ABSTRACT OF THESIS

Name of Candidate L.B.Roberts

Address [redacted]

Degree Ph.D. *Date* 20th.October 1972

Title of Thesis Cholesterol metabolism in the rat liver

The results obtained from the perfusions of normal rat livers have been used as a control in assessing the effects of bile drainage, thyroidectomy and the administration of ethyl chlorophenoxyisobutyrate(CPIB) upon cholesterol equilibria in the isolated blood and liver system. All these modifications are considered to have an effect on the intra-hepatic synthesis of cholesterol.

I) Biliary drainage is known to stimulate hepatic cholesterol synthesis and although the exact mechanism is unknown it is believed to be related to the decreased absorption of cholesterol from the intestine which occurs as a result of a depleted intestinal bile acid pool. The effect of biliary drainage, for periods of up to 44h, on the rates of appearance of radioactive cholesterol in the perfusate of the isolated liver preparation, have been examined. The results showed that stimulation of cholesterol synthesis in the liver had no significant effect on the exchange of cholesterol between liver and blood. However the results did suggest that a significant proportion of the newly synthesised cholesterol was excreted in the bile as bile acids.

2) Hypothyroidism is known to affect both cholesterol synthesis and the concentration of cholesterol in the blood. Perfusion studies on livers taken from thyroidectomised rats showed that hepatic cholesterol synthesis was reduced and that the site of inhibition lay at some point beyond the rate limiting step in the biosynthesis. This effect resulted in an apparent change in the kinetics of the exchange of cholesterol between liver and blood. This change was shown not to be a real one by carrying out simulation studies using the model derived for normal liver perfusion. It was finally concluded that hypothyroidism has no effect on the rate of exchange of cholesterol between liver and blood.

3) The hypocholesterolaemic drug CPIB is widely used in the treatment of atherosclerotic conditions and has been shown to reduce the concentration of cholesterol in the blood. The mode of action of the drug is still conjectural, but two theories have been advanced. Firstly that hepatic cholesterol synthesis is reduced, and secondly that the rate of release of newly synthesised lipoproteins(which contain cholesterol) is diminished. A study of the effect of the drug on the isolated perfused rat liver was performed. The results suggested that the primary action of CPIB is by inhibition of cholesterol synthesis at some point before the major rate limiting step. No convincing evidence could be found to support the suggestion that the rate of release of lipoprotein cholesterol may be diminished. It was established that the action of the drug was very rapid, inhibition of cholesterol synthesis occurring within 30min of administration of CPIB.

In conclusion it seems that changes in the rate of hepatic cholesterol synthesis have little effect on the exchange of cholesterol between liver and blood.

Use other side if necessary.

CHOLESTEROL METABOLISM IN THE RAT LIVER

By

Leslie Barry Roberts

A thesis submitted for the degree of
Doctor of Philosophy, University of Edinburgh, 1973

Index.

RESULTS.

SUMMARY

Cholesterol is synthesised in all nucleated mammalian cells. The liver is considered to be the most active tissue, both in terms of the rate at which cholesterol is synthesised, and also in that it has the major contribution to whole body cholesterol when measured on the basis of organ weights. The fate of newly synthesised liver cholesterol is either distribution to extrahepatic tissues, excretion as cholesterol in the bile, or degradation to bile acids. The blood is the carrier medium by which cholesterol synthesised in the liver ultimately equilibrates with the cholesterol in the extrahepatic tissues.

This study set out to examine the equilibria (for cholesterol) which exist between liver and blood. In the whole animal it is difficult to obtain an estimate of the exchange rate between liver and blood for two reasons, firstly because other tissues contribute cholesterol to the blood cholesterol pool, and secondly because the exchange is considered to be very rapid. These problems were overcome by using an isolated perfused rat liver preparation which allowed a study of cholesterol equilibria in isolation from extrahepatic sources of endogenous or exogenous cholesterol. At the same time the preparation allowed many perfusate samples to be taken over a short time period. This would have been difficult in a whole animal. The perfusate consisted of whole homologous blood to preserve the complex relationships which exist between biochemical compartments of cholesterol in this system. The cholesterol synthesised *de novo* in the liver was labelled by the addition of (2-^{14}C)DL mevalonic acid to the perfusate. A number of perfusions

were also performed in which sodium (^{3}H) acetate was used as the precursor of cholesterol.

Using this preparation a study has been made of the rates at which cholesterol synthesised in rat liver appears in some biochemical and anatomical compartments of this isolated blood and liver system. A compartmented model has been adopted to simulate the biological system. The simplest model used consisted of six compartments, plasma unesterified and ester cholesterol, liver unesterified and ester cholesterol, erythrocyte cholesterol and biliary cholesterol.

The data obtained from the perfusion studies have been fitted to this model to give information about exchanges occurring in the system. The model simulations were performed on an analogue computer which allowed estimates to be made of the absolute rate of transfer (i.e. the amount of cholesterol) between compartments in the system, some of which were inaccessible to experimental sampling. For example the liver could not be sampled easily at different time points because steady state conditions would be disturbed. No direct measurement of the changes in the amount of radioactivity in the cholesterol compartments of the liver could therefore be made. These changes could however be predicted from the simulation studies on the computer.

Subsequently the model was extended by the provision of a further compartment to represent the radioactive precursor of cholesterol, (2^{14}C)DL mevalonic acid. This extended model gave a better fit between the experimental and simulated data and also allowed prediction of the rate at which the labelled mevalonic acid was utilised. The results of the simulation studies showed that the most rapid turnover of cholesterol in the blood and liver system occurred between erythrocytes and plasma.

This was followed by turnover between liver and blood and the slowest rates were found in the hydrolysis and esterification of cholesterol occurring within the plasma or within the liver. This was taken to indicate that the liver was the primary source of plasma cholesterol esters in the rat.

Since cholesterol synthesis is intracellular it was thought that the rate at which radioactive cholesterol appears in the perfusate, after de novo synthesis, might be influenced by the intracellular distribution with time. A study in vivo of the rate at which newly synthesised cholesterol moves between intracellular organelles did suggest some element of compartmentation.

Three pools of intracellular cholesterol could be identified, namely in the soluble fraction, in the nuclear fraction and in the combined microsomal and mitochondrial fractions. The model was not extended to allow for this effect because it would have been difficult to obtain a reliable solution to such a complex model.

The results obtained from the perfusions of normal rat livers have been used as a control in assessing the effects of bile drainage, thyroidectomy and the administration of ethyl chlorophenoxyisobutyrate (C.P.I.B.) upon cholesterol equilibria in the isolated blood and liver system. All these modifications are considered to have an effect on the intrahepatic synthesis of cholesterol.

1) Biliary drainage is known to stimulate hepatic cholesterol synthesis and although the exact mechanism is unknown it is believed to be related to the decreased absorption of cholesterol from the intestine which occurs as a result of a depleted intestinal bile acid pool. The effect of biliary drainage, for periods of up to 44h, on

the rates of appearance of radioactive cholesterol in the perfusate of the isolated liver preparation, have been examined. The results showed that stimulation of cholesterol synthesis in the liver had no significant effect on the exchange of cholesterol between liver and blood. However the results did suggest that a significant proportion of the newly synthesised cholesterol was excreted in the bile as bile acids. In addition it would appear that newly synthesised cholesterol is the preferential precursor of bile acids rather that the pre-existing cholesterol.

2) Hypothyroidism is known to affect both cholesterol synthesis and the concentration of cholesterol in the blood. Perfusion studies on livers taken from thyroidectomised rats showed that hepatic cholesterol synthesis was reduced and that the site of inhibition lay at some point beyond the rate limiting step in the biosynthesis. This effect resulted in an apparent change in the kinetics of the exchange of cholesterol between liver and blood. This change was shown not to be a real one by carrying out simulation studies using the model derived for normal liver perfusion. It was finally concluded that hypothyroidism has no effect on the rate of exchange of cholesterol between liver and blood.

3) The hypocholesterolaemic drug C.P.I.B. is widely used in the treatment of atherosclerotic conditions and is known to reduce the concentration of cholesterol in the blood. The mode of action of the drug is still conjectural, but two theories have been advanced. Firstly that hepatic cholesterol synthesis is reduced, and secondly that the rate of release of newly synthesised lipoproteins (which contain cholesterol) is diminished.

A study of the effect of the drug on the isolated perfused rat liver was performed. The results suggested that the primary action of C.P.I.B. is by inhibition of cholesterol synthesis at some point before the major rate limiting step. No convincing evidence could be found to support the suggestion that the rate of release of lipoprotein cholesterol may be diminished. It was established that the action of the drug was very rapid, inhibition of cholesterol synthesis occurring within 30min of administration of C.P.I.B.

In conclusion it seems that changes in the rate of hepatic cholesterol synthesis have little effect on the exchange of cholesterol between liver and blood.

The thesis has been written according to the instructions (1972) issued by the Biochemical Society to authors of papers to be presented in the Biochemical Journal.

Definitions used in this study.

Specific Radioactivity	is the ratio of the amount of radioactive tracer to that of the unlabelled substance and in this study is expressed as nanoCuries per milligram of mother substance (nCi/mg).
Blood/liver system	in the context of this thesis this term is meant to indicate that the liver and its attendant blood supply are considered in isolation from the other tissues of the whole animal.
Compartment (Pool)	this is a chemically distinguishable phase or volume which sometimes, but not always, conforms to an anatomical distribution, e.g. cholesterol in erythrocytes has an anatomical distribution, esterified cholesterol in the liver has both an anatomical and biochemical disposition.
Transfer	implies a unidirectional movement of substance from one compartment to another.
Exchange	designates simultaneous and equal transfer into and out of a compartment, or between compartments.

Rate Constant	indicates the proportion of a compartment being turned over per unit time, e,g, $0.004min^{-1}$ shows that 4/1000 of the compartment (j) is being turned over per minute.
Turnover Rate	is the amount of substance being added to or removed from a compartment per unit time. Units are mass per unit time.
Turnover Time	is defined as the time required for an amount of substance to be transferred into or out of a compartment, which is equal to the compartment size. It is the reciprocal of the rate constant and has the dimensions of time, (t^{-1}).
Steady State	means that the rate of loss from a compartment is balanced by the rate of input.
Machine Unit	is a term used in analogue computing and represents a voltage. One machine unit is considered as the maximum operating voltage for the instrument in use. All operations where numbers are represented by voltage must be such that this value is never exceeded.

Caternary Model	is a model where the compartments are arranged in a chain and tracer substance only exchanges (or transfers) with adjacent compartments.
Mammillary Model	is a model where peripheral compartments exchange (or transfer) tracer substance with a central common compartment and not between themselves.
Patching Diagram	is a diagram representing the program of an analogue computer.

Mathematical notation and nomenclature correspond to those used in the Faculty of Mathematics, The Open University, Bletchley, Buckinghamshire.

Commutative Diagram	is a diagram in which elements or sets of elements are connected by arrows (representing operations or functions) and which permits alternative paths from the same starting point to the same finishingpoint.
Domain	is a set of elements upon which an operation may be performed.
Image	of an element of the domain is the element assigned to it by the operation.

$\square$ or 0 or $\triangle$ is the notation for a binary operation and indicates the general case for an operation on the elements of a set. For example a binary operation can be addition or multiplication. Different operators are represented by different notation, e.g. $\square$ could be equivalent to addition and 0 equivalent to multiplication.

$f : x \longmapsto$ means the mapping (function) f assigns the value of y to elements in the domain of x.

Symbols used in this study.

Q_j = amount of substance in compartment j.

q_j = amount of tracer in compartment j.

k_{pj} = rate constant for transfer into compartment j from compartment p.

a_j = specific radioactivity of substance in compartment j.

λ_i = exponential constant in the i^{th} exponential term.

X_i = amplitude of the i^{th} exponential term.

t = time.

n = number of compartments.

INTRODUCTION

Cholesterol is a secondary alcohol found in a group of substances of animal and plant origin with the generic name of sterols. Sterols in plants are known as phytosterols and in animals as zoosterols. The sterols contain between twenty seven and twenty nine carbon atoms and are distinguished from other secondary alcohols by virtue of being crystalline solids with melting points in the 100 - 200°C range. All sterols have a ring structure based on cyclo-pentanoperhydrophenanthrene and the formula for cholesterol proposed by Wieland and Dane (1932) is shown in Fig. 1. Cholesterol is derived in a biosynthetic chain starting with 'acetate' (Fig. 2).

Synthesis of Cholesterol.

