol were hydrolyzed by the Abell-Kendall[42] procedure in the case of smaple preparation for total cholesterol determination. For free cholesterol, an isopropanol extract of serum was used. The mobile phase for total cholesterol was isopropanol-acetonitrile (50:50,v/v), for free cholesterol it was isopropanol-acetonitrile-water (60:30:10;v/v). The cholesterol was quantified by comparing the peak areas of the eluted cholesterol of the sample with those of the cholesterol standards. The method gives linear response over a concentration range of 0.25-5.00 g/l (0.65-12.93 mmol/l) of cholesterol. The lower limit of detection with the method is 0.25 μg ml^{-1} (0.008 mmol/l) of cholesterol.

The HPLC method provides analytical results which compare favourably with those obtained with the widely used Abell-Kendal[42] method. Steroids with a structure similar to that of cholesterol have been found to interfere.

2.2.3 Near Infrared Reflectance Analysis[56-61]

Near infrared spectrometry has been applied[56] to cholesterol determination and this has led to the development of near infrared reflectance analysis(NIRA).

Near infrared reflectance analysis is a new concept based on the near infrared reflectance widely used in the food and agricultural industries[58]. The infrared reflectance method, applied to serum cholesterol determination, requires preliminary calibration with a reference chemical analysis performed on human sera whose cholest-

erol concentration is to be measured. The calibration makes it possible to select characteristic wavelengths where the parameter, cholesterol, has specific absorption bands in the near-infrared region.

NIRA is directly performed on serum without the need for extraction or reagent. The apparatus measures reflectance(R) as the ratio of reflected energy from the sample and the incident radiation. The logarithm of reciprocal R was measured at different wavelengths and entered into a computer[57]. The principle of the measurement depends on a linear mathematical algorithm associating sample concentration with the different reflectance values. The algorithm is as follows:

$$C_1 \text{mmol/L} = A_o + a_1 \log \frac{1}{R_1 - 1} + \ldots + A_n \log \frac{1}{R_n - 1}$$

$$C_p \text{mmol/L} = A_o + a_1 \log \frac{1}{R_1 - P} + \ldots + A_n \log \frac{1}{R_{n-p}}$$

The calculation of the constants A_o, ... A_n is performed with a training set having a precise rating and covering the whole range of cholesterol concentrations. These concentrations are made with a precise reference technique [60]. This gives a matrix of the type shown above in the algorithm. The method of linear regression[61] is used to determine the values of the constants. The results obtained correlate well with the commonly used Liebermann-Burchard[45] method and with the enzymatic Trinder[59] assay.

NIRA offers an outstanding advantage of eliminating extraction procedures and the use of chemical reagents. Nevertheless it suffers a serious shortcoming in so far as the method requires about 30 samples having precise ra-

tings and covering the whole range of cholesterol concentrations.

2.2.4 Definitve Methods (Isotope Dilution/Mass Spectrometric Methods) [62-64]

A candidate reference method has been proposed[62] which is definitive and based on the isotope dilution/mass spectrometry (ID/MS) technique.

Application of mass fragmentography to serum cholesterol determination has led to a highly accruate and precise procedure. The basic principle is that the ratio between unlabeled molecules and molecules labeled with ^{2}H, ^{13}C, or ^{14}C can be determined with high accuracy and precision with the use of mass spectrometry. Isotopically-labeled molecules of the same kind as the molecules that are to be determined, are added in a fixed amount to a fixed volume of serum or urine. After extraction and eventual chromatographic purification, the ratio between the molecules is determined with a mass spectrometer equipped with a multiple-ion detector[63].

Because the labeled molecules have masses that differ from the unlabled molecules only by a few mass units, the ratio between labled molecules and unlabeled molecules can not be changed during extractions and chromatography. In multiple ion detector analysis there is little possibility of interference, because there is little chance for compounds to be present in the purified material that have both the same chromatographic properties as the molecule to be determined and the same specific ions in the mass spectrum. Bjorkem and his coworkers[63] have reported an

isotope dilution-mass spectrometric technique for determination of cholesterol in serum giving an analytical range of 5 µg-100 µg/l. In this technique d_4-cholesterol is employed and the intensities of the molecular ions are monitored at m/z 389, and 386. The Cohen[64] group, on the other hand, used d_7-cholesterol and the intensity ratio of silylated molecular ions at m/z 465 and 458 was measured for each sample and for calibration mixtures.

The esters are hydrolyzed and the cholesterol is separated and converted into the trimethylsilyl ether derivatives for measurement by gas chromatography-mass spectrometry. A weight ratio for the sample is obtained by linear interpolation of the ion-intensity ratios, and then the total cholesterol is calculated.

Apart from the need for expensive and sophisticated instruments, this ID/MS method for determination of cholesterol appears to be the most accurate with possible error in pipetting not greater than 2 %.

2.2.5 Enzymatic Methods for Determination of Cholesterol [65-85]

Enzymatic Analyses of cholesterol are generally based on measuring, directly or indirectly, either 4-cholestene-3-one or hydrogen peroxide produced from enzymic oxidation of cholesterol, and also on measuring the oxygen consumed in the oxidation process.

2.2.5.1 Ultraviolet Radiation Absorption Measurement

Interest in microbiological enzymes capable of degrading cholesterol dates back many years[65,66], and Flegg [67] and Richmond[68], after nearly two decades, have shown

that certain enzymes may be applied to the assay of cholesterol. Flegg[67] used "cholesterol dehydrogenase" isolated from Nocardia erythropolis[65] for the assay of total cholesterol by incubating saponified serum extracts with the enzyme for as long as 2 hrs and measuring the isopropanol extract of the produced 4-cholestene-3-one by its absorption at 240 nm. Richmond[68] isolated a cholesterol: oxygen oxidoreductase from another species of Nocardia and applied the purified enzyme to the direct assay of cholesterol in saponified serum.

The following sequence of reactions is involved generally in all of enzymatic assys of total cholesterol:

$$\text{cholesterol ester} + H_2O \xrightarrow{\text{cholesterol esterhydrolase}} \text{cholesterol} + \text{fatty acids}$$

$$\text{cholesterol} + O_2 \xrightarrow[\text{oxidase}]{\text{cholesterol}} \text{4-cholestene-3-one} + H_2O_2$$

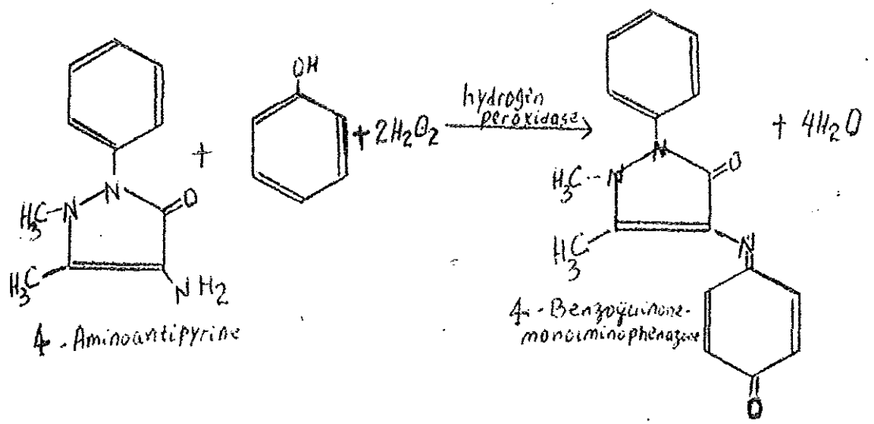
It is thus evident that cholesterol can be assayed by measuring either of the two products, 4-cholestene-3-one or H_2O_2, or the oxygen consumption in the reaction.

The isolation of cholesterol oxidase and esterase has changed the direction of research conducted in seeking analytically useful methods for determination of cholesterol; cholesterol methodologies have concentrated on enzymatic assays. There have been various versions of enzymatic methods of cholesterol detemination most of which involve measurements based on the reaction of H_2O_2 produced from oxidation of cholesterol. The main versions of enzymatic analysis of cholesterol are given below.

2.2.5.2 Visible Radiation Absorption Measurement

A colour system has been developed for cholesterol

determination involving two enzymes for free cholesterol and three for total cholesterol[69,70]. Hydrogen peroxide generated by cholesterol oxidase is measrued by oxidative coupling of 4-aminoantipyrine and phenol[59]. The reaction is given below.



The quinoneimine dye shows a broad maximum absorption band centered at about 500 nm, and its absorption is proportional to the concentration of total cholesterol.

Steroids present in serum and having structures similar to that of cholesterol interfere in this method. The method, however, is more specific than the nonenzymatic cholesterol assays of the time.

2.2.5.3 Fluorimetric Measurements

Hwang and coworkers[71] have developed a fluorimetric method in which hydrogen peroxide can be detected in concentration as low as 10^{-8}M. This kinetic method involves a flow-injection procedure and use of a reagent containing p-hydroxyphenyl acetic acid, peroxidase and ammonia. p-Hydroxy phenyl acetic acid is oxidized by H_2O_2 to form a fluorescent dimer:

This procedure as automated by the Lazaras grou[72], could be applied to the determination of cholesterol[73] with the hydrogen peroxide being produced from cholesterol.