Cholesterol is synthesised in all nucleated mammalian cells with some tissues being more active than others (Dietschy and Siperstein, 1967). The actual intracellular site of cholesterol synthesis is unknown but synthesis is accomplished *in vitro* by a combination of the supernatant and microsomal fractions (Bucher and McGarrahan, 1956; Bucher *et al.*, 1959). The supernatant fraction acts by virtue of its glycolytic activity, which serves as a source of ATP and reduced co-enzymes, and by its ability to convert acetate to acetyl-Co-A. It is also required for the cyclisation of squalene to lanosterol (E.C.1.99.1.13. squalene cyclohydroxylase). The microsomal fraction contains the enzyme β -hydroxy- β methylglutaryl reductase and catalyses the reduction of hydroxymethylglutarate to mevalonate (E.C.1.1.1.34. mevalonate : NADP oxidoreductase). This fraction is also responsible for the conversion of lanosterol to cholesterol (Tchen and Block, 1955). A further suggestion that the mitochondria may be involved arose from the findings of Bucher *et al.* (1960) who showed that the condensing enzyme,

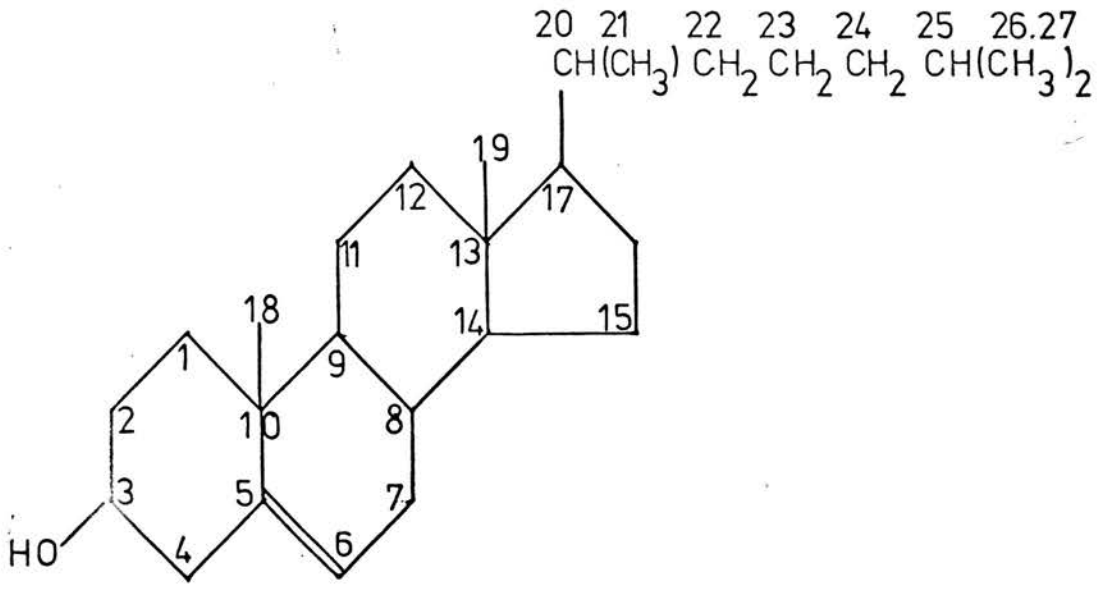


Fig.I. Formula for cholesterol.

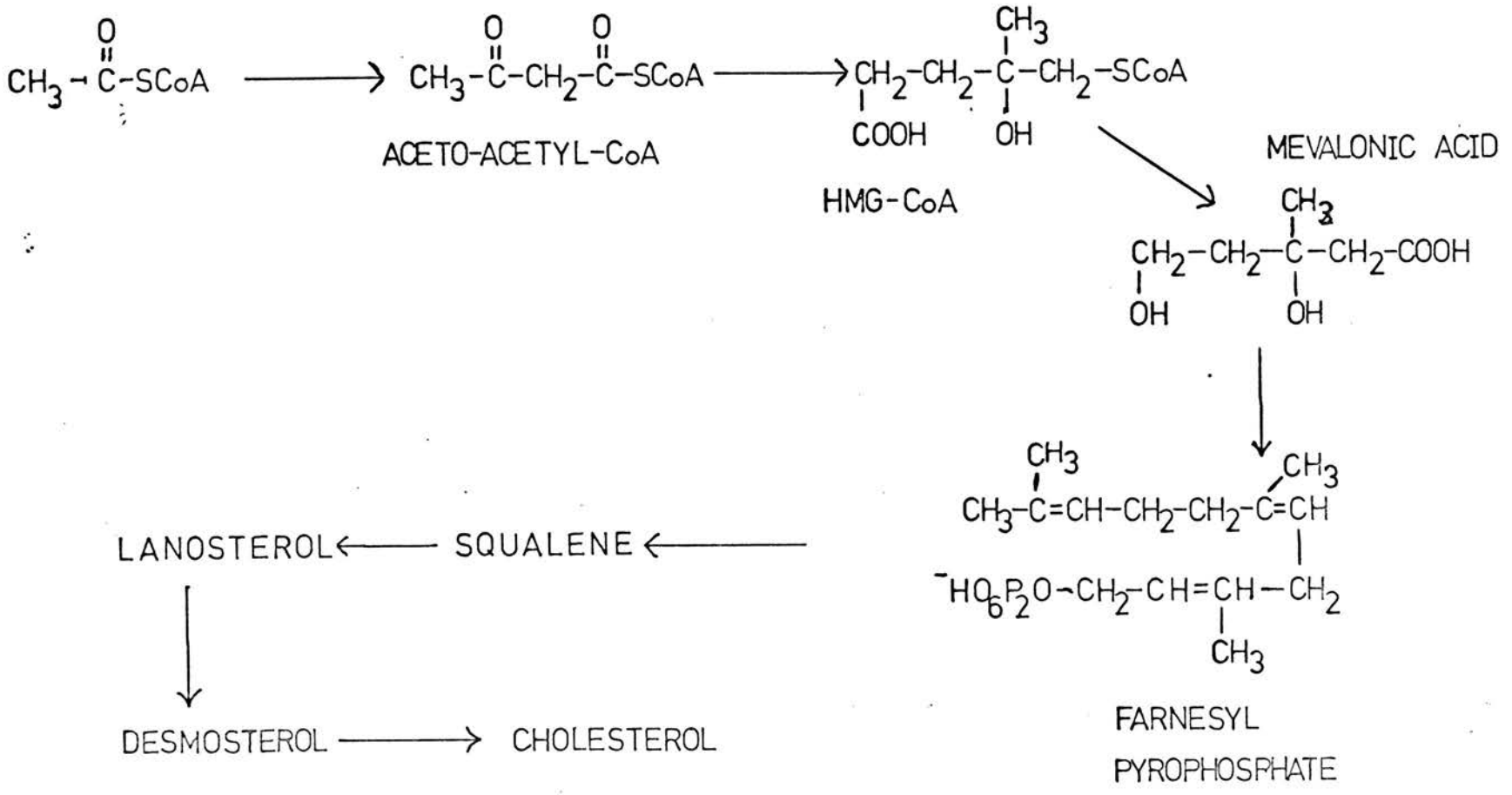


Fig.2. The biosynthesis of cholesterol from 'acetate'.

hydroxymethylglutaryl Co-A synthetase (E.C.4.1.3.5. 3-hydroxy-3-methyl-glutaryl - Co-A acetoacetyl Co-A-lyase) is largely present in the mitochondria. This would explain the greater efficiency of the heavier microsomal fractions.

Srere et al. (1950) showed that (^{14}C) acetate is actively incorporated *in vitro* into cholesterol in a wide range of tissues and this was confirmed *in vivo* by Popjak and Beeckmans (1950) who demonstrated cholesterol synthesis in extra-hepatic tissues on intact animals. Dietschy and Siperstein (1967) examined the relative rates of sterol synthesis in seventeen tissues of rat and concluded that the liver was the most active tissue. The ileum was also very active with a rate about 65% of that of the liver. Contribution to whole body synthesis, when organ weights were taken into account (Dietschy and Wilson, 1968), was mainly attributed to the liver which demonstrated 82% of the whole body sterol synthesising capacity.

Formation of Cholesterol Esters.

Cholesterol can be esterified at its free hydroxyl group with fatty acids, the relative proportion of different fatty acid esters being different for different tissues, e.g. in man the predominant fatty acid in serum is linoleic acid and in adrenals is oleic acid. There is also a species difference for the same tissue, e.g. human adrenals show a preponderance of oleic acid and rat adrenals of arachidonic acid (Goodman, 1965).

The esterification of cholesterol has been shown to occur in liver, pancreas, intestine (Swell et al.,1955) and in plasma (Sperry, 1935). The liver esterifying mechanism is related particularly to the mitochondrial and microsomal fractions (Deykin and Goodman, 1962; Goodman *et al.*, 1964). Co-factors ATP and CoA are required but can be replaced by the addition of pre-

preformed fatty acyl-CoA-ester. The enzyme is therefore designated a fatty acyl-CoA cholesterol acyltransferase.

The presence of an enzyme catalysing the formation of cholesterol esters in the plasma was first suspected in 1935 when Sperry showed that the incubation of human plasma for three days at 37°C resulted in a marked decrease in unesterified cholesterol with no change in the total concentration. The effect could be abolished by heating the serum at 55 to 60°C for 1 - 2 h. The first suggestion that lecithin was involved in the reaction came from Le-Breton and Pantaleon (1947). Subsequently Glomset et al. (1962b) found that rat or human plasma at 37°C showed a molar increase of esterified cholesterol equivalent to to the molar decrease of unesterified cholesterol and lecithin. Incubation of plasma with labelled free fatty acids (FFA) did not produce esterified cholesterol, whereas incubation of plasma with lecithin esterified with linoleic acid formed cholesteryl linoleate (Glomset, 1962a). The enzyme responsible has been called lecithin - cholesterol - acyl transferase (L.C.A.T.). The fatty acid composition of plasma cholesterol esters in different species is probably related to two factors, firstly, the type of fatty acids available on the plasma lecithin, and secondly, the specificity of the acyltransferase for the particular fatty acid substrate. There is also a significant difference between the reactivity of different classes of serum lipoprotein with L.C.A.T. which may result from differences in the relative proportions of unesterified cholesterol and lecithin on the lipoprotein surface (Nichols and Gong, 1971).

Degradation of cholesterol.

The major quantitative pathway for the degradation of cholesterol is the formation of bile acids in the liver. Chaikoff et al.(I952) found that in the rat 80-90% of the radioactivity derived from an intravenous injection of (4-^{I4}C)cholesterol was present in the acid fraction of faeces collected during the first fifteen days after administration. In a similar experiment using(26-^{I4}C) cholesterol a large proportion of the radioactivity was recovered in the CO_2 of expired air. The authors concluded from these two experiments that 80-90% of the cyclopentanoperhydrophenanthrene nucleus was metabolised to form bile acids and that the remainder was excreted as neutral sterols.

Cholesterol is converted to the primary bile acids, cholic and chenodeoxycholic acids. These are then conjugated by the formation of an amide bond at position twenty four with either taurine or glycine. The enzyme systems responsible for the degradation of cholesterol to form bile acids are localised in the microsomal fraction(Mendelsohn and Staple, I963 and in the mitochondria, with co-factors in the I00,000g supernatant(Berseus and Danielsson,I963).

The hydroxylation reactions are catalysed by NADPH-dependent enzymes in the liver microsomal fraction and involve 7α and I2α hydroxylation steps(Einarrson and Johansson,I969). Secondary bile acids, derived from the dehydroxylation of the primary bile acids by bacteria, are formed in the intestine (Fig.3.) Absorption of the secondary bile acids from the intestine is followed by conjugation with either glycine or taurine and secretion

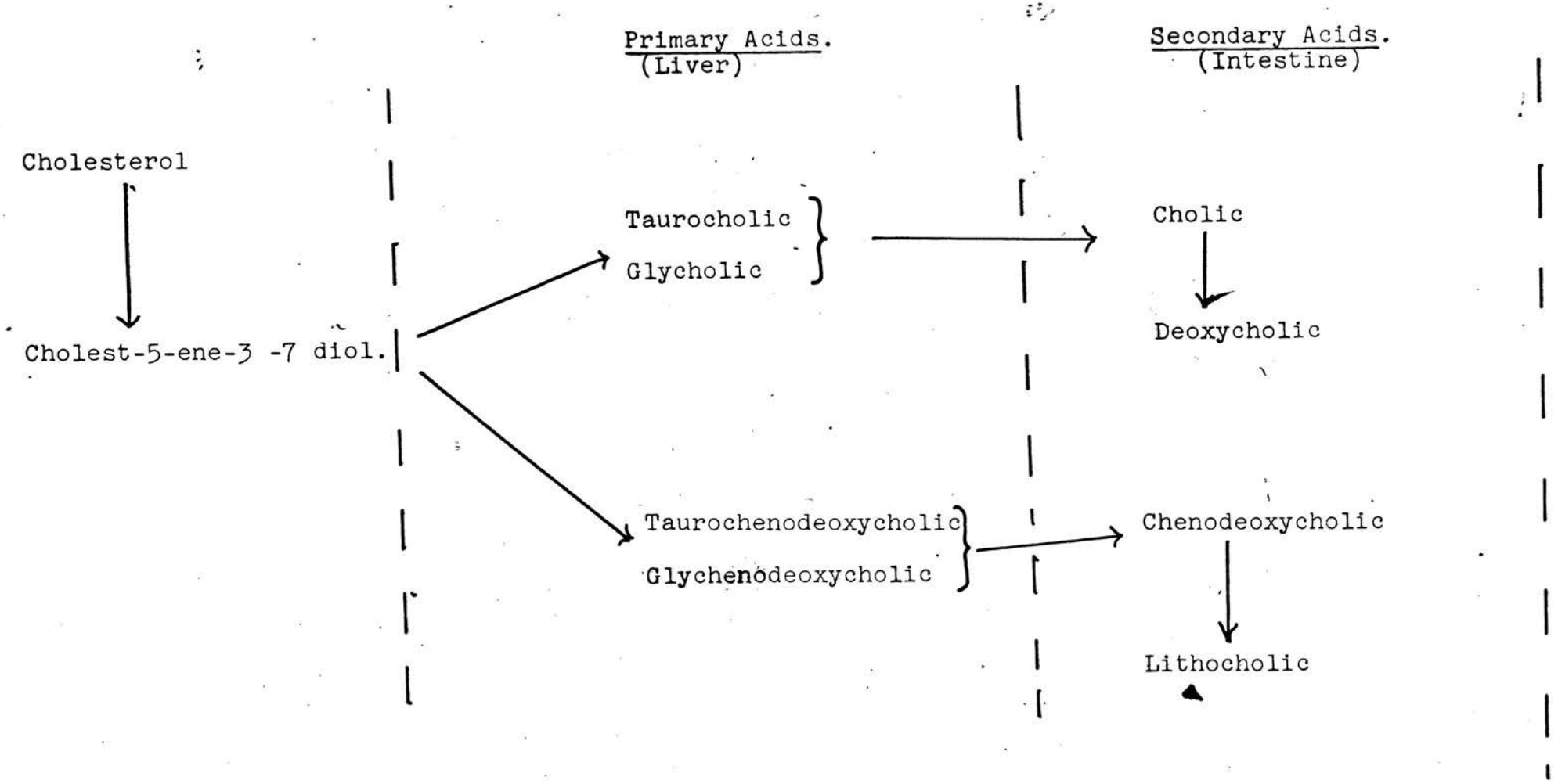


Fig.3. Formation of primary and secondary bile acids from cholesterol.

into the bile, thus forming an enterohepatic circulation analogous to that for cholesterol (Bergstrom, 1962). Approximately 95% of the bile acids secreted into the bile are absorbed from the intestinal tract and are then re-excreted by the liver. The remaining 5% is excreted in the acid sterol fraction of the faeces.