The Peinado group[74,75], on the other hand, has developed a sensitive kinetic fluorimetric system in which a suitable reagent, 2-hydroxy-naphthaldehyde thiosemicarbazone(HNTS) is chosen to give a fluorescent product in its reaction with H_2O_2. The hydrogen peroxide produced by enzymatic oxidation of cholesterol was reacted with HNTS in an ammoniacal medium and the reaction was catalyzed by manganese(II). This system gives a fluorescent product (λ_{ex}= 390nm, λ_{em}= 450 nm) and makes possible the determination as little as 50 pmol of H_2O_2. The procedure does not require peroxidase enzyme and takes no longer than 5 minutes.

Cholesterol has been determined in egg yolk, cold liver oil, and horse serum by using this method. The optimum range of concentration for cholesterol measurement was 0.33-37 μM. Cholesterol esters were hydrolyzed with alchoholic

alkali solution prior to the measurement.

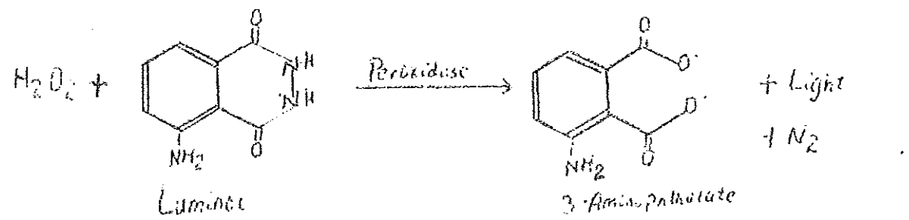
These spectrofluorimetric methods employed for determination of cholesterol in common samples appear to be sensitive, but not free from rigid control of the experimental conditions since they are based on kinetic procedures.

2.2.5.4 Chemiluminescence Measurement

The hydrogen peroxide produced from enzymatic oxidation of cholesterol has been quantified by chemiluminescence[75-80]. Malavolti et al[76] have proposed a method for the determination of cholesterol based on the reaction of H_2O_2 with luminol at $p^H 9.0$ catalyzed by horseradish peroxidase. The reaction produces chemiluminescent light the intensity of which is related to the cholesterol concentration. The sequence of the reaction is given below:

cholesterol + H_2O ester $\xrightarrow{\text{chol. ester hydrolase}}$ cholesterol + fattyacid

cholesterol + O_2 $\xrightarrow{\text{chol. oxidase}}$ 4-cholestene-3-one + H^2O_2



The detection limit in this method is 0.2 mg/100 ml or 5 μM.

The Taniguchi[75] group has introduced into the Malavolt's method a Fourier-transform digital filter to produce peak-height measurements and to increase the rapidity

and precision. The Fourier transform digital filter processes the light emission data. The sample volume required is 0.2 µl serum only. The methods are rapid but suffer from interferences[76].

2.2.5.5 Electrochemical Measurements

Researchers have used their imagination and creativity to solve the cholesterol assay problem by electrochemical procedures as well. They have concentrated on the H_2O_2 produced from enzymatic oxidation of cholesterol.

Noma and Nakayama[81] have proposed a polarographic method for rapid microdetermination of cholesterol. Cholesterol in serum was subjected to oxidation by cholesterol oxidase, in which process oxygen is consumed.

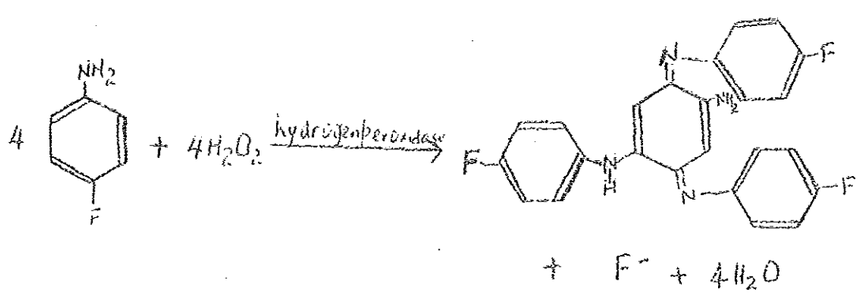
$$\text{cholesterol} + O_2 \xrightarrow{\text{chol.Oxidase}} \text{4-cholestene-3-one} + H_2O_2$$

A polarographic oxygen analyzer was used along with a circuit modified to record simultaneously the amount and rate of oxygen consumption. The concentration of cholesterol and the oxygen consumption were linearly related. The volume of sample that has been used was 10 µl. Total cholesterol has also been assayed with a hydrolysis step employing cholesterol esterase before the assay. The method is rapid but needs some sophisticated apparatus.

The other polarographic method proposed for the determination of cholesterol is that of Clark[82], in which the cholesterol esters are hydrolyzed by the esterase and the cholesterol is simultaneously oxidized by oxidase. The H_2O_2 produced is measured by a polarographic anode covered with an acetate/polycarbonate membrane. The membrane allows H_2O_2 to diffuse to the Pt-anode, where it is oxidized,

but prevents the diffusion of ascorbic acid, uric acid, and bilirubin to the electroactive surface. The method seems to be attractive for its simplicity, but preventing other electroactive species from reaching the electrode surface is difficult particularly species with the same size as that of H_2O_2.

Siddiqi[83] has used organofluorocompounds to indirectly measure the hydrogen peroxide released from enzymatic oxidation of cholesterol. The method is based on the peroxidase-catalyzed rupture of the covalent C-F bond in certain organofluoro-compounds in the presence of H_2O_2 and the measurement of liberated F^- by a fluoride ion-selective electrode. The concentration of cholesterol is directly proportional to that of F^- measured. Analytically useful reactions were observed with 4-fluoroaniline, 5-fluoro-2-methylaniline, 4-fluorophenol, 2,3,5,6-tetrofluorophenol, pentafluorophenol, and 3-fluoro-DL-tyrosine. The method requires samples of only a few µl and takes only 2 minutes. The reaction between H_2O_2 and 4-fluoroaniline is given below:



In the method as many as three enzymes are used which increases the cost of analysis.

Amperometric methods for the determination of total and free cholesterol in a fast flow system have been developed recently. Masoom[84] and Yao[85] have made use of immobilized enzyme technology and have benefited from the advent of fast flow systems in their independent efforts to devise amperometric methods for cholesterol determination. A flow injection system for assays of total and free cholesterol has been described. For the total cholesterol concentration about 2 µl sample was injected into the packed-bed reactors of immobilized cholesterol esterase and cholesterol oxidase covalently bound to silica. The free cholesterol was assayed with the same system except that the cholesterol esterase reactor was not necessary. In this method an amperometric peroxidase electrode is used. In the common electrochemical methods the H_2O_2 enzymatically produced from cholesterol is monitored amperometrically in a flow through platinum electrode. As the anodic oxidation of H_2O_2 is irreversible, the electrode operates at a high potential(0.6-0.7 v vs Ag/Ag^+). At such a potential, other electroactive species (ascorbic acid, uric acid, etc.) in biological fluids are oxidized. Therefore, the hydrogen peroxide generated in the enzyme reactor is converted to hexacyanoferrate(III) in peroxidase immobilized electrode by the following reaction:

$$H_2O_2 + 2\ Fe(CN)_6^{4-} + 2H^+ \xrightarrow{\text{peroxidase}} 2Fe(CN)_6^{3-} + 2H_2O$$

As the hexacyanoferrate(III) can be measured amperometrically at a low potenital (-50 mv vs Ag/Ag^+) there is little interference from other constituents normally present in blood serum.

The peak current is linearly related to cholesterol concentration in the range 2-160 mg/100 ml. The lowest concentration that can be detected by this flow system is 0.02 mg/100 ml. Dihydrocholesterol and 7-dihydrocholesterol have been found to interfere in the system. Also, oxidizable species (ascorbate, urate, tyrosine, cysteine, glutathione, and bilirubin) in serum may reduce some of the hexacyanoferrate(III) which is produced by the peroxidase electrode.

2.3. Extraction and Isolation of Cholesterol

The most commonly used nonenzymatic methods of cholesterol determination employ an extraction step[24]. Direct methods in which serum is added directly to colour reagents have been developed, but they all are compared with those involving extraction procedures to establish their accuracy.

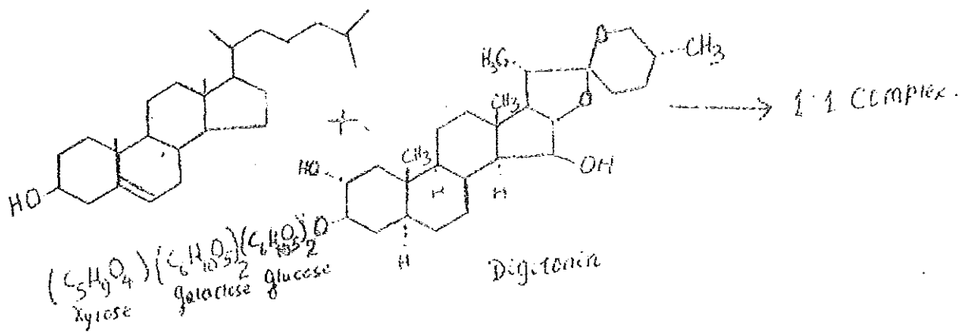
Complete extraction of cholesterol from blood serum requires dissociation of the lipid-protein bond. Chloroform extracts only a small fraction of serum cholesterol. Equipment has been devised[86] which dries serum on filter paper and extracts cholesterol in a high yield with chloroform in one continuous operation. Ether also extracts only a small fraction of cholesterol from normal serum[87]. Petroleum ether on the other hand completely extracts free and esterified cholesterol from serum[88]. The ethanol-ether system, which was introduced by Bloor in 1914 [89] effects complete extraction of cholesterol from serum.

Other solvents have also been used successfully in

the determination of cholsterol: acetic anhydride-dioxane[90], acetic anydride[41], acetone, acetone-ethanol[40], acetic acid[91], chloroform-sulfuric acid[92], chloroform-methanol [93], carbon tetrachloride [94], methanol, methanal-methanol [95], ethanol[96], isopropanol, and ethylacetate-ethanol[97].

Cholesterol extraction and isolation from blood sera and gallstones have also been accomplished by column chromatographic procedures[98].

The organic solvent extracts contain cholesterol and its esters plus other substances. Many cholesterol methods include a step in which cholesterol is selectively isolated by precipitation[24]. Digitonin has been the most commonly used precipitating agent for cholesterol. This naturally occurring glucoside forms a 1:1 complex with cholesterol.



The cholesterol-digitonin complex is quite insoluble in most solvents. The complex can be decomposed and cholesterol recovered by treatment with pyridine, acetic anhydride or acetic acid[99,100]. Cholesterol esters do not form

digitonides since they do not have free hydroxyl groups. Only naturally occurring sterols having a 3-hydroxyl group form digitonides[24]. The precipitation of cholesterol digitonide has been facilitated by aluminium hydroxide [101] and Al^{3+} in acidic solution[102]. On the other hand, digitonin itself reacts in the Liebermann-Burchard reaction [47,103] causing a positive error unless the digitonin is removed prior to colour formation.

3. OBJECTIVE OF THE PRESENT WORK

The literature review in the previous section reveals that quite a large number of methods, enzymatic and non-enzymatic, exist for the quantitative determination of cholesterol. The nonenzymatic chemical methods used for routine analysis of total and free cholesterol in serum[44,46] are rather non-specific and susceptible to various interferences, and entail the use of strongly acidic, corrosive and hazardous reagents, particularly in the mostly used Liebermann-Burchard[44] method. It should be recalled that total and free cholesterol can be determined by enzymatic methods with photometric measurement of either 4-Cholestene-3-one[68] or H_2O_2[70] formed in the reaction sequence given in section 2.2.1 The measurement of 4-cholestene3-one by its UV radiation absorption is not favoured because of the inherent interference from other compounds in the biological samples showing strong absorbance in the UV region.

Chemiluminescene[76] or fluorimetric[72] measurements of H_2O_2 produced by cholesterol oxidase make possible the cholesterol assay in blood sera and other common samples. Cholesterol oxidase-dependent assays involving the electrochemical quantification of oxygen consumption[87] or H_2O_2 production may also be used; however, they require sophisticated and carefully adjusted instrumentation to provide reliable results.

In one of the enzymatic methods, hydrogen peroxide produced from the enzymatic oxidation of cholesterol is reacted with 4-aminoantipyrine dye[70]. This is regard-

ed as the method of choice among the enzymatic methods[15]. However, this method as well as all enzymatic methods involving use of cholesterol oxidase are stated [104] to suffer from interference since the enzyme oxidizes not only cholesterol but also other steroids present in blood sera or other common samples. Furthermore, problems have been revealed in standards, blanks, and the extent of hydrolysis and completeness of oxidation[104]. Cooper et al[104] in examining the most preferred enzymatic method have detected intereferences by colour developing reagents, lack of comparability of chemical reaction rates between standards and serum, and lack of reproducibility of activity of commercial enzymes. Further studies by the same group on standards, the extent of hydrolysis, the effect of colour developing reagents on cholesterol reaction products have generated uncertainity about the extent of hydrolysis of cholesterol esters. Difficulty was experienced in obtaining linearity of calibration curves.

The gas chromatographic and high performance liquid chromatographic techniques proposed for cholesterol assay are not free from draw backs. The GC method is susceptible to minor changes in experimental conditions whereas the HPLC suffers from interferences[55]. Near infrared reflectance analysis also has a serious practical disadvantage as it requires sera from about thirty persons.

Only the ID/MS method seems to recieve no criticism except that the instruments are expensive and the operation needs skilled personnel.

It is therefore, obvious that a simple, accurate, and precise method for the determination of cholesterol

in serum and/or other common matrices is still required. This is evident from the extent of research which has been and is being conducted.

The objective of this work is thus to develop a simple and precise method for the determination of cholesterol using a suitable chemical reaction of cholesterol that involves a procedure in which common laboratory facilities (i.e., reagents and instruments) are sued.

Dichromate is used for clinical determination of the alcohol level of blood[105]. In the procedure, alcohol in a microdiffusion unit is isolated by allowing it to diffuse into a compartment containing acidified dichromate solution. The change in light absorption of the dichromate solution is monitored spectrophotometrically. The analytical application of the dichromate oxidation of the alcoholic functionality has not been extended to cholesterol as can be seen from literature survey, hence it could form the basis for the development of a method for the quantitative determination of cholesterol.

The reaction between cholesterol and dichromate has been studied at various temperatures ranging from 0 to 87^{o}C in acetic acid-benzene[1,106,107]. Fieser[106,107] obtained various organic products from the oxidation reaction.

The major organic product at temperatures below 15^{o}C is 4-cholestene-3,6-dione. At higher tempeatures carboxylic acids are produced from cholesterol whereas at all temperatures the reduction product of dichromate is chromium(III). The chromium(III) gives a coloured system absorbing light in the visible region. The goal of the

present work has been to study wether the light absorption of chromium(III) produced from oxidation of cholesterol could be correlated with concentration of cholesterol, and wether this could be developed into a method for the analysis of blood sera.

4. EXPERIMENTAL

4.1 Equipments

A Beckman UV-VIS Spectrophotometer Model 24 equipped with 1-cm cuvettes and Beckman Recorder were used for absorption measurements. A Vortex Stirrer (Griffin & George) and Centrifuging Machine (LABOFUGE, GMBH) were used for sample preparation. A Gallenkamp water bath with temperature regulator was used for maintaining constant temprature.

4.2 Reagents and Chemicals

Standard Cholesterol Solution. A standard solution of cholesterol (10 mg ml^{-1}) was prepared by dissolving 250 mg cholesterol (BDH high puirty grade) in 25 ml benzene (BDH, reagent grade); or 100 mg cholesterol was dissolved in 25 ml (4 mg ml^{-1}) petroleum ether 40-60^{o}C(BDH, Analar).

Dichromate Solution. A 0.25 M dichromate solution was prepared by dissolving 15.5024 g reagent grade sodium dichromate dihydrate in 100 ml of glacial acetic acid (May & Baker, Analar 99.7 %).

Digitonin Solution. A 1 % (w/v) solution of digitonin (Sigma, reagent grade) was prepared in water-ethyl alcohol (1:1,v/v).

Alchoholic Potassium Hydroxide, 0.5 N. A stock solution of 50 % (w/v) aqueous solution of KOH was prepared by dissolving 50 g of KOH in 100 ml of distilled water. Just before saponification of serum, the stock solution was diluted 16-fold with 96 % ethyl alcohol.

4.3 Sample Preparation

(i) Sample Collection. Sera collected from the National Research Institute of Health, Addis Ababa, Ethiopia, were stored at $4^{o}C$ in a refrigerator and analyzed within four days. The sera were vortexed for two minutes before use.

(ii) Procedure for Separation of Free Cholesterol. A serum sample (0.2 ml) was transferred into a 15 ml centrifuge tube with a glass stopper and treated with 3 ml of acetone-ethanol) (1:1,v/v) mixture. To this was added 1 ml of 1 % digitonin solution in the 1:1 water-ethanol and the tube was kept at $0^{o}C$ for 30 minutes to ensure complete precipitation of free cholesterol as digitonide. The tube was removed from the refrigerator and centrifuged for 15 minutes. The supernatant liquid was decanted and the precipitate was washed with distilled water to remove the alcoholic solvent.

The digitonide was then broken down by addition of 1.0 ml acetic acid followed by incubation at $60^{o}C$ for 30 minutes. Ten milliliters of petroleum ether ($40-60^{o}C$) was added to the reaction mixture and the mixture swirled until the acetic acid was mixed evenly with the petroleum ether and the precipitate of digitonin was suspended evenly. The digitonin was separated from the cholesterol solution by centrifuging for about 15 minutes. The supernatant liquid transferred to another test tube and the digitonin was washed with 5 ml petroleum ether. The combined petroleum ether extracts were concentrated to 1 ml. The petroleum ether extract of cholesterol was either used as such or evaporated to dryness and the residue was dissolved in benzene to give a suitable sample for the assay, for the spectrophoto-

metric determination of cholesterol.

(iii) Procedure for Separation of Total Cholesterol. A 2 ml aliquot of serum was transferred into a 40-ml centrifuge tube. To this was added 25 ml of 0.5 N alchoholic KOH. The contents were mixed by inverting several times. The alkaline serum was then placed in a water bath at 60°C for 40 minutes. After cooling to room tempeature, the saponified serum was transferred into a separatory funnel, and 15 ml water was added to it. The cholesterol was extracted with 10 ml of petroleum ether by shaking vigorously for 2 to 3 minutes. The petroleum ether layer was then transferred to a 25-ml volumetric flask and the aqueous phase was washed with 10 ml of petroleum ether. The extracts were combined and then diluted to 25 ml with petroleum ether in the volumetric flask. A 2.5 ml aliquot of this solution was treated as described in section 4.3(ii).