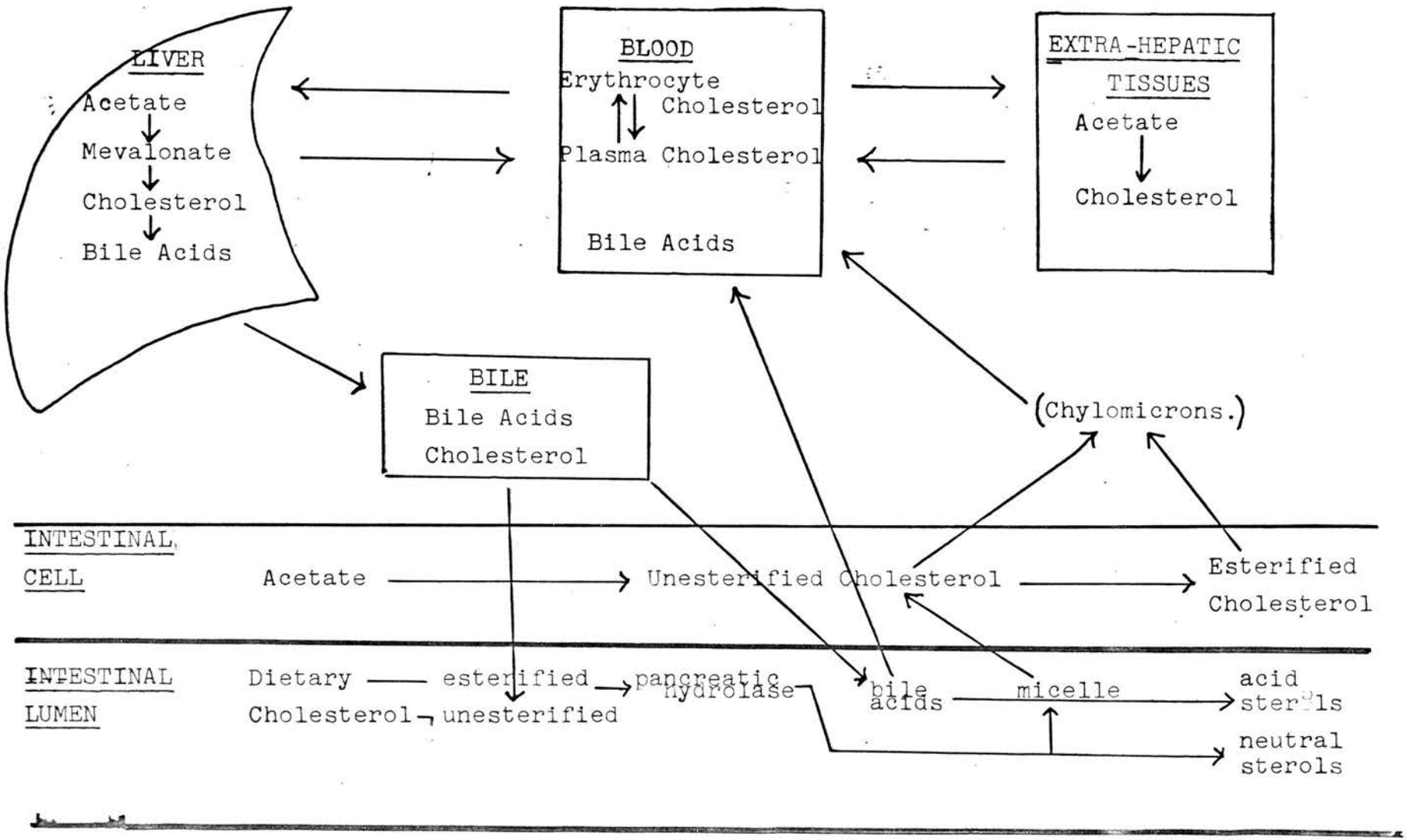
In addition cholesterol is metabolised to form steroid hormones, but le Roy (1957) estimated that only 0.5% to 1% of the plasma unesterified cholesterol is utilised daily in this way.

Physiology of Cholesterol absorption and excretion.

Dietary cholesterol mixes in the intestinal lumen with cholesterol derived from biliary and intestinal secretions (Fig. 4). Any esterified cholesterol is converted to the unesterified form by a pancreatic hydrolase. It is believed that the fraction which is absorbed must be incorporated into a mixed micelle along with free fatty acids (FFA) and monoglycerides (MG) under the solubilising influence of bile acids. The micelle is brought into intimate contact with the brush border of the intestinal epithelial cell and the micellar cholesterol is absorbed. Unabsorbed cholesterol is excreted with the neutral sterol fraction in the faeces. After absorption, the cholesterol mixes with the intracellular intestinal pool and 70% is esterified (Daskalakis and Chaikoff, 1955). The intracellular, esterified and unesterified cholesterol is incorporated into the chylomicrons which are released into the intestinal lymph, and ultimately enter the systemic vascular system by way of the thoracic duct (Goodman, 1965; Biggs et al., 1951).

In the blood cholesterol is distributed amongst the erythrocytes and the plasma lipoproteins. Four major classes of lipoprotein are recognised, i.e.chylomicrons

Fig.4. Cholesterol movement in the whole mammalian organism.



with a specific gravity less than 0.95; very low density proteins (VLDL) with a specific gravity in the range 0.95 - 1.006; low density proteins (LDL) with a specific gravity in the range 1.019 - 1.063; and the high density lipoproteins (HDL) having specific gravities between 1.063 and 1.210.

The composition of these classes is summarised in Table 1. It can be seen that 60% of the plasma cholesterol is carried by the β and pre-β globulins with a smaller proportion being carried by the HDL. The HDL may however be important in so far as they carry most of the plasma lecithin, which is a potential donor of fatty acids, and may be involved in the intravascular esterification of cholesterol (Glomset, 1968; Nichols and Gong, 1971).

The plasma unesterified cholesterol exchanges with erythrocyte cholesterol which is wholly unesterified, and is confined to the plasma membrane (Nelson, 1967). The exchange occurs both in vivo (Eckles et al., 1955; Gould, 1951), and in vitro (Bruckdorfer and Green, 1967; Hagermann and Gould, 1951). It is dependent upon the pH, (Bruckdorfer and Green, 1967), upon the relative concentration of the lipoprotein classes present in the plasma (Quarfordt and Hildermann, 1970), and possibly upon the concentration of bile salts present in the plasma (Cooper and Jandl, 1969).

The esterified cholesterol on the other hand is preferentially taken up by the liver so that 85 to 90% of the ester is within the liver twenty minutes after release from the lymphatics into the blood (Goodman, 1962). Following hepatic capture, the cholesterol ester is slowly hydrolysed and equilibrates with the unesterified cholesterol in the liver and blood. This, in turn, ultimately equilibrates with the cholesterol in the extrahepatic tissue.

Fraction	Sf	Density g/ml	Mobility	% Cholesterol	
				Unesterified	Esterified
Chylomicrons	400	0.95	Origin	1 - 3	2 - 4
V.L.D.L.	20 - 400	0.95 - 1.006	Pre-	10	5
L.D.L.	0 - 12	1.019 - 1.063		8	37
H.D.L.	-	1.063 - 1.21		15	22

Table 1

Physical and biochemical characteristics of the plasma lipoproteins.

Contribution to the unesterified cholesterol pool in the liver is also made de novo from acetate. The cholesterol at this point may either be degraded to form bile acids which, along with unesterified cholesterol, are excreted in the bile or the cholesterol may be released into the hepatic vein in association with the lipoproteins and, in the systemic blood, is in rapid equilibrium with other tissues (Avigan et al., 1962). Some of the cholesterol secreted in the bile is reabsorbed in the small intestine and an enterohepatic circulation of cholesterol is achieved.

Control of Cholesterol synthesis.

The rate of synthesis of cholesterol from acetate (Fig 2) can be controlled by feeding cholesterol. This causes marked depression of hepatic cholesterol synthesis (Gould, 1951; Weiss and Dietschy, 1971). The site of this feedback mechanism was shown by Gould and Popjak (1957) to be located prior to mevalonic acid. Subsequently, Siperstein and Fagan (1964) were able to show that the major site of feedback control is located at the reaction responsible for the conversion of β -hydroxy- β - methyl glutarate (HMG) to mevalonate, i.e. an inhibitory effect of cholesterol on β -hydroxy- β -methyl glutaryl reductase. The intracellular site of this reaction is localised in the membrane fraction of the microsomes, although Bucher et al. (1960) showed that the mitochondria contain the greater portion of this intracellular enzyme. It must be emphasised that this feedback mechanism is predominantly associated with the liver, other tissues not showing such large inhibition of the synthesis rate when the animal is fed cholesterol (Dietschy and Wilson, 1968).

The feedback mechanism is probably controlled by a

specific lipoprotein complex (D < 1.019)(Siperstein and Fagan, 1964), although Cooper and Morgalis (1971) found that incorporation of acetate into cholesterol by isolated rat hepatocytes was inhibited to some extent by all of the lipoprotein classes. There was a measure of selectivity in that the VLDL produced 70% inhibition, HDL produced 55% inhibition and the LDL, 35% inhibition. Boyd and Onajobi (1969) demonstrated the presence in rat and human serum of an apoprotein of the α-lipoproteins which inhibited cholesterol synthesis in rat liver in vitro. A specific lipoprotein complex seems more likely because the in vitro inhibition of cholesterol synthesis has not been demonstrated using 'free' cholesterol.

Although the major rate-limiting step is believed to be between HMG and mevalonate, two other subsidiary sites of control have been demonstrated. The first of these is the conversion of mevalonate to farnesyl pyrophosphate, and the second is at the conversion to farnesyl pyrophosphate to squalene (Gould and Swyrd, 1966). The reduced activity of these sites is regulated by cholesterol feeding but diminished enzymatic activity at the conversion of HMG to mevalonate is also found in the fasting state (Bucher et al., 1960). This reduction in the rate of sterol synthesis has been found in other tissues (Dietschy and Wilson, 1968), but the liver shows the greatest sensitivity.

There is strong evidence that bile acids are implicated in regulating tissue cholesterol synthesis. Thus, diversion of the bile flow by an external bile fistula leads to a rapid increase in the rate of cholesterol synthesis both in the liver (Economou et al., 1958) and in the small intestine (Dietschy and Siperstein, 1965). Oral administration of compounds which form complexes with bile acids (e.g. quaternary amino compounds or neomycin) show

the same pattern of increased sterol synthesis (Tennent et al., 1959; Powell et al., 1962). Conversely, expansion of the intestinal bile acid pool by oral administration of cholic acid (Beher and Baker, 1959) leads to a reduction in hepatic sterol synthesis.

It has been suggested that hepatic bile acid concentrations are the controlling factor (Fimognari and Rodwell, 1965); deoxycholic and taurodeoxycholic acids exhibiting the greatest inhibitory activity with liver slices. The implication of this study is that bile acids exert a direct feedback regulation of cholesterol synthesis. Conversely, Swell et al., (1968) showed that taurocholate stimulated the incorporation of mevalonic acid into the cholesterol synthesised in an isolated perfused rat liver. They also showed that a greater secretion of cholesterol and bile acids, into bile, occurred in these experiments.

An increased rate of hepatic cholesterol synthesis has also been demonstrated after biliary obstruction (Fredrickson et al., 1954; Weiss and Dietschy, 1971) when the concentration of bile acids is increased in blood (Boyd et al., 1966) and in the liver (Weiss and Dietschy, 1969).

The concept of control of hepatic sterol synthesis by bile acid is still valid if one assumes an indirect, rather than a direct relationship. In the model proposed by Weiss and Dietschy (1969) hepatic cholesterol synthesis is controlled by regulation of the intestinal absorption of cholesterol which itself is a function of the intestinal bile acid pool size. The active principal in the control of hepatic sterol synthesis should therefore be the chylomicrons absorbed into the intestinal lymph and Weiss and Dietschy showed that diversion of the intestinal lymph flow produced increased hepatic cholesterol synthesis, whilst infusion of the chylomicrons depressed the synthesis rate. Against this should be set the report of Cooper and

Morgalis (1971) who noted that the in vitro incorporation of acetate into cholesterol in isolated rat hepatocytes was not affected by inclusion of chylomicrons in the incubating medium.

By far the major part of the circulating cholesterol is derived from the liver (Eckles et al., 1955; Hellman et al., 1955). Some of this cholesterol is endogenous, being synthesised de novo and some derived from the esterified cholesterol, absorbed in the form of chylomicrons and then taken into the liver, with subsequent intrahepatic hydrolysis. Cholesterol is released from the liver in combination with an apoprotein synthesised in the liver ribosomes (Bungenberg de Jong and March, 1968). The plasma unesterified cholesterol is equally distributed between the major lipoprotein classes (Kook and Rubenstein, 1969; Roheim et al., 1963). The cholesterol esters on the other hand do show differential specificity for incorporation into lipoproteins, the most rapid transfer being to the $D < 1.019$ class in a non-selective manner with regard to their fatty acid composition (Roheim et al., 1963).

Turnover of Cholesterol.

As early as 1933, Schonheimer and Breusch employed balance studies to demonstrate a remarkable degree of cholesterol homeostasis in whole animals. There are three basic techniques used for studying cholesterol turnover; firstly a chemical balance method where the input and output of cholesterol and its degradation products are measured; secondly an isotope balance method; and finally the analysis of cholesterol decay curves in serum after intravenous administration of a single pulse dose of radioactive cholesterol.