4.4 Procedure for Sepectrophotometric Determination of Cholesterol

A 5 ml aliquot of dichromate solution (0.5 M) was transferred into a 50 ml Pyrex conical flask and the cholesterol solution in benzene (1 ml) was added to it. The solution was diluted to 10 ml with glacial acetic acid. Alternatively, 1 ml of the cholesterol solution in petroleum ether was added to 10 ml of 0.25 M dichromate solution in glacial acetic acid. The solution was heated at 80°C for 1 hour, and then cooled to room temperature. The absorbance of the solution was measured at 585 nm against the reagent blank.

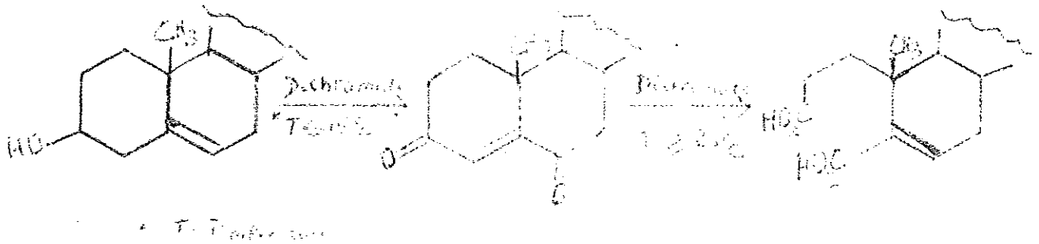
For a calibration curve, 0.2, 0.4, 0.6,0.8, and 1.0 ml

samples of the standard cholesterol solution in benzene (10 mg ml^{-1}) or in petroleum ether (4 mg ml^{-1}) were treated as in the above procedure.

5. RESULTS AND DISCUSSION

5.1 Colorimetric Reaction

In Fieser's study [106,107] of the oxidation of cholesterol with dichromate at various temperatures and with varying amounts of the oxidant, 4-cholestene-3,6-dione was found to be the chief product obtained from the oxidation at temperatures below 15°C. However, as the temperature rised above 25°C the dione was converted to acids. Furthermore as the amount of dichromate was increased relative to the amount of cholesterol, the more highly oxidized products, diacids, were obtained.



The Fieser's experiment is briefly described below with due emphasis given to factors controlling the yields of the products.

A solution of 51.2 g (172 mmoles) sodium dichromate dihydrate in 200 ml acetic acid was cooled to 15°C. Cholesterol solution was prepared by dissolving 20 g (52 mmoles) of cholesterol in 200 ml of benzene, and then cooled to 10°C; diluted with 200 ml of acetic acid and the temperature was adjusted to 15°C. The cooled dichromate solution was added into the cholesterol solution. The reaction mixture was placed in a refrigerator (8 °C) and let

stand overnight. After separation and purification of the neutral product, 8.6 g (42%) 4-cholestene-3,6-dione was collected. Fieser pointed out that this enedione was found to be totally oxidized to acids in the same reaction period when the temperature was allowed to rise to 25°C. Under the optimum conditions for formation of the enedione, two diacids namely, Diels acid (3,4-seco-5-cholestene-3,4-dioic acid) and 3-keto-Δ^4-6,7-secodioic acids were also found to occur together with the enedione. A separate experiment on the oxidation reaction with smaller quantity of sodium dichromate dihydrate (20.4 g instead of 51.2 g) was allowed for 1.5 h at 20°C. The purified acid fraction gave 1.9 % Diels acid. In another oxidation conducted at 25°C for 6 h the yield of the acid was 2.9%.

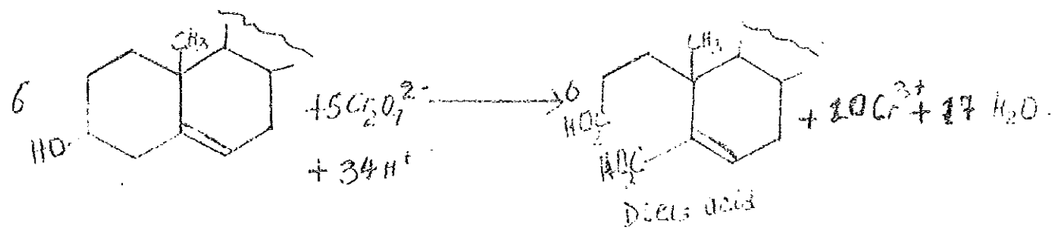
These experiments showed that the higher yields of the diacid were obtained at higher reaction temperatures and with longer reaction times.

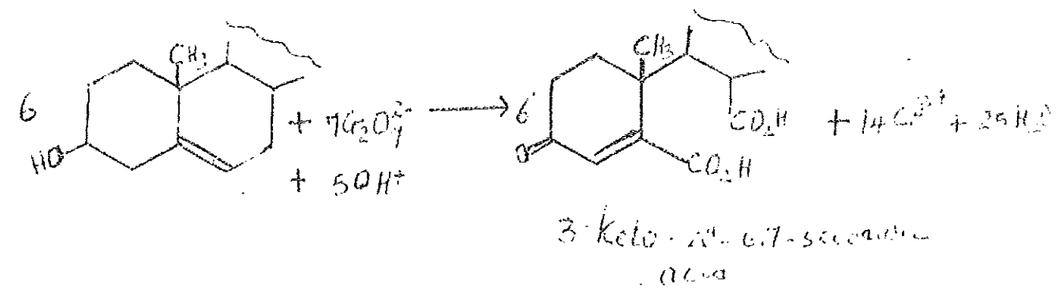
Fieser carried out also the oxidation by employing a molar ratio (1.3:1.0) of dichromate to cholesterol lower than that (3.4:1.0) used in the procedure given above and obtained a product that is not oxidized as highly as 4-cholestene-3,6-dione. A cholesterol solution (5 g in 200 ml acetic acid) cooled to 20°C was mixed with a dichromate solution (5.1 g $Na_2Cr_2O_7{\cdot}2H_2O$ in 50 ml acetic acid) and let stand at 16-20°C for 5 h. About 8 % 4-cholestene-6β-ol-3-one was obtained along with 4-cholestene-3,6-dione (about 20 %).

Thus it can be obviously seen that the more highly oxidized products were obtained by using higher molar ratios of dichromate to cholesterol, by conducting the reaction at higher temperatures, and with longer reaction

times. The stoichiometry was found to be a function of these experimental parameters.

In the present investigation the molar ratio of dichromate to cholesterol and the reaction temperature were much higher than those used in Fieser's experiments mainly because of the necessity of completeness of tbe reaction and of consistent products to be monitored for quantitative assay of cholesterol. However, no specific set of experiment was carried out to establish the stoichiometry. The oxidation reaction was codnducted at 80°C and with dichromate concentration of about 100 times that of the cholesterol. By comparison with Fieser's [106,107] experiments it is believed that the present system gives consistently the same and highly oxidized species, Diels acid and/or 3-keto- Δ^4-6,7- secodioic acid. The reaction scheme can be represented as:





Because of the practical difficulties in the quantitative separation and measurement of the organic (oxidation) products, the reduction product, i.e., chromium(III), was selected as the species for photometric measurement at 585nm. The degree of absorption of chromium(III) formed the basis of the determination of the concentration of cholesterol in the reaction system.

5.2 Absorption Spectra

The absorption spectrum of dichromate in acetic acid has been recorded both in the absence and in the presence of cholesterol (Fig. 3). The spectra show that in the presence of cholesterol the band at 440 nm decreased in intensity and a new band appread at 585 nm indicating the reduction of dichromate to chromium(III) by cholesterol i.e., the oxidation of cholesterol by dichromate. The spectrum of dichromate in the presence of cholesterol has been compared with that of chromium(III) solution in acetic acid (Fig. 4). The shape of the absorption band in the region 575-585 nm has been found to be identical in the two spectra with a small shift in the wavelength of absorption maximum confirming the absorption band at 585 nm in the spectrum of dichromate with cholesterol is that of chromium(III).

The regular octahedral hexaaquo complex of chromium in the oxidation state + 3, $[Cr(H_2O)_6]^{3+}$, is well known [109] in aqueous solutions and numerous salts such as $[Cr(H_2O)_6]Cl_3$. In aquo ion the electronic transition bands are found at 575, 404 and 270 nm. The acetate complex of chromium in the oxidation state +3 is also known and is established [109] to have the basic unit $[Cr_3O(CH_3COO)_6L_3]^+$, where L