Using the last technique in man, Goodman and Noble (1968) analysed the specific radioactivity decay curve for plasma cholesterol and concluded that the turnover of

plasma cholesterol conformed to a two pool model, Fig.5. They called the pool with the more rapid turnover, pool A, and the slower pool, B. This study complemented the earlier work of Avigan et al.(1962) who demonstrated different rates of entry of radioactive cholesterol into various tissues after a single oral pulse dose of (^{14}C) cholesterol. In this study comparison of the ratios of the specific radioactivity of the tissue cholesterol with that of the plasma cholesterol indicated rapid equilibrium between the plasma cholesterol and cholesterol in the liver, spleen, heart and lung, and slower rates of equilibrium for kidney, muscle and brain. The study of Chobanian et al. (1962) in general confirmed these two arbitrary groupings with the exception that they found the lung to be in the group of tissues with the slower rate of turnover vis-á-vis the plasma. These two groupings would correspond to the mathematical model proposed by Goodman and Noble (1968). Avigan et al. (1962) also calculated the rate constants for the bidirectional flow of cholesterol between the plasma and each of the tissues examined, but concluded that the rapid exchange of cholesterol between plasma and liver (less than one day) did not permit a reliable determination of the rate constants for these tissues. The very rapid interchange of cholesterol between liver and plasma in dogs has also been recorded by Eckles et al. (1955). A slower rate has been noted in man, although the liver to plasma exchange in this case was still the most rapid when compared with other tissues (Field et al., 1960).

In the studies of Avigan et al. (1962) and Goodman and Noble (1968) the experimental time course extended in excess of seven weeks and very frequent sampling in the early part of the work was omitted. It is possible that more accurate delineation of the early part of the decay curve might have allowed interpretation of the

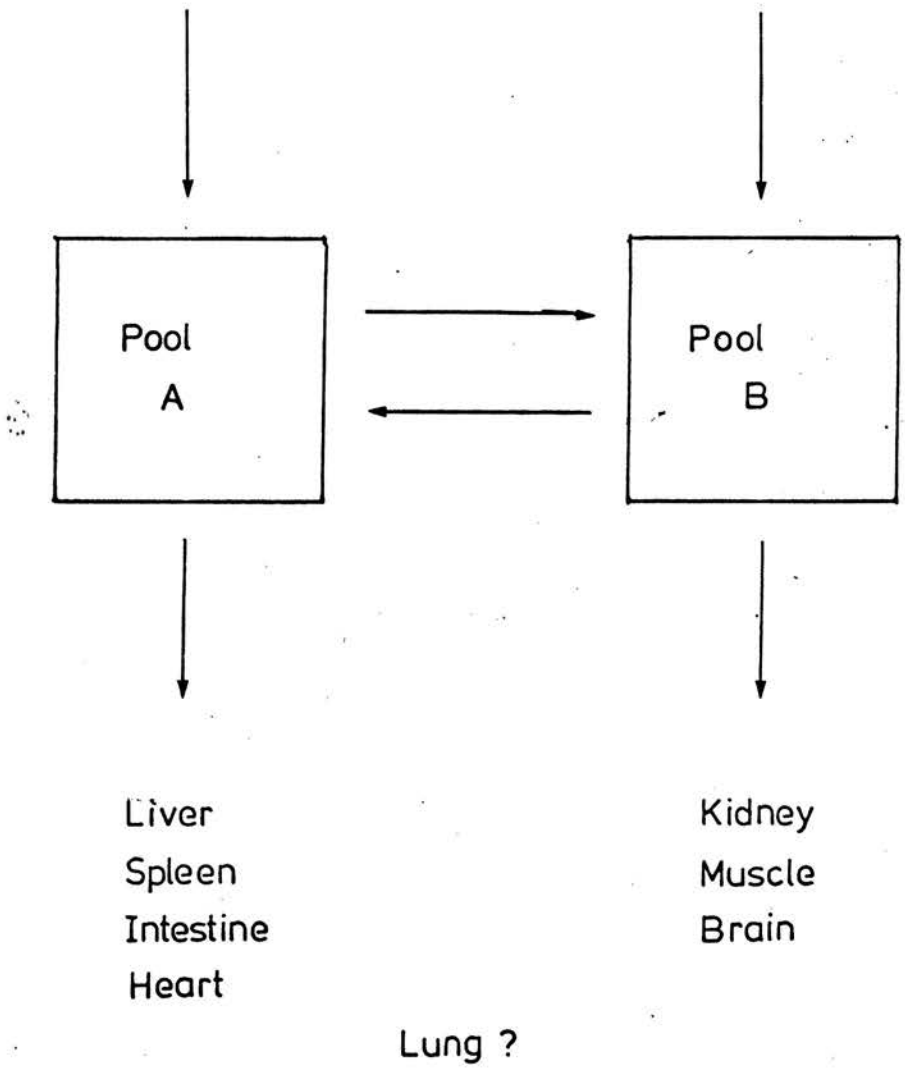


Fig.5. Two pool model of cholesterol turnover in the whole mammalian organism.

kinetics associated with the tissues having the most active exchange processes, i.e. Pool A (Fig. 6). In this context Porte and Havel(1961) studied the plasma cholesterol curve in dogs, after intravenous administration of a lipoprotein artificially labelled with (^{14}C) cholesterol, for periods of up to 120h with frequent sampling in the first 12 h.

Three other important cholesterol exchange processes may contribute to the shape of the early part of the plasma cholesterol decay curve. Firstly, rapid exchange of cholesterol between plasma and erythrocytes has been demonstrated by Hagerman and Gould (1951). A similar study was performed by Quarfordt and Hilderman (1970) who analysed the results in greater detail. These authors showed that even in this relatively simple system there are different rate constants describing cholesterol exchange between erythrocytes and high density lipoproteins and between erythrocytes and low density lipoproteins. Therefore this minor exchange process could well be a small exponent 'buried' in the plasma cholesterol decay curve derived from experiments on whole animals.

Secondly, cholesterol esters turn over at rates different from unesterified cholesterol. In vivo studies in man by Field et al. (1960) showed that esterified cholesterol in plasma reached equilibrium with liver esters more slowly than plasma unesterified cholesterol equilibrated with liver unesterified cholesterol (unesterified cholesterol = 2.5 days, esterified cholesterol = 16 days). However the equilibrium between liver and plasma cholesterol esters was still more rapid than any of the exchanges of cholesterol or its esters between plasma and other tissues. The rate of release of ester cholesterol from the isolated perfused rat liver is slower than that for the unesterified form (Swell and Law, 1971). These latter

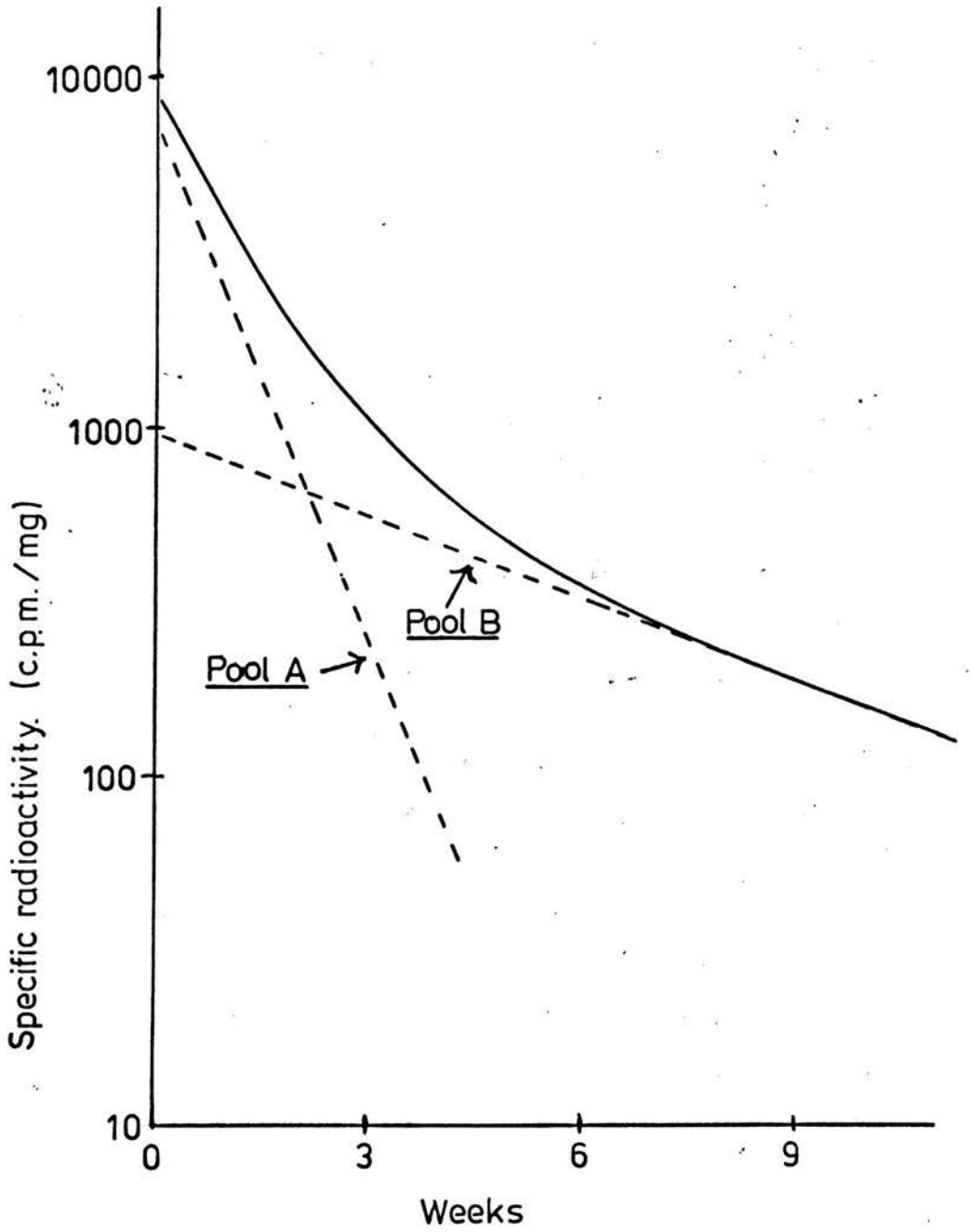


Fig.6. The specific radioactivity decay curve of blood cholesterol after a single intravenous priming dose in man. (According to Goodman and Noble 1968)

authors also showed that esters with different fatty acid substituents were incorporated into the major lipoprotein classes at different rates. This is supported by Quarfordt and Goodman (1969) who found a consistent preferential incorporation of cholesterol esters into VLDL during a three hour perfusion of an isolated rat liver.

Thirdly, if there is intracellular compartmentation of cholesterol then the intracellular pool cannot be considered as homogeneous and may well have an effect on the shape of plasma cholesterol decay curves. For example, if cellular uptake or release of cholesterol is governed by a rate limiting step in the intracellular distribution of newly absorbed cholesterol then this will be evidenced in the components of the decay curve. Some evidence of an intracellular compartmentation has been noted, for example, Chesterton (1966) found that within two minutes of intravenous administration of (2^{14}C)DL mevalonic acid there was a greater incorporation into cholesterol of the nuclear, mitochondrial or soluble fractions. Subsequently the relative activity in the microsomes compared to the nuclei decreased but increased relative to the mitochondria. The time span of these experiments was thirty minutes. Swell and Law (1966) also found a preferential incorporation of mevalonic acid into the liver microsomes during a three hour isolated liver perfusion. Similar findings for brain have been noted by Kahn and Folch-Pi (1967).

It has also been reported that the rough and the smooth fractions of the endoplasmic reticulum showed differential incorporation of (2^{14}C)DL mevalonic acid into cholesterol, the rough and smooth II fractions being equally more active than the smooth I fraction (Glaumann and Dallner, 1968). Suzuki *et al.* (1969) agreed with this principle of differential incorporation but found the smooth endoplasmic reticulum more active than the rough in sequestering (^{14}C) cholesterol from the plasma.

In longer term studies (up to 42 days), the specific radioactivity of the cholesterol in the sub-cellular fractions, after intravenous administration of labelled cholesterol, was virtually the same for all fractions with the exception that the value for the soluble fraction was consistently lower (Hollander and Kramsch, 1967).

Interpretation and correlation of kinetic data from different authors must be made with care, not only because of species differences but also because other factors influence the kinetics of cholesterol metabolism. Thus, Givner and Dvornik (1965) found that cholesterol synthesis, particularly in liver and brain, is markedly dependent on age. In addition Kritchevsky et al. (1961) demonstrated well defined differences in cholesterol oxidation and biosynthesis in livers of male and female rats. Finally, Back et al. (1969) reported that there is a diurnal variation in cholesterol synthesis in rat liver from acetate but not from mevalonate. This would imply rhythmical changes in the pattern of enzyme activity required in the biosynthetic pathway leading to mevalonate. Such changes in HMG - CoA reductase activity have been demonstrated by Shapiro and Rodwell (1969), who found maximal enzyme activity at midnight and this co-incides with the maximum synthesis rates found by Back et al. (1969).

Although the rate constants between blood and various tissues of the body have been recorded (Avigan et al.,1962), the rapid exchange of total cholesterol between blood and liver has prevented the presentation of kinetic data on this biological system. The problem is further compounded by exchanges taking place between individual pools of cholesterol present within the blood and liver system and the rate constants describing the simple exchange of

cholesterol between liver and blood are composite parameters made up of subsidiary rate constants. For example, the cholesterol will exchange between plasma and erythrocyte, and between the plasma ester and unesterified pools, these functions should be part of the rate constant describing exchange between liver and blood.

The possible exchanges in the blood/liver system are summarised in Fig. 7. As can be seen there is a complex interchange system. In the liver the relative proportions of esterified and unesterified cholesterol depend upon the activity of the esterifying enzyme and the availability of fatty acid substrate. In turn the bile acids are synthesised from the intrahepatic cholesterol pool, although the actual precursor pool in unknown. It is likely that these three biochemical components are distributed amongst the anatomical structures of the liver cell. Unesterified cholesterol, and possibly esterified cholesterol undergo a reciprocal exchange with the blood. In the vascular compartment unesterified cholesterol is in equilibrium with lipoproteins, erythrocytes and with esterified cholesterol. This last equilibrium is dependent upon enzyme specificity and substrate availability. Esterified cholesterol also exchanges between lipoproteins. There is no evidence of an intravascular synthesis of bile acids, but it seems reasonable to assume a bidirectional flow between liver and blood.

The determination of the rate constants describing the movement of cholesterol in the blood and liver system would be difficult <u>in vivo</u> because of the contribution of extrahepatic tissues to the blood cholesterol pool. Ideally the liver should be isolated from the whole animal. There would however, be deficiencies in this experimental situation because the feedback control on cholesterol synthesis, exerted by the intestinal absorption rate, would be absent. In addition the enterohepatic recirculation

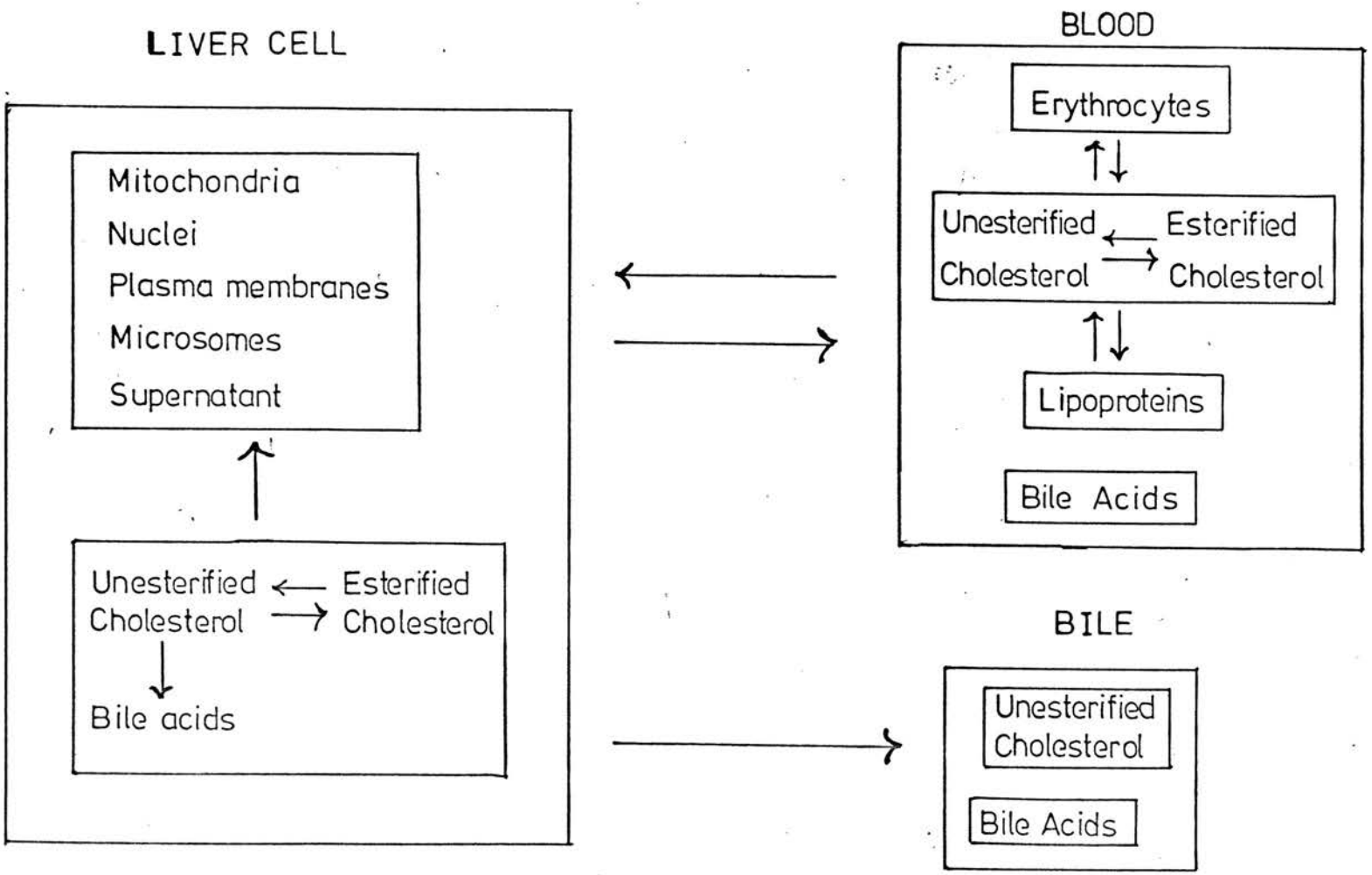


Fig.7. Illustrates the disposition and movement of cholesterol in the blood and liver system.

of bile acids is also absent. Bearing these criticisms in mind, the use of isolated organ perfusion should allow the determination of the rate constants describing the transfer of cholesterol within the blood and liver system and it would be necessary to sample each anatomical and biochemical compartment associated with cholesterol. This is not possible because the actual process of sampling may perturb the relationship between the compartments. Sequential sampling of the liver during the course of an experiment is also impractical for technical reasons and this therefore precludes access to pools of cholesterol existing within the liver.

The only way of examining the kinetics of exchanges taking place in such a system is to construct a biological and mathematical model which would explain the changes taking place in an easily sampled compartment of the system. If the model adopted is consistent with the data derived from this compartment then the whole model may allow quantitation of exchanges occurring in other experimentally inaccessible parts of the biological system. An example of the type of physical model which may represent the cholesterol exchanges occurring in the blood and liver system is shown in Fig. 8.

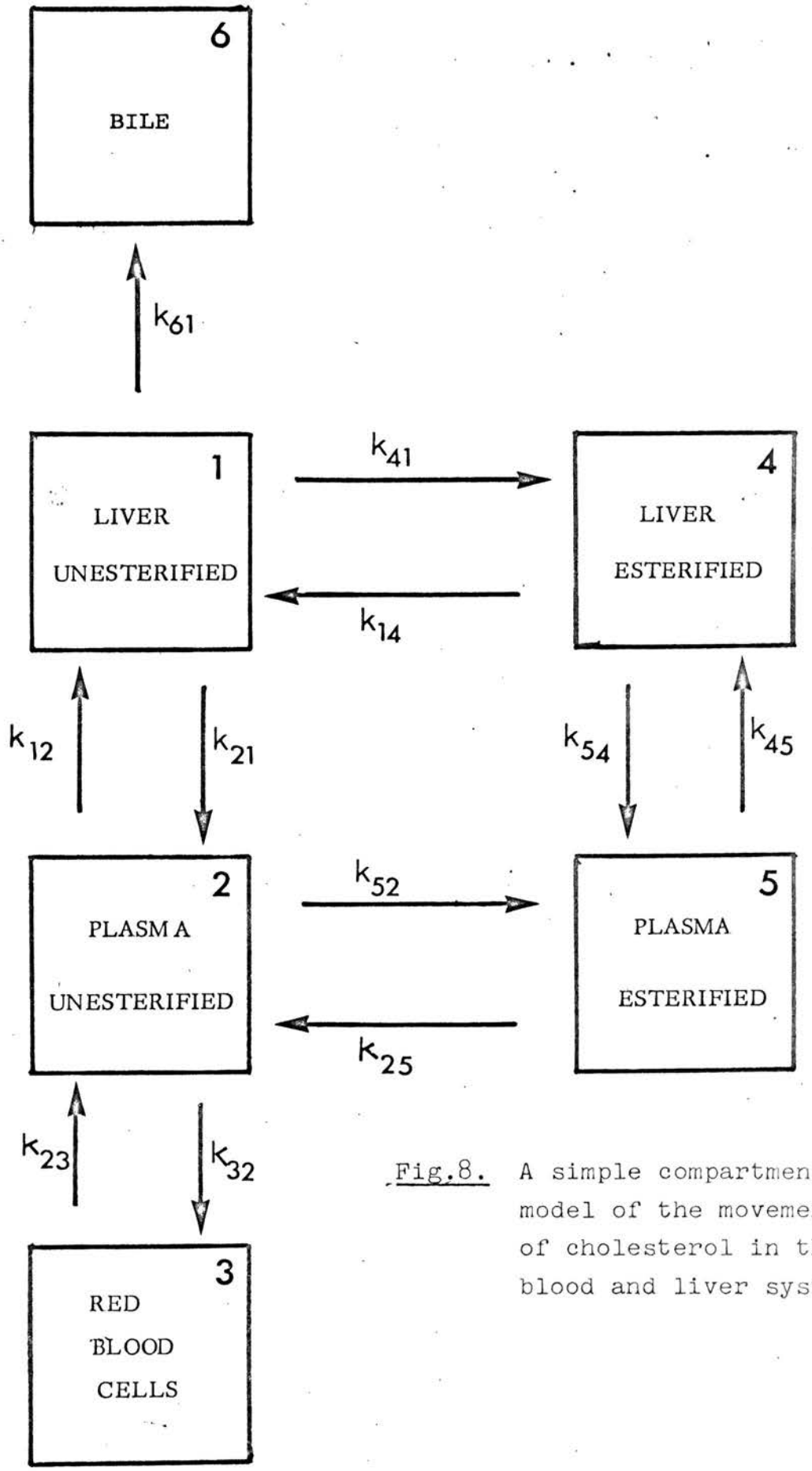
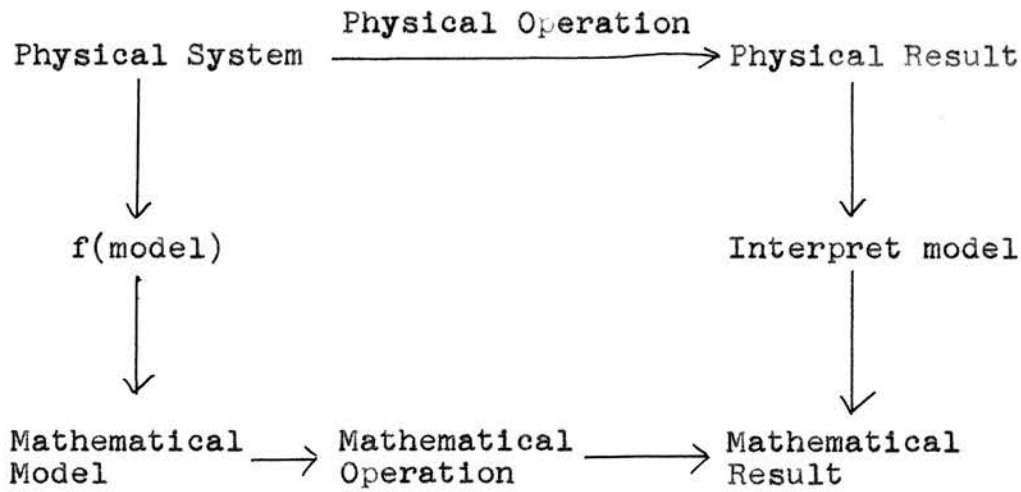


Fig.8. A simple compartmented model of the movement of cholesterol in the blood and liver system.

Derivation and Construction of Models.

A model can have two meanings. It can represent a physical system or be a description in mathematical terms which corresponds to the physical system. This is usually a function of time.

The idea of modelling may be expressed in a commutative diagram:-

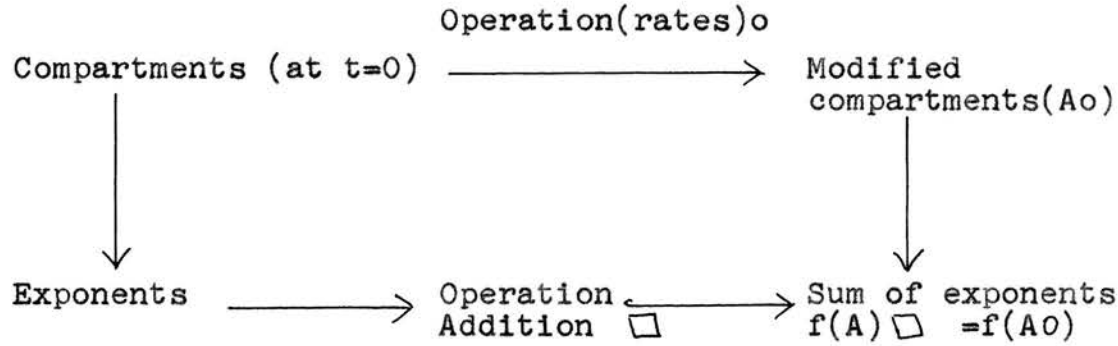


Here the physical system (e.g. the kidney) in operation produces a physical result (production and excretion of urine). If the individual processes responsible for this production can be represented by mathematical functions then a combination of these functions would represent a mathematical model. When the mathematical model is operated by inputting data a mathematical result is obtained which should correlate with the physical result.

In theory the relationship between the physical result and the mathematical model should be perfect but in practice the mathematical model will only represent certain features of the physical solution and for most 'real life' operations will be an approximation. The number and

values of the parameters which define the mathematical model must have correspondence with the physical model, if this is not so then either the physical or mathematical model must be altered to obtain this correspondence. For example, in compartmental analysis the number of functions in the mathematical model must be equal to the number of compartments judged to be present. It is possible however that the mathematical model will correspond to more than one physical model, thus, although the number of compartments may be correct they may be connected in different ways.

The most common approach in the formulation of models has been that of compartmentation, i.e. the disruption of the physical system in terms of a series of linked compartments between which the substance under investigation can move (Ackerman et al., 1967; Robertson, 1957). The commutative diagram in this instance is often of the following form:-



o and □ are operators where □ ≡ addition and o is unspecified but is intrinsic to the physical system.