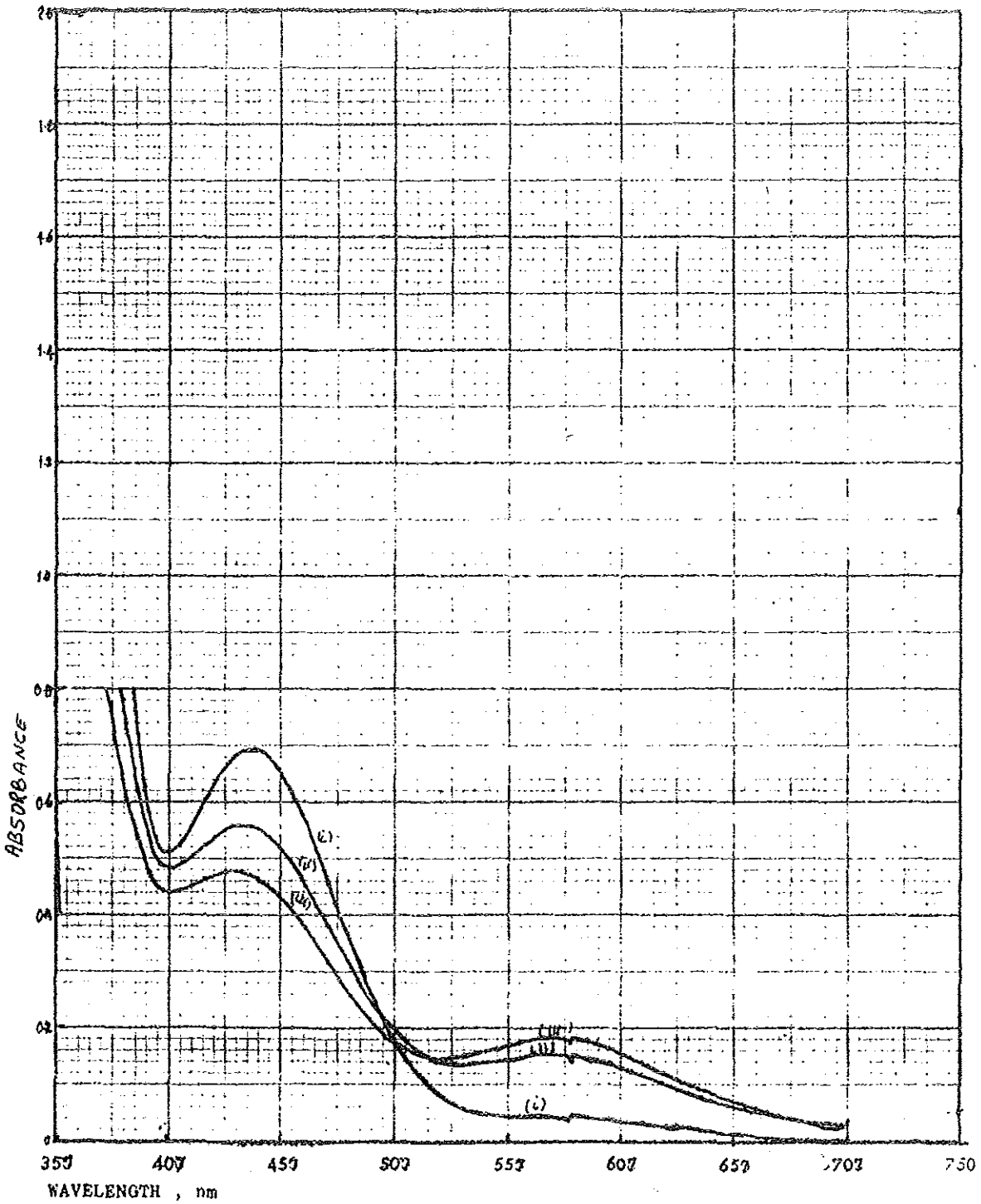


Fig. 3 Absorption spectra of (i) 0.025 M $Cr_2O_7^{2-}$ solution in benzene-acetic acid system, (ii) 0.025 M $Cr_2O_7^{2-}$ + 3.6 x 10^{-4}M cholesterol in benzene-acetic acid, and (iii) 0.025 M $Cr_2O_7^{2-}$ + 4.0 x 10^{-4} M cholesterol in benzene-acetic acid solvent system.

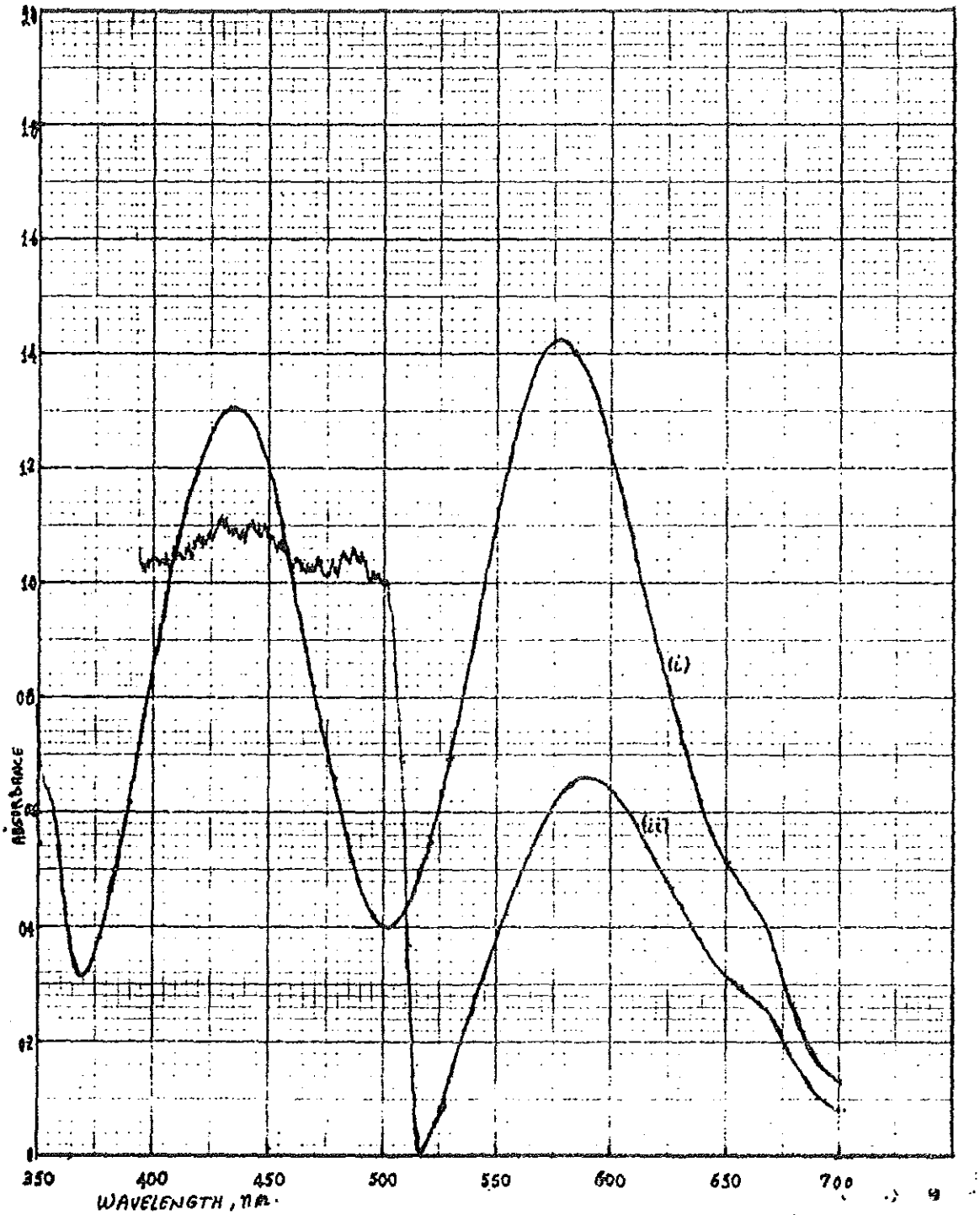


Fig. 4 Absorption spectra of (i) 0.04 M $CrCl_3.6H_2O$ in acetic acid recorded against solvent and (ii) 0.05 M $Cr_2O_7^{2-}$ + 2.59 x 10^{-4} M cholesterol in benzene-acetic acid recorded against reagent blank, 0.05 M $Cr_2O_7^{2-}$ solution.

stands for the ligand molecule. In the present system the solvent is acetic acid and the thermal equilibrium gives rise to acetate ions which will form the complex species with chromium in the oxidation state +3. Thus "chromium(III)" is meant to refer to this species throughout this thesis.

The shift in the positions of transition bands (Fig. 4) from those of the $[Cr(H_2O)_6]^{3+}$ can be accounted by considering the location of the two ligands (acetate and H_2O) in the spectrochemical series, the stabilization ability of the ligands, and the steric effects. In $[Cr_3O(CH_3COO)_6L_3]^+$ there is an equilateral triangle of Cr atoms with an oxygen atom at the center. There are two bridging CH_3COO groups across each edge of the triangle. Finally, a molecule L is coordinated to each Cr atom so that it has a distorted octahedral coordination.

There is also a wavelength shift of about 12 nm (Fig. 4) between the band at 577 nm in the spectrum of $CrCl_3.6H_2O$ in acetic acid and the band at 589 nm in the spectrum of the chromium(III) liberated from the oxidation reaction. This may be attributed to the effect of the anions which in the former case may lead to a complex that has one or more chloride ions held within the inner sphere as in $Cr_3O(CH_3COO)ClL_2$, and to solvent effect since in the latter system a small amount of benzene was introduced.

5.3 Effect of Experimental Variables

Both the accuracy and precision of the photometric determination depend on the completeness of the colorimetric reaction and this in turn depends on the proper adjustment of the experimental varibales. Hence the effect of se-

veral experimental varibables have been studied in order to establish the optimum conditions. The effect of a particular variable was studied by measuring the absorbance of the system following the procedure described in the experimental section, keeping all other experimental variables constant, except the one under study.

5.3.1 Stability of the Reagent. The stability of the reagent (dichromate solution) was studied by measuring its absorbance at 585 nm and 440 nm at different time intervals before it was used for oxidation of cholesterol. The absorption at 585 nm was measured using two different concentrations (0.025 M and 0.5 M) of dichromate covering the concentration range giving a linear response in the optimized procedure. The absorption of 0.5 M dichromate solution at 440 nm was too high for measurement with a 1 cm cell.

Table 2. Stability of dichromate solution with time at room temperature.