The biological system is modelled on movement of material between compartments. This movement may be governed by first order kinetics and the compartments can therefore be represented by a series of exponential

functions. The summation of these functions gives an equation based on time which should yield data equivalent to that found experimentally.

The compartments need not conform to anatomical boundaries and may be related to biochemical or physiological concepts. Distribution of the material under investigation between the compartments is usually followed by tracers, particularly radioactive tracers, (Robertson, 1957).

The physical model chosen can be derived in two ways, firstly based on a priori knowledge of the system under investigation, or secondly, by prediction from the equation defining the mathematical model. In practice it is usually a combination of both approaches. It is possible to derive a multi-compartment model based upon sampling from a single compartment, but in this instance the model is less likely to be unique and ideally all conceivable compartments should be sampled. There are situations however where this is not possible, or where at most a single datum point is obtained for some of the compartments. For example, in the study of Bamber-Riley et al. (1961) who examined the rate of disappearance of bromosulpthalein from plasma in man, frequent sampling of the liver would be inappropriate on ethical grounds, although a single needle biopsy would be acceptable.

Assuming that only one compartment is sampled, then the number of possible compartments can be predicted by analysis of the tracer decay curve for that compartment. This is accomplished by finding an equation which describes the decay curve and this function can be, for example, a power series, an exponential summation, or a multiple regression. Fortunately many biochemical or physiological processes are described by first order kinetics and the physical model can often be represented

by a set of linear differential equations. For example, the rate of movement of substance A from a single compartment is given by:-

$$\frac{dQ_A}{dt} = -kQ_A$$

where Q is the concentration of A at any time, t

This is equivalent to $Q_A = Q_A(o)\, e^{-\lambda t}$

where $Q_A(o)$ is the initial concentration of substance A and $\lambda = k$

For a two compartment model with exchange, two differential equations are required. The solution of these two differential equations takes the form:-

$$Q_j = X_j e^{-\lambda_1 t} + X_{j-1} e^{-\lambda_2 t}$$

and represents the mathematical model of the two compartment system. Thus the number of exponential terms found in the curve should indicate the number of compartments in the system. This is very useful if a priori knowledge of the system is limited. Even if the number of exponentials in a curve can be well defined, determining the correspondence of the individual exponential functions to the physical system can be difficult and depends to a great extent upon the intuitive knowledge of the investigator who can make reasonable suggestions as to the correspondence.

The validity of this approach depends upon the accuracy with which exponential terms may be obtained from the curve, particularly after long time intervals. Two

techniques are available for determining the number of exponents in a curve, firstly 'curve peeling', utilising a graphical approach. The inaccuracies of this method have been discussed and exemplified by Ottaway (1971). The technique is simple to perform; the experimental data is plotted as a logarithm against time and a straight line fitted to the terminal portion of the plot, which should be linear, assuming that the larger exponents have died away. The values at each time point on this line are subtracted from the original data and a new plot constructed from this difference value. The process is then repeated until all the terms are separated (see Fig.33). The graphical technique is highly subjective and with imprecise data such as that derived from biological experiments it is difficult to fit more than three exponential terms. In any event calculation of model parameters using this method can give results which are up to 30% inaccurate (Crawley et al., 1970).

The second method is to assume a mathematical model (i.e. an equation defining the physical system) and fit the function to the experimental data by minimising the sum of residual squares. Using this method any number of exponential terms can be used and the fit becomes better as the number of exponents taken increases, but at the cost of instability in the values of the parameters defining the equation. This instability becomes apparent when two or more of the components have approximately the same exponential constants. Glass and Garretta (1967,1971) studied the effect of data error, exponent ratio and the number of data points on the mean parameter error in a bi-exponential function. For ten data points and an accuracy of $\pm$ 3% the mean parameter error rose from 14% with an exponent ratio of 4 :1 to 104% when the exponent ratio fell to 2 : 1. In a tri-exponential

function the mean parameter errors were even greater. Collection of more data reduced the errors, e.g. increasing the data points from twenty to sixty over the same time interval reduced the errors by a factor of two. Therefore it may be concluded that if the system under investigation contains more than three compartments, then prediction of the actual number of compartments is unrealistic unless large numbers of accurate data are available.

Using sums of exponential terms derived from the analysis of the decay curve for a single compartment, one can obtain values for the parameters of the physical model. These parameters may not satisfy the data obtained from the sampling of other compartments in the system. This is exemplified in the study of Ackerman et al.(1967) who showed that imposition of model parameters derived from the analysis of the decay curve of one compartment onto a three compartment model did not accord with the data derived from sampling all three compartments. A compromise solution had to be adopted to obtain the best fit to all three decay curves and this was derived using an iterative guessing technique to obtain the values of the physical model parameters directly. Small errors in the fitted curve can lead to large errors in the computed model parameters.

The'correctness' of a model is judged by whether the correspondence obtained between the simulated and experimental data is both consistent and unique (Berman, 1963). This means that if systematic deviations between experimental data and simulated data are noted, then the primary model is incorrect and must be modified. Systematic deviations may be ignored if the fit obtained is within the bounds of experimental repeatability. If the correspondence is good but there is instability in the values of the model parameters then the model is probably not unique.

For example, consider the equation

$f : x \longmapsto \sin x$ where x is the domain and sin x is the image of x under the function of sin x

For a restriced domain (-0.4, 0.4) this equation (system) can be approximately modelled by any of the following equations.

$f : x \longmapsto x$

$f : x \longmapsto \cos (\pi/2 - x)$

$f : x \longmapsto x \ \frac{-x^3}{6}$

i.e. if x = (-0.4, 0.4) then all of these functions have the same image as $f : x \longmapsto \sin x$. The data generated by f(sin x) does not therefore have a unique model.

One way of testing the uniqueness of a model is to apply a perturbing stimulus whose effect might be reasonably forecast; if alterations in the value of the primary parameters accomodate this displacement then it is likely that the original physical model is correct.

In practice the usual method of solution of a projected physical model is to set up a series of differential equations. These should be consistent with the number of compartments predicted from an analysis of the decay curve for one compartment, in conjunction with information available about the system from previous investigators. It may be assumed, initially, that all compartments are interconnected with bi-directional transfer between each, or the model may be constrained by a priori information, e.g. the model may be caternary or mammillary with some elements of unidirectional transfer.

Having established a primary model the differential equations may then be written. Fig. 9 shows a simple three compartment system with all of the tracer (q) initially in compartment A. The equations which describe this model are:-

$$\dot{q}_A = q_B k_{AB} - q_A k_{BA} + q_C k_{AC}$$

$$\dot{q}_B = q_A k_{BA} - q_B k_{AB} - q_B k_{CB}$$

$$\dot{q}_C = q_B k_{CB} - q_C k_{AC}$$

The mathematical model then takes the form

$$Q_j = X_j e^{-\lambda_1 t} + X_{j-1} e^{-\lambda_2 t} + X_{j-2} e^{-\lambda_3 t}$$

For simple systems the differential equations may be solved by analytic methods, e.g. by the use of an operator to produce a set of linear algebraic equations which is solved as for any set of simultaneous equations (Atkins, 1971). Alternatively the parameters of a sum of exponentials may be used to calculate rate constants directly by matrix algebra (Atkins, 1971). However when the systems or models of the system become more complex then numerical methods are more suitable and may be implemented on analogue or digital computers.

Use of Digital Computers.

The differential equations may be solved using a numerical iterative technique which uses the rate constants as they appear in the required equations. This is in contrast to the analytic method which is far more rigid in approach and requires restructuring each time details of the model are changed. Numerical analysis uses a

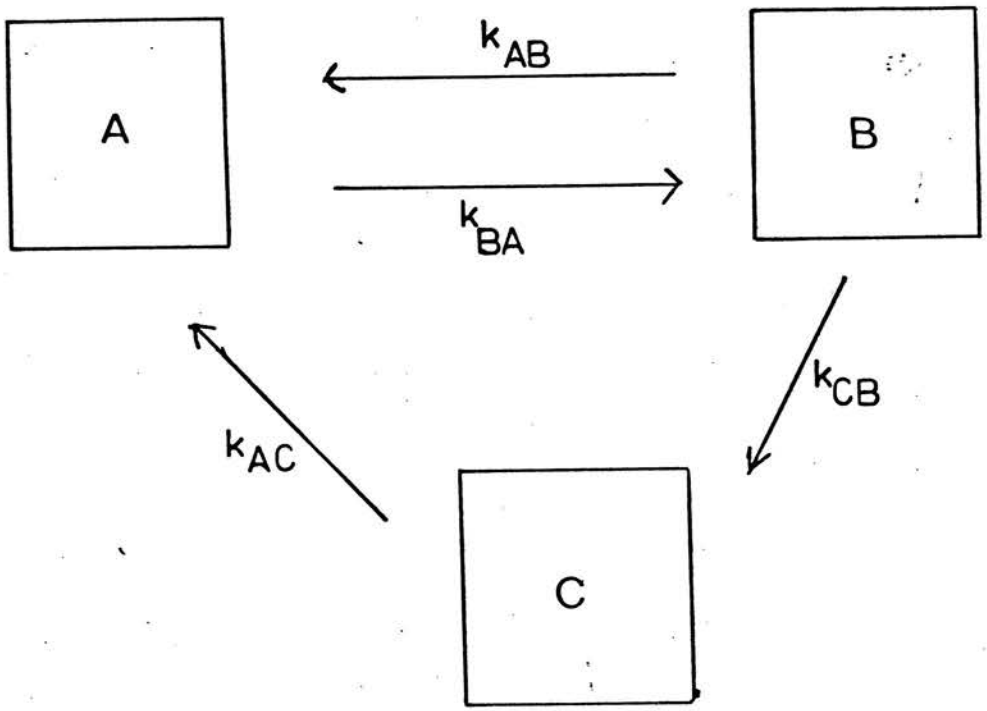


Fig.9. A simple compartmented model showing features of exchange and transfer.

converging method to obtain acceptable rate constants, providing reasonable initial estimates are given. An assessment of whether the iterative procedure will converge, given a particular initial estimate, can in theory be made by comparing the error bound intervals of the initial value and that of f(x), if this ratio is less than the initial value, then the procedure will converge. Although there are a number of curve fitting programs available, e.g. those of Atkins (1971) and Taylor (1970) the only general modelling program available is that developed by Berman and Weiss (1967) and called SAAM 23, i.e. Simulation, Analysis and Modelling Program, version 23. Its limitations are discussed in a brief general assessment by Prescott (1971) and Ottaway (1971).

As the digital computer requires good initial estimates of the parameters of the mathematical or physical model in order to converge to a solution, an initial assessment of the validity of the projected model can be made by preliminary studies with an analogue computer.

Use of Analogue Computers.

Reference should be made to Phillips and Taylor (1969) for an introduction to the principles underlying analogue computing. A more detailed account of the method of usage of this type of computer is given by Taylor (1971). An introduction to analogue computer programming can be found in a book by Charlesworth and Fletcher (1970).

By implication an analogue computer functions by representing the values of the parameters in terms of some physical analogue, e.g. length, volume or more usually voltage in modern electronic-analogue computers. It treats equations of the form $y = f(x)$ and is particularly suitable for solving differential equations because of the correspondence of this function with the behaviour of voltage

curves found in systems connecting capacitators and resistors.

Some analogue problems require manipulation of the system equations to obtain relationships of the type $y = f(x)$, which are termed machine equations. However in compartmental analysis the machine equations can be written directly from the proposed model and a patching diagram can be drawn up. This may require further modification if there is any incompatibility in the magnitude of the values of the parameters of the physical model. This procedure known as scaling, is achieved by adding appropriate feedback on the operational amplifiers or by the addition of potentiometers in the circuit. For experiments which accumulate data over long or short time periods the simulation time can be changed by altering the integrator time constant or by time scaling the machine equations. Where continuous solutions are required the output of the computer can be to a trace oscilloscope or to an x-y plotter.

The parameters of the physical model, i.e. rate constants, are usually represented in the model by potentiometers which allow the operator to change the value of any rate constant and subsequently examine the effect on the continuous solution. If the model is fairly simple then one can quickly see if a solution will be found in reasonable computer time. For more complex models (Heimets, 1969) the number of permutations of the rate constants increases rapidly and the problem becomes unwieldy. For example, if an imbalance is introduced into a simulated system by altering one rate constant then the other rate constants must be modified to retain the status quo. Searching for optimal values in a complex system can therefore be very time consuming. One answer to this problem is to attempt to reduce the complexity of the

system by experimental means and to derive data for the functioning of a smaller part of the system which can be analysed independently. This data may be subsequently incorporated into the larger model.

The main advantage of the analogue versus the digital computer is that it is faster in solving differential equations and allows a more intimate relationship between operator and machine, thereby allowing more rapid convergence of a solution in real time. The major disadvantages lie in the lack of precision, limited logical capacity and in some instances, insufficient hardware to accomodate complex models (Taylor, 1971; Garfinkel, 1969).

One of the ways to validate a model is to test its ability to predict an experimentally observed effect by manipulation of the integrated functions describing the model. For example, in a hypothetical model describing the absorption, metabolism and excretion of a drug, modification of the excretory rate should result in changes in the shape of the decay curve associated with the blood concentration. If this change is experimentally observed when the excretory rate is manipulated in a real subject by therapeutic means, then the model can be said to be unique.

Factors affecting Cholesterol Metabolism.

Several instances arise in cholesterol metabolism where changes in synthesis rates or in the concentration of cholesterol in certain compartments or in excretory rates may be produced by experimental design. Three factors affecting cholesterol metabolism can be considered:-

1. Bile diversion

In the intact animal diversion of bile from the intestine results in a decreasing bile flow and falling biliary excretion of cholesterol and bile acids. Biliary diversion for periods of more than fourteen hours results

in increased hepatic cholesterol synthesis (Myant and Eder, 1961), and increased biliary excretion of cholesterol and bile acids was noted when drainage was longer than thirty hours (Eriksson, 1957a). The total serum cholesterol concentration was not found to change over a nine day period when the fistula was operative (Eriksson, 1957a). Because of the rapid exchange of plasma and liver cholesterol (Eckles et al.,1955), this would imply that either the increased hepatic cholesterol synthesis is balanced by the stimulated biliary excretion of bile acids and cholesterol, or that intestinal absorption is reduced. An additional factor may be that the sterol synthesising capacity of other tissues (principally the intestine) is reduced, or that newly synthesised cholesterol is stored in extrahepatic tissues. Studies by Kay and Entenman (1961) and Percy-Robb and Boyd (1970), showed an increased rate of bile acid secretion during perfusion of livers taken from rats which had been subjected to biliary drainage for forty four hours. At the same time the perfusate total cholesterol concentration did not change. This would imply that the increased sterol synthesis was balanced by an increased secretion of bile acids and possibly of cholesterol.

Furthermore, Percy-Robb and Boyd (1970) suggested that the rate of equilibration of newly synthesised cholesterol between liver and plasma was increased in conditions where hepatic cholesterol synthesis was stimulated.

2. Thyroid Status

It is well established that patients with myxoedema have increased plasma cholesterol concentrations. Experiments with radioactive cholesterol in man do not provide evidence of an increase in total body cholesterol in myxoedematous subjects (Gould, 1959) and it may well be that there is a redistribution of total body cholesterol.

This is supported in part by de Matteis (1969) who noted small increases in liver cholesterol concentration in thyroidectomised rats.

The mechanism underlying the plasma cholesterol elevation is not clear but the thyroid hormones are undoubtedly involved. There is a fall in plasma cholesterol concentration associated with a marked increase in faecal excretion of neutral sterols when L-thyroxine is administered (Miettinen, 1968a). This was accompanied by a variable increase in the secretion of bile acids and excretion of unabsorbed dietary cholesterol. Eriksson (1957b) however, found increased bile acid and cholesterol excretion in thyroxine treated rats.

Stimulation of hepatic cholesterol synthesis from (^{14}C) acetate by thyroid hormone has been demonstrated by Gould et al. (1955), and in 1969 Schweppe and Jungman showed that L-thyroxine increased the rate of cholesterol ester synthesis by rat liver microsomes. These studies were supported by that of de Matteis (1969) who found decreased incorporation of (^{3}H) acetate into liver cholesterol using slices of liver taken from thyroidectomised rats. Gould et al. (1955) also found little incorporation of (^{14}C) acetate into plasma cholesterol in their group of myxoedematous subjects. A reduced catabolism of cholesterol to bile acids was demonstrated in the study of Hellstrom and Lindstedt (1964), who reported a reduced turnover of cholic acid in myxoedematous subjects; this could be increased by administration of D-triiodothyroxine.

The best explanation for the effects of thyroid hormone on cholesterol metabolism seems to be a change in hepatic cholesterol synthesis or in bile acid and cholesterol excretion rates. Thus Miettinen (1968a) showed that the reduction in serum cholesterol concentration in myxoedematous patients undergoing therapy with L-thyroxine, correlated closely with the increase in faecal neutral

sterols and bile acid output ($r = 0.87$).

If the absorption of dietary cholesterol is assumed to be unchanged, then the thyroid hormones must act by controlling excretion of biliary cholesterol, either directly or by influencing hepatic cholesterol synthesis or both. An effect on hepatic cholesterol synthesis is the most likely explanation because Gries et al. (1962) demonstrated a significant induction of the enzyme β - hydroxy- β -methyl glutaryl reductase in the liver cell after injection of triiodothyroxine into the perfusate of an isolated rat liver preparation.

There is little information available as to whether the thyroid hormones act after the rate limiting step of HMG to mevalonic acid. In this context it is interesting to note that Percy-Robb (1968) found that the incorporation of (2^{14}C)DL mevalonic acid into cholesterol in the whole blood perfusate of an isolated rat liver perfusion was different when comparing livers taken from normal and thyroidectomised animals. The initial rates of incorporation were similar for both groups of animals, but after two hours the rate decreased markedly in the thyroidectomised group.

3. Effects of Drugs.

The correlation between plasma cholesterol concentration and the incidence of coronary heart disease has led to a search for agents which would reduce the plasma cholesterol concentration. Reduction in plasma cholesterol is primarily dependent upon the relationship between the entry of dietary or newly synthesised cholesterol into the circulation and the catabolism of plasma cholesterol. In the absence of dietary restriction therefore, the potential sites of action for a therapeutic agent are either i) to increase catabolism or excretion of cholesterol or ii) to inhibit cholesterol synthesis.

Various non-absorbable agents have been shown to bind bile acids *in vitro*, for example, cholestyramine (Tennent *et al.*, 1960), or lignin (Eastwood and Girdwood, 1968). They act by sequestering bile acids and cholesterol i.e. an *in vivo* mimic of biliary drainage. Hypocholesterolaemia is the result. Neomycin, another non-absorbable agent acts by directly reducing absorption of cholesterol or bile acids from the gut, thereby reducing the plasma cholesterol concentration (Powell *et al.*, 1962). Nicotinic acid administration causes a reduction in serum cholesterol level, probably by two independent mechanisms. Firstly by stimulating an increased excretion of neutral sterols of endogenous origin (Miettinen, 1968b), and secondly, by inhibition of cholesterol synthesis from (^{14}C) acetate (Holmes, 1964). Unfortunately the dose levels required in some subjects cause unacceptable side-effects.

The drug which has aroused major interest in terms of its hypo-lipidaemic effect is ethyl chlorophenoxyisobutyrate, (Atromid S, Clofibrate, CPIB; I.C.I. Pharmaceuticals Ltd.). This drug was first described by Thorp and Waring (1962) when they reported that its administration to rats caused large reductions in the plasma cholesterol concentration. Subsequently the drug was found to be effective in man (Hellman *et al.*, 1963; Oliver, 1963). The time course of this effect is remarkably short. In rats, an intraperitoneal injection of Atromid results in a significant reduction in plasma cholesterol concentration within six hours (Pereira and Holland, 1970).

The mode of action of this drug has been the subject of intensive investigation and a number of hypotheses have been formulated to explain its effect on the circulating plasma cholesterol concentration. The best available evidence points to a block in hepatic cholesterol synthesis between acetate and mevalonate, and to a

decreased rate of release of lipoprotein from the liver. Thus, Avoy et al. (1965) showed both in vivo and in vitro that the hepatic synthesis of cholesterol from acetate was greatly diminished whereas the incorporation of mevalonate was relatively unaffected. This was supported by the in vitro study of White (1970), who also demonstrated a small reduction in the incorporation of mevalonate into cholesterol. Investigations on further sites of action of the drug were extended when Azarnoff et al.(1965) reported that liver from rats fed Atromid for periods up to twenty one days showed marked reductions in the incorporation of mevalonic acid into cholesterol. This reduction may be a secondary effect related to the dosing period used. Grundy et al. (1969) have also produced evidence that Atromid acts to stimulate release of cholesterol from storage sites with a proportional increase in the excretion of faecal neutral sterols.

There is some suggestion that CPIB also reduces the rate of triglyceride, and therefore lipoprotein release by the liver (Duncan et al., 1964) without a concomitant decrease in the uptake of palmitate substrate. Azarnoff et al. (1965) confirmed this effect and also reported that cholesterol release from the liver was impaired. These two findings would tend to explain the marked increase in liver weight found in animals treated with CPIB (Pereira and Holland, 1970).

These three effects on cholesterol metabolism can be introduced into experiments using isolated organ perfusion, either directly e.g. addition of the drug to the perfusate, or indirectly by previous biliary drainage or by surgical thyroidectomy. It would be of interest to establish whether these changes could be predicted by the model or if the model could be accomodated to observed experimental data in these changed circumstances.

Objectives of the Study.

There are few studies which have been performed specifically to examine the rate of exchange of cholesterol between liver and blood, and where this has been achieved the results are open to question because the studies have been performed *in vivo* and the element of exchange between extrahepatic tissues and the blood has been unavoidably uncontrolled. This thesis describes experiments examining the kinetic distribution of cholesterol between the blood and liver (Fig. 8) using an isolated rat liver perfusion technique in which the system was open to experimental control and extrahepatic tissues were not involved. In these investigations the rate of appearance of radioactive, ester and unesterified cholesterol in plasma, erythrocytes and bile was determined and an attempt was made to construct a feasible biological and mathematical model to correspond to the data obtained. Simulation of the model using an analogue computer allowed estimates to be made of the values of the model parameters. An examination of the kinetics of the intra-cellular distribution of newly synthesised liver cholesterol was performed to establish if there were any time-dependent distributions which would be reflected in the data derived from whole liver perfusions.

Because of the problems involved in formulating and testing compartmental models with more than three compartments, the biological system was 'dissected' at the experimental stage and estimates of the parameters in the *in vitro* exchange of cholesterol between plasma and erythrocytes obtained before simulating the whole liver perfusion. This allowed preliminary estimates of the values of the parameters of a small portion of the larger model and reduced the difficulties in modelling the complete data. An assessment of the validity of the model and of the values of the parameters was made by comparison with other studies.

Having established a 'reasonable' model for the normal situation the study was extended to examine the effect of thyroid status and previous biliary drainage on the rates of appearance of radioactive cholesterol in the perfused system. Where necessary the new data obtained was accomodated to the normal model to see if the actual point or site of change, produced by manipulation of the physical system, could be elucidated.

Finally a study of the effects of the hypocholesterolaemic drug, ethyl chlorophenoxyisobutyrate (Atromid-S500, I.C.I. Pharmaceuticals Ltd.) on the isolated liver system was performed, to determine whether the hypocholesterolaemic effect was related to changes in hepatic synthesis or in the distribution between liver and blood. An attempt was made to establish the time course of the action of the drug.

METHODS

1. Quantitative determination of Cholesterol.

The Wistar strain of rats used throughout this study were known to have a mean plasma total cholesterol concentration of about 500µg/ml (Percy-Robb, 1968). The experiments to be carried out would necessitate the use of as small blood samples as possible to determine the concentration and specific radioactivity of cholesterol. A further limitation imposed upon the amount of cholesterol available for analysis arose from the requirement that a differentiation should be made between esterified and unesterified cholesterol. Consequently it became necessary to have the most sensitive, specific and practicable method available.

The methods available for determining cholesterol can be divided into three main groups:-

i. methods where the specimen is treated directly with reagent without the removal of non-cholesterol chromogens e.g. proteins, bilirubin (Zlatkis et al.,1953).

ii. methods where a preliminary extraction of cholesterol from the specimen is performed and the reaction carried out either directly on this extract or upon the residue obtained after evaporation of the solvent.(Franey and Amador, 1968).

iii. a further refinement of ii. is to separate the cholesterol from the solvent extract by either precipitation as a complex or by absorption chromotography.(Schoenheimer and Sperry, 1934; Hirsch and Ahrens, 1958). The cholesterol can then be determined in the purified fraction using a colorimetric method or by ultra-violet spectrophotometry (Weigensberg et al. 1959).

In so far as meeting the analytic requirements of this study most of the above options were open to criticism. The direct method is not specific for cholesterol and was not considered further. The second approach was more

acceptable but again problems of specificity arose and the methodology is not easily adaptable to the differentiation of 'free' and ester cholesterol. The last group of methods was the one chosen and the precipitation procedure was adopted primarily because it was more suitable for analysis of large batches.

Apart from consideration of specificity another factor is present which in any event would restrict the choice of method to the last group. Most of the specimens for analysis would contain an unknown quantity of a labelled precursor of cholesterol synthesis. It was important that any of this labelled precursor not used in synthesis should be separated from the newly synthesised cholesterol so that accurate estimations of specific radioactivity could be made.

Ultraviolet method

The non-destructive ultra-violet method described by Weigensberg et al. (1959) was examined. This method had the advantage of being able to determine the radioactivity on the same specimen that was used for the determination of concentration. Three factors mitigated against its use:-

1. the unavailability of a suitable intrument to measure reliably at 205nm.
2. the coefficient of variation of the method was greater than 8% when the concentration of cholesterol in the sample being analysed was less than 50µg/ml. This was unacceptable.
3. the linearity was poor below cholesterol concentrations of 50µg/ml in the sample extract.

Criticisms 1 and 2 were probably due to instrumental instability.

Colorimetric reaction.

The more important colorimetric chromogenic reactions are listed in Table 2 (after Richterich, 1969). The Pearson reaction was discounted because of the interference by even small quantities of digitonin (Chiamori and Henry, 1959). The Tschugaeff reaction is said to be very sensitive but requires stringent exclusion of moisture. Most widely used is the Liebermann-Burchard reaction, although it is susceptible to the effect of time, temperature, solvent and water.

The method of choice therefore, was that of Zlatkis et al. (1953) and a comparison was made of the sensitivity of this reaction with that of Liebermann-Burchard. The comparison was made using amounts of cholesterol ranging from 0 - 100µg per sample and the final volumes adjusted to be equal for both reactions. Absorbance values at published maxima indicated that the Zlatkis reaction is four or five times more sensitive than that of Liebermann-Burchard. This confirms the results of Zlatkis et al. (1953).

The problems of differential chromogenicity of the esterified and unesterified forms of cholesterol were not considered as all of the estimations in this study were performed on the unesterified form of cholesterol, i.e. the specimens were hydrolysed prior to analysis.

Zlatkis reaction: conditions for optimum sensitivity.

Variations in reagent composition, temperature and time of reaction were investigated to optimise the sensitivity of the reaction. A solution of recrystallised cholesterol (see p 44) was prepared in methanol at a concentration of 100µg/ml. This solution was used in the investigation of the Zlatkis method.