Time hr.	Absorbance of 0.025 M $Cr_2O_7^{2-}$		Absorbance of 0.5 M $Cr_2O_7^{2-}$
	585 nm	440	at 585 nm
0	0.208	0.645	0.264
½	0.218	0.642	0.272
1	0.215	0.640	0.271
2	0.221	0.638	0.273
3	0.220	0.640	0.270
4	0.218	0.640	0.272
6	0.220	0.639	0.273
12	0.220	0.640	0.271
24	0.220	0.638	0.274
36	0.235	0.630	0.285
48	0.267	0.616	0.325

Results obtained (Table 2) show that the dichromate solution in acetic acid remains stable for 24 hours only indicating that fresh solution should be prepared to be used within one day.

5.3.2 Effect of Temperature and Heating Time

The dichromate oxidation has been followed photometrically at different temperatures with various heating times by keeping other parameters constant (Table 3). Constant results were obtained with a heating time of one hour at 80°C and hence this temperature was used throughout all subsequent experiments. This result indicates that the complete oxidation of cholesterol to the final products can only be achieved by heating the reaction mixture for 1 hr at 80°C.

Table 3. Effect of temperature and heating time

$[Cr_2O_7^{2-}]$ = 0.25 M; [Chol] = 2.59 x 10^{-3}M

Time (hr)	*Absorbance at 585 nm vs. blank			
Temp.	**20°C	40°C	60°C	80°C
½	0.342	0.572	0.634	0.781
1	0.360	0.636	0.659	0.859
2	0.385	0.720	0.740	0.854
3	0.402	0.760	0.790	0.854

*Measurement immediately after ooling to room temperature.

**Room tempeature.

5.3.3 Time of Measurement

The time of the measurement of the colour was studied by measuring the absorbance of the solution at different

time intervals, and it was found that provided the oxidation was made at 30°C, the absorbance of the product solution remained constant for up to 48 h. The results are summarized in Table 4. These results indicate that with a reaction temperature of 80°C the time interval before the absorbance measurement is not critical provided that the solutions are properly closed and kept at room temperature. With oxidation at lower reaction temperatures the absorbance continued to increase with time indicating that the reaction was not complete and it continued slowly even at room temperature.

Table 4. Time of absorbance measurement
Heating time = 1 hr.; $[Cr_2O_7^{2-}]$ = 0.25 M; [Chol] = 2.59×10^{-3} M.

*Time (h)	Abs. at 585 nm.			
Temp.	20°C	40°C	60°C	80°C
½	0.359	0.636	0.654	0.854
1	0.372	0.647	0.679	0.853
3	0.385	0.682	0.706	0.854
6	0.415	0.736	0.721	0.854
12	0.442	0.751	0.786	0.852
24	0.493	0.772	0.803	0.854
48	0.515	0.785	9.815	0.854

*Interval between oxidation and absorbance measurement.

5.3.4 Effect of the Dichromate Concentration

The effect of dichromate concentration on the reaction system was investigated by keeping the concentration of cholesterol constant (2.59×10^{-3} M) and varying the dichromate concentration over a wide range. It was found that the absorbance value of the system remain-

ed constant for oxidations with dichromate concentration in the range 0.05-0.25 M. With lower dichromate concentrations, the absorbance of the system decreased indicating that the reaction was incomplete whereas with higher concentrations of dichromate the absorbance remained constant indicating that the excess of dichromate had no effect on the reaction system. The results are given in Table 5.

Table 5. The effect of dichromate concentration on the oxidation. Reaction temperature = 80°C; Heating time = 1 hr. [Chol] = 2.59×10^{-3} M

$[Cr_2O_7^{2-}]$	$[Cr_2O_7^{2-}]$:[CHOL]	Abs at 585 nm
0.01	4	0.536
0.02	8	0.536
0.05	20	0.844
0.10	40	0.850
0.15	60	0.854
0.25	100	0.850

5.3.5 Effect of Solvent Composition

Fieser[108] used benzene to dissolve cholesterol in his study of the oxidation of cholesterol by dichromate in acetic acid. In the present investigation was also used benzene to introduce cholesterol into the reaction system. With the aim of broadening the choice of suitable solvents and avoiding the use of benzene, the potentially carcinogenic solvent, several other solvents such as carbon tetrachloride, chloroform, hexane and petroleum ether were tested. Among these solvents only petroleum ether was found to be suitable. Hexane has been omitted because a pre-

cipitate of dichromate appeared when the chol. esterol solution in hexane was added to the dichromate solution in acetic acid. It is interesting that hexane and petroleum ether gave different results. This may be due to the high volatility of petroleum ether (bp 40-60°) compared with hexane (bp 69°). Petroleum ether vaporized at the reaction temperature (80°C) whereas hexane remained in the reaction system perhaps because of the boiling point elevation effect from the acetic acid. Hexane in the solution lowered the polarity of the medium and hence the precipitate appeared. Chloroform was ruled out because of the very high absorbance of the blank even at room temperature. Carbon tetrachloride also gave a blank with high absorbance (Table 6).

Furthermore the effects of varying the percentage of benzene in the benzene/acetic acid mixture have been studied. The results are given in table 7.

Table 6. Solvent choice

[Chol] = 2.59 x 10^{-3} M; [$Cr_2O_7^{2-}$] = 0.25 M

Solvent	Reaction temperature	*Abs. at 585 nm		Remark
		Blank	Test soln	
Hexane	room temp.	-	-	precipitation
$CHCl_3$	room temp.	2.480	2.640	not suitable
CCl_4	80°C	0.638	1.020	low sensitivity
Pet.ether	80°C	0.325	1.165	Suitable
Benzene	80°C	0.250	1.100	Suitable

*Measured against solvent.

Table 7. Effect of the composition of the benzene/acetic acid mixture.
$[Cr_2O_7^{2-}]$ = 0.25 M; [Chol*] = 2.59×10^{-3} M
Total volume = 10 ml;
Temp. = 80°C, Heating time = 1 hr.

Volume of benzene (ml)	Volume acetic acid (ml)	%(v/v) benzene	Abs at 585 nm.
0.0	10.0	0.0	0.850
0.5	9.5	5.0	0.851
1.0	9.0	10.0	0.850
1.5	8.5	15.0	0.850
2.0	8.0	20.0	0.848
3.0	7.0	30.0	0.830
3.5	6.5	35.0	0.808

*Introduced in the form of solution in the stated volume of benzene except in the zero percentage case, where 10 mg solid cholesterol was added directly.

It was found that the system gave constant absorbance up to 20% (v/v) benzene. At higher percentages of benzene the dichromate began to precipitate.

5.3.6 Optimum Experimental Conditions

On the basis of the results obtained by studying the effects of experimental variables on the reaction system, the optimum experimental conditions are summarized in Table 8.

Table 8. Optimum experimental conditions

Parameter	Condition
Temperature	80°C
Heating time	1 hr.
Percentage composition of benzene	$\leq$ 20%
$[Cr_2O_7^{2-}]$	0.05-0.25 M
Measurement time	up to 48 h.

These results show that the system is relatively free from the rigid control of experimental variables.

5.4 Photometric Characteristics

The coloured system was found to obey the Beer's law in the concentration range 0.01-2.00 mg ml^{-1} (10-2000 ppm) of cholesterol in the benzene-acetic acid solvent system and 0.01-1.0 mg ml^{-1} (10-1000 ppm) of cholesterol in the petroleum ether-acetic acid solvent system. The results are given in Tables 9 and 10 and the calibration curves are shown in Figs. 5 and 6.

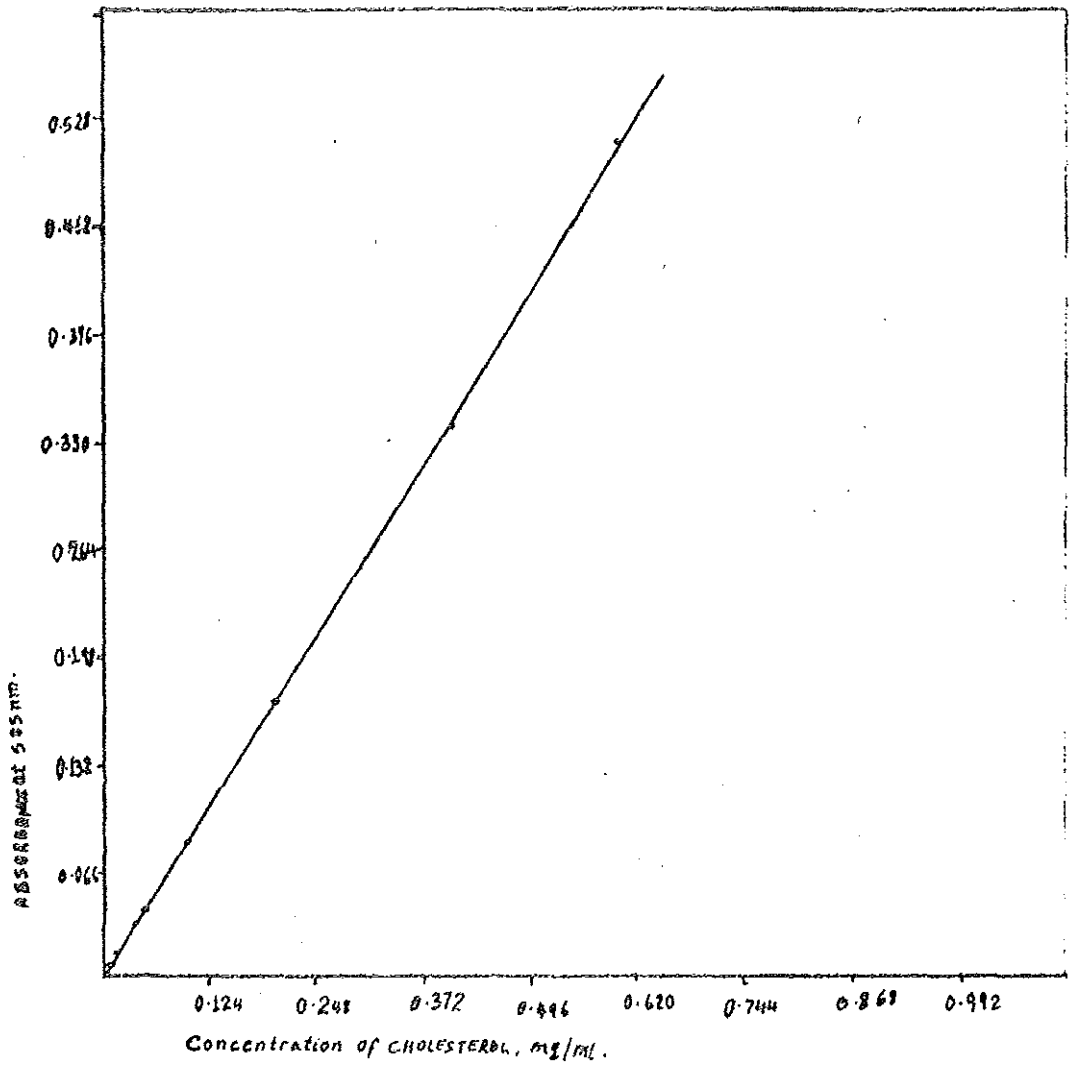


Fig. 5 Calibration curve for determination of cholesterol in benzene-acetic acid solvent system.

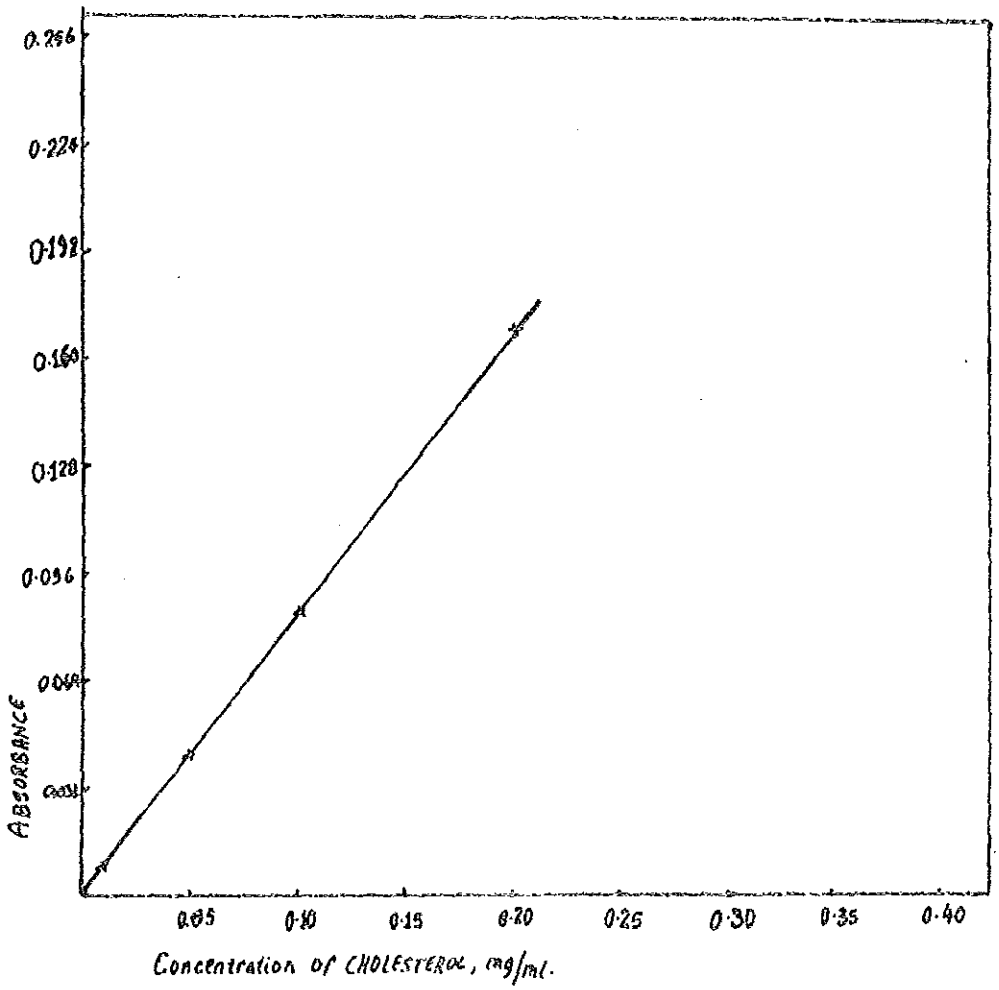


Fig.6 Calibration curve for determination of cholesterol in petroleum ether-acetic acid solvent system.

Table 9. Calibration curve data for the benzene-acetic acid solvent system.
$[Cr_2O_7^{2-}]$ = 0.25 M; Reaction temp = 80°C
Heating time = 1 h

Concentration of Cholesterol		Abs at 585 nm
$Mg.ml^{-1}$	ppm	
0.01	10	0.009
0.02	20	0.017
0.04	40	0.034
0.05	50	0.043
0.10	100	0.085
0.20	200	0.171
0.40	400	0.342
0.06	600	0.512
0.08	800	0.682
1.00	1000	0.854
1.50	1500	1.280
2.00	2000	1.6950

Table 10. Calibration curve data for the petroleum ether-acetic acid solvent system.
$[Cr_2O_7^{2-}]$ = 0.25 M; Reaction temp = 80°C;
Heating time = 1 h

Concentration of cholestrol		Absorbance at
$mg.ml^{-1}$	ppm	585 nm
0.01	10	0.008
0.05	50	0.041
0.10	100	0.084
0.20	200	0.168
0.40	400	0.330
0.60	600	0.498
0.80	800	0.666
1.00	1000	0.842

The optimum concentration range, i.e., the concentration limits within which the relative photometric error is less than 1 % as, evaluated from the Ringbom plot [110] (% transmittance versus logarithm of molar concentration)

for the determination of cholesterol was found to be 0.16-1.00 mg ml^{-1} both in the benzene-acetic acid and petroleum ether-acetic acid solvent systems. The plots are shown in Figs. 7 and 8.

The photometric sensitivity [111], i.e., the concentration that gives an absorbance A = 0.001 measured in a cuvette of 1 cm pathlength was found to be 1.1 µg ml^{-1} (1.1 ppm) of cholesterol in benzene-acetic acid solvent system and 1.25 µg/ml (1.25 ppm) cholesterol in the petroleum ether-acetic acid solvent system.

The lower limit of determination [112], that is the concentration which gives an absorbance that is equal to three times the standard deviation of blank determination, was found to be 5.4 µg ml^{-1} cholesterol using benzene-acetic acid and 4.72 µg ml^{-1} of cholesterol using petroleum ether-acetic acid. The results are given in Table 11.

Table 11. Evaluation of lower limit of determination $[Cr_2O_7^{2-}]$ = 0.25 M; No cholesterol

Solvent system	Number of analysis	Abs at 585 nm	Mean Abs	Standard deviation	Lower Limit of Determination
Benzene-acetic acid	3	0.271 0.275 0.273	0.273	1.63×10^{-3}	5.4 µg ml^{-1}
Pet. ether-acetic acid	3	0.326 0.329 0.329	0.3273	1.26×10^{-3}	4.72 µg ml^{-1}

The precision of the method has been evaluated by triplicate analyses of samples each containing 1 mg ml^{-1} of cholesterol. The relative standard deviation was found to be 0.3 %. The results are summarized in Table 12.