The reaction was sensitive to the amount of ferric chloride present in the final reaction volume. Fig. 10 shows that the increase in sensitivity fell off rapidly

Author		Reagents	Code
Salkowski	1872	Chloroform + Sulphuric acid	Red
Thudichum	1878	Chloroform + Sulphuric acid	Red
	1884	Sulphuric acid + Glacial acetic acid	Red
Liebermann	1885	Acetic anhydride + Sulphuric acid	Green
Burchard	1890	Chloroform + Acetic anhydride + Sulphuric acid	Green
Pearson et al.	1953	Toluene-p-sulphonic acid + Acetic anhydride + Glacial acetic acid + Sulphuric acid	Green
Carpenter	1957	Trichloro-ethane + Acetic anhydride + Sulphuric acid	Green
Tschugaeff	1900	Acetyl chloride + Glacial acetic acid + Zinc chloride	Red
Trinder	1952	Acetyl chloride + Dichloro-ethane + Sulphuric acid	Red
Zlatkis et al.	1953	Acetic acid + Sulphuric acid + Iron chloride	Red
Brown	1959	Acetyl chloride + Dichloro-ethane + Perchloric acid	Red
Searcy and Berquist	1960	Glacial acetic acid + Sulphuric acid + Iron sulphate	Red

Table 2. After Richterich, 1969.

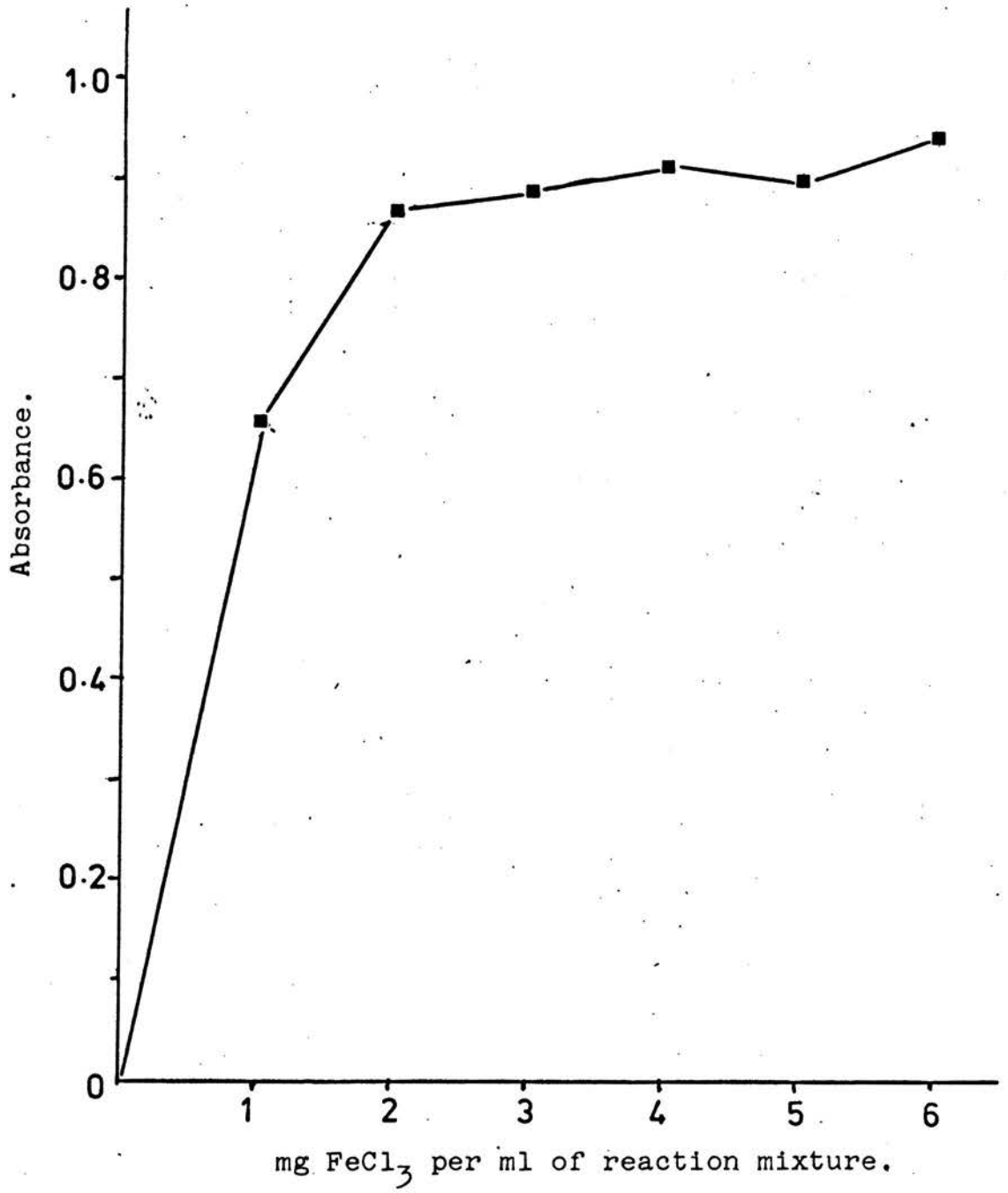


Fig.IO. The effect of different concentrations of ferric chloride on the sensitivity of the Zlatkis reaction.

when more than 3mg of ferric chloride were present. The amount of sulphuric acid in the final reaction volume was also critical. Fig. 11 shows that the sensitivity rose to reach a constant level at about 1.2ml acid in the reaction mixture. Full development of the colour was also dependent upon the time of reaction and it is clear from Fig. 12 that at least 45min must be allowed to develop the full colour. The absorbance remains constant for at least six hours after the full reaction has developed. The final method adopted is shown below.

Recommended procedure.

Stock ferric chloride solution	2.5g $FeCL_3 6H_2O$ dissolved in glacial acetic acid made up to 100ml.
Working ferric chloride solution	Dilute 4ml stock solution to 100ml with glacial acetic acid.
Colour reagent	Working $FeCl_3$ solution with H_2SO_4 in the ratio of 1:1. Allow to cool.

Test — Should contain a dried residue of cholesterol ranging between 0 and 50µg. This is dissolved in 0.5ml methanol.

Blank — 0.5ml methanol.

Standards — 0.1, 0.2, 0.3, 0.4, 0.5ml of the standard cholesterol solution.
Each standard made up to 0.5ml with methanol.

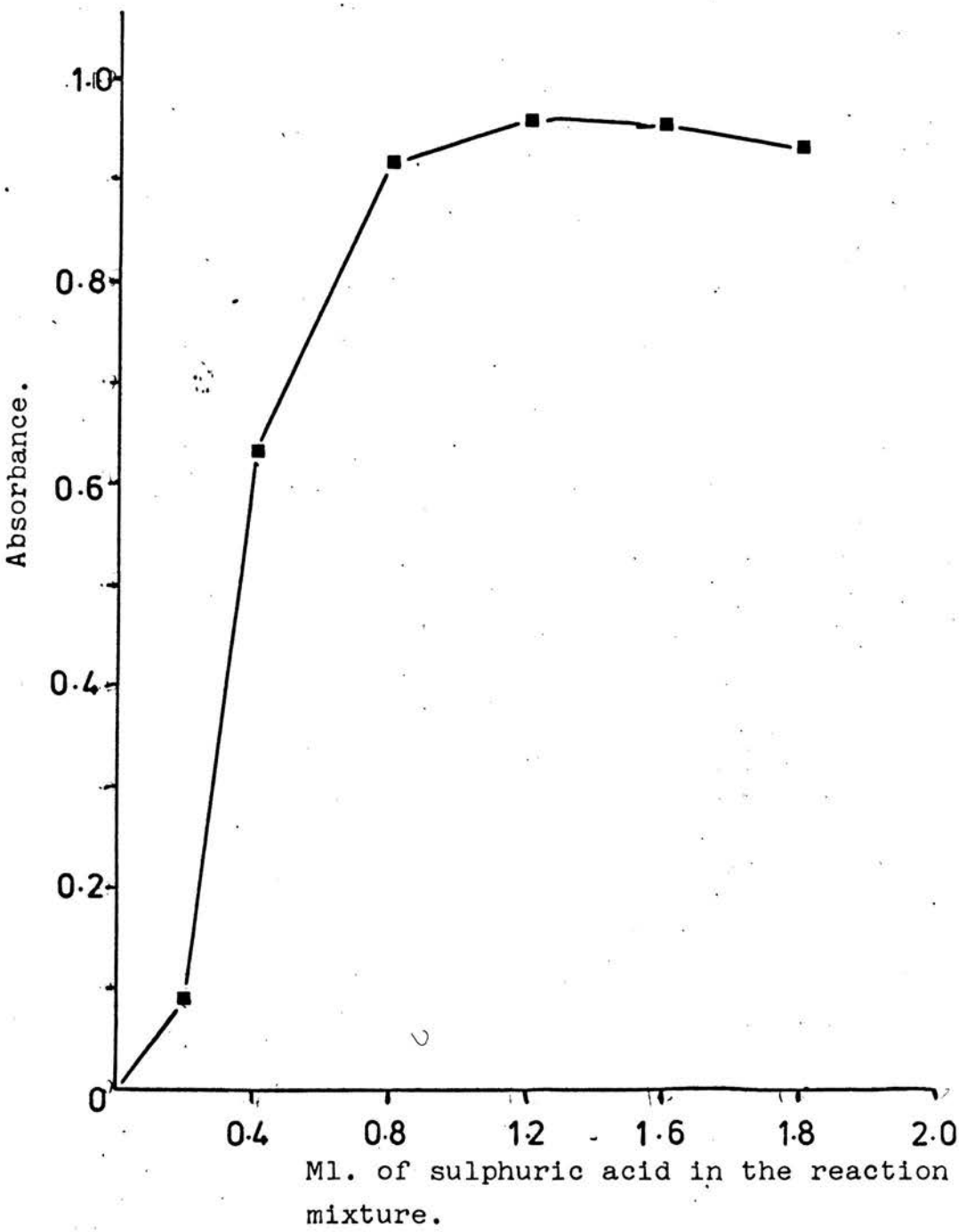


Fig.II. The effect of different amounts of sulphuric acid on the sensitivity of the Zlatkis reaction.

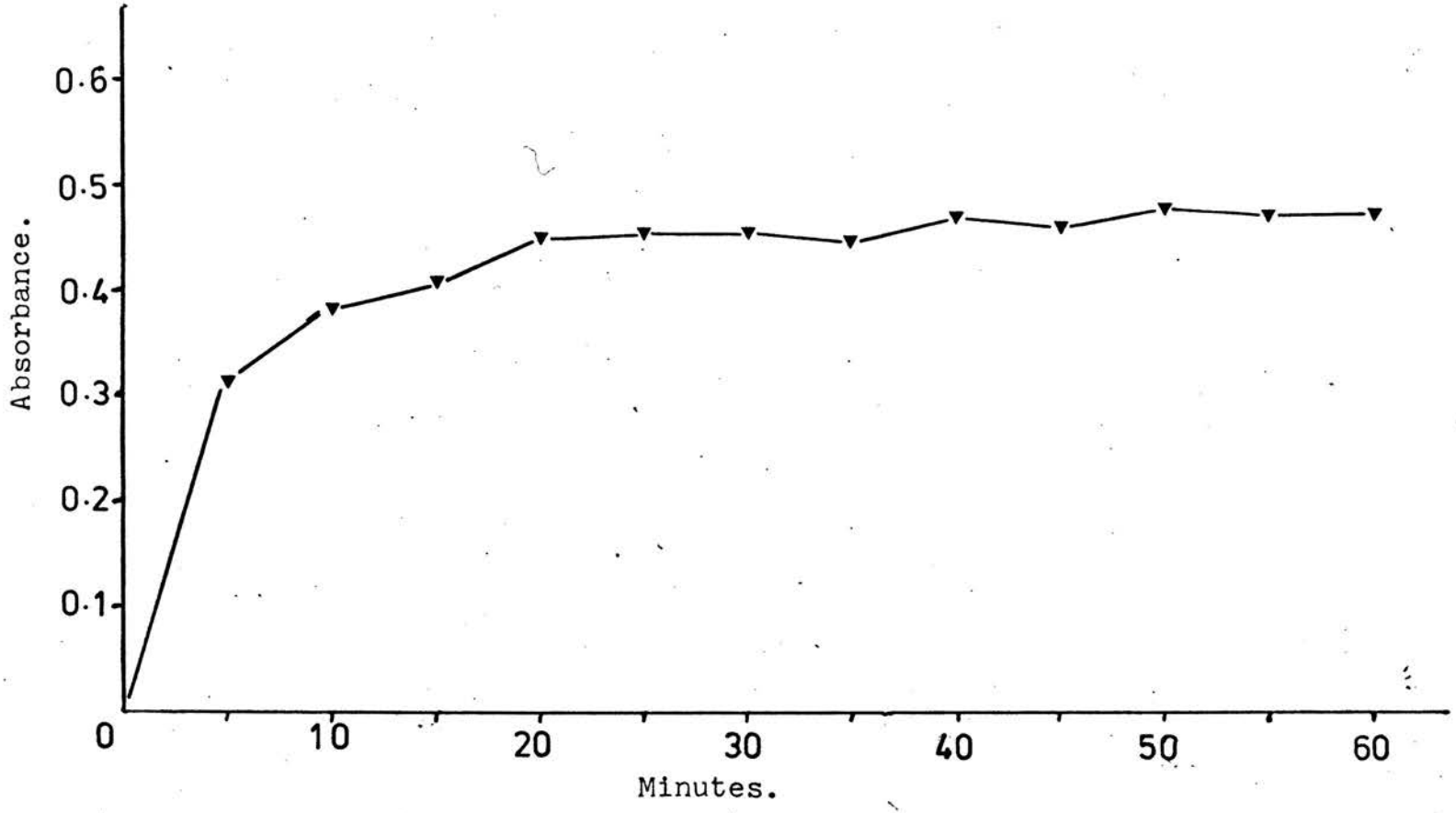


Fig.I2. Time required to obtain the maximum absorbance at 560nm in the Zlatkis reaction.

To each tube was added 3.0ml of ice cold colour reagent. When all the additions had been made, the tubes