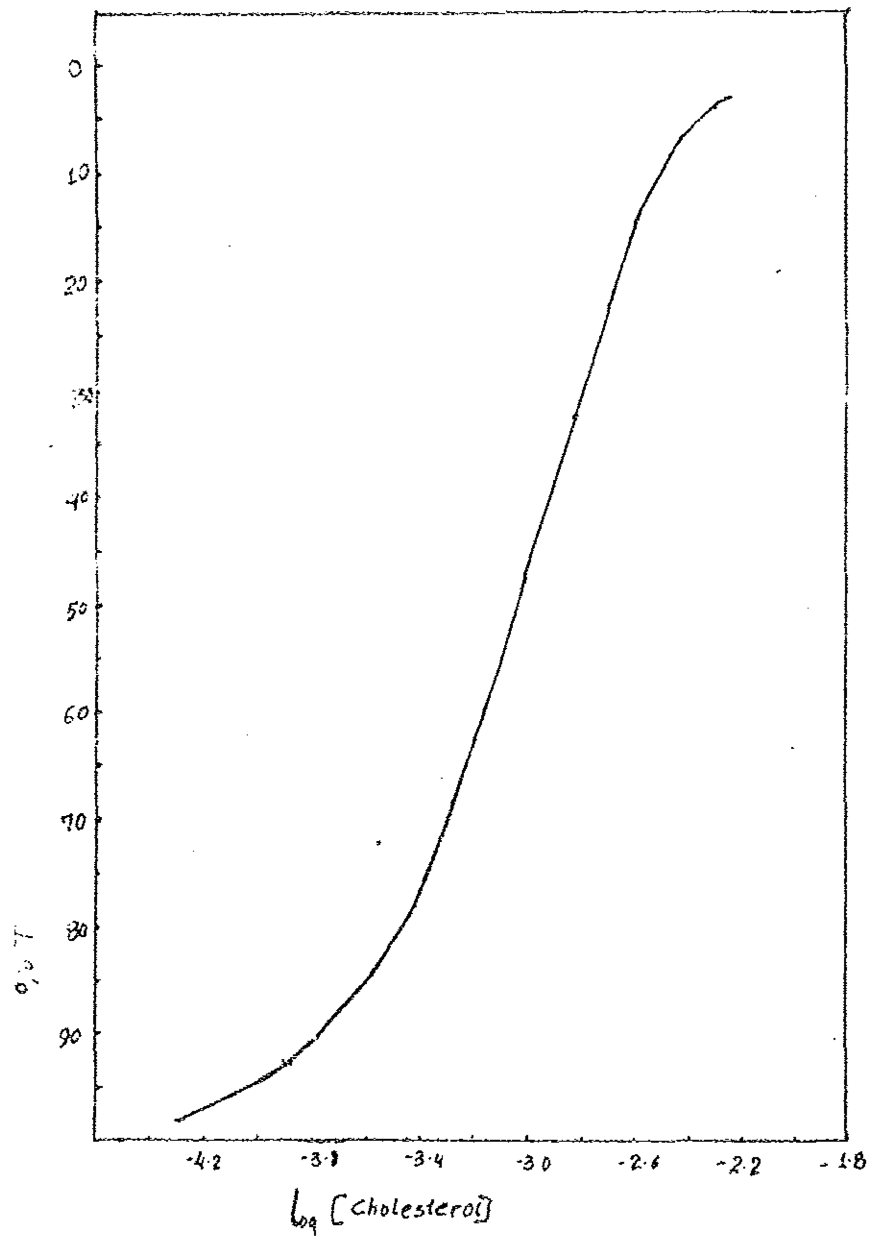


Fig. 7 Ringbom's plot for the determination of cholesterol, [illegible] acid system

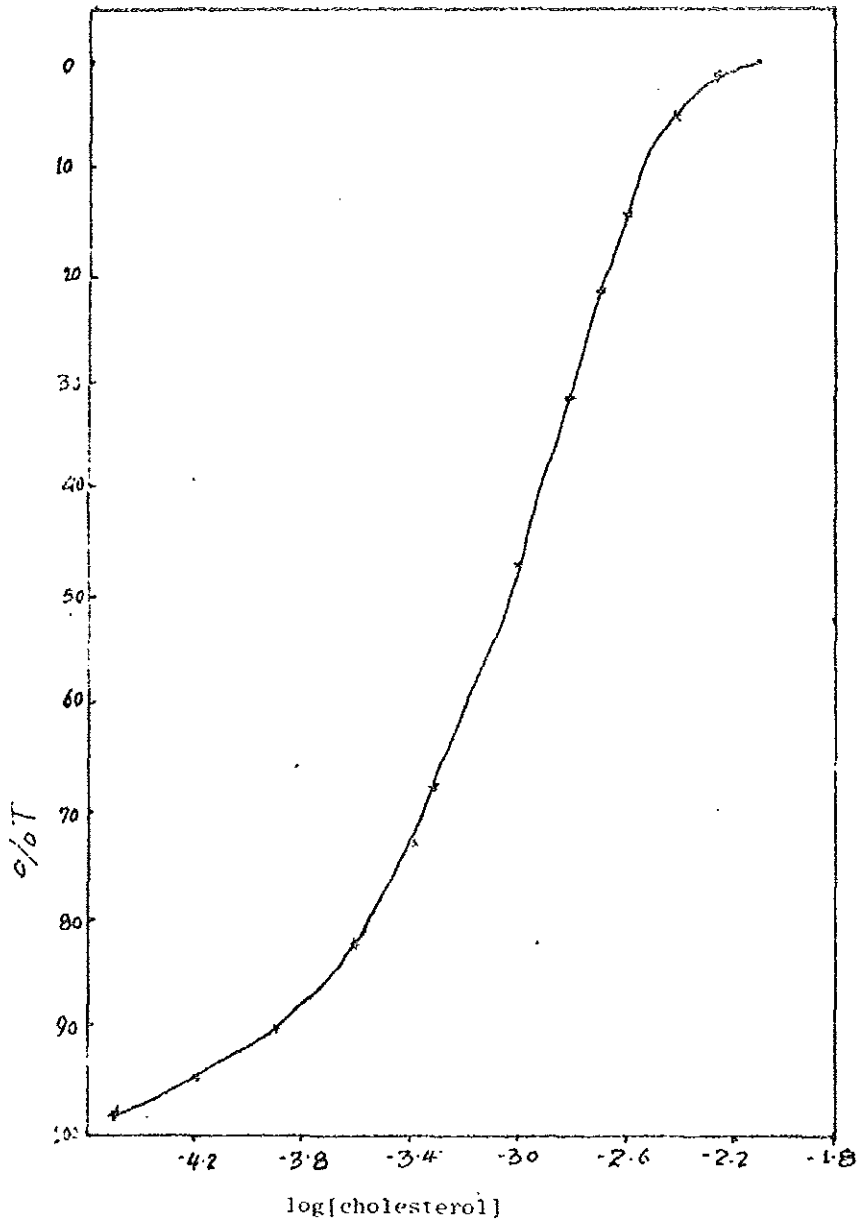


Fig. 8 Ringbom's plot for the determination of cholesterol in petroleum ether-acetic acid.

Table 12. Evaluation of the precision of the method cholesterol = 10 mg/10 ml; solvent system: benzene-acetic acid. Heating time = 1 hr.

Number of Analysis	Abs at 585 nm	Mean Abs	Standard Dev.	Relative standard Dev.
3	0.855 0.858 0.861	0.858	2.48×10^{-3}	0.3 %

These results indicate that the proposed method is fairly sensitive and gives reproducible results. It should be noted that the data given in each Table in the thesis are the average of tripliate measurements and are within ±1 % error.

5.5 Application of the Method to Blood Serum

In order to asses the analytical potentialities of the proposed method, the method was applied to the determination of free and total cholesterol in serum samples. Ten samples taken from different persons were collected from the National Research Institute of Health, Addis Ababa, Ethiopia. Cholesterol was determined by the proposed method after separating it by using digitonin following Sobel's[100] procedure, described in the Experimental Section. The total cholesterol was detemined after hydrolyzing the esterified cholesterol in serum with alcolic KOH according to the procedure given in the Experimental Section. The results of the total cholesterol obtained by the proposed method have been compared with the clinical results obtained by the Pearson's [112) method. This me-

thod is employed for routine analysis of total cholesterol in the National Research Institute of Health (NRIH), Addis Ababa, Ethiopia, wherefrom the clinical results (Table 13) used for comparison were collected.

The principle of the Pearson's procedure is based on the Liebermann-Burchard reaction [114]. In the procedure, a solution of p-tolene sulphonic acid in glacial acetic acid and acetic anhydride is added to serum. The mixture is allowed to stand until the material cools and concentrated sulphuric acid is then added. The colour measurement is made after 20 minutes at 560 nm. The simplicity of the procedure accounts for its use in the routine clinical analysis of cholesterol.

The results given in Table 13 show that there is a good agreement between the proposed method and the Pearson's method since the relative errors between the results of the two methods are less than 2 %. Hence the proposed method can be applied to the analysis of blood sera for cholesterol.

Table 13. Results of cholesterol determination in blood sera.

Sample	Free cholesterol by proposed method. (mg/100 ml)	Total cholesterol by proposed method. (mg/100 ml)	Total cholesterol by clinical method. (mg/100 ml)
64/10	58	195±1	196
44/11	54	209±1	211
55/11	47	157±2	155
74/11	73	261±3	258
6/10	51	157±1	160
17/10	52	178±2	175
26/10	54	171±2	168
53/10	49	179±1	180
2/11	61	208±2	204
34/11	60	208±3	211

6. CONCLUSION

A method has been developed for quantitative determination of free and total cholesterol in blood sera. The method is based on spectophotometric measurement of chromium(III) produced from oxidation-reduction reaction taking place between cholesterol and dichromate in acetic acid medium. It is simple, inexpensive, and precise. The chemicals and equipments necessary to carry out the analysis of cholesterol by the proposed method are those which are available in most common laboratories. The interference from other substances except the companion sterols has been circumvented by selectively separating cholesterol using digitonin. The sterols normally accompanying cholesterol in blood do not exceed 3 % of the cholesterol content. Hence the proposed method can be applied to the analysis of blood serum for cholesterol.

In the Liebermann-Burchard reaction which is the basis of the routinely used methods, the measured colour system is unstable and measurement is made at a fixed time. This step makes most of the routinely used methods susceptible to low precision and poor reproducibility. In contrast, the proposed method gives reproducible results and is precise. The other advantages of the method over those based on the Liebermann-Burchard reaction are that the reagents and solvents used are less corrosive and the digitonin used for precipitating cholesterol can be recovered.

The disadvantages of the proposed method are the requirement that fresh solution of dichromate must be prepared to be used within one day and the fact that isolation of cholesterol by precipitation with digitionin is laborous and involves manipulative processes.

To develope the proposed method further, toluene can be tested wether it can be used as an alternative solvent to benzene and petroleum ether so that the problem of hazards from the carcinogenecity of benzene and the problem of concentration changes of solutions in petroleum ether (40-60^{o}C) because of its volatility will have been solved. Moreover, its application to the analysis of cholesterol in gallstones can be studied.

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Advisor's Approval

This thesis has been submitted for examination with my approval as University Advisor.

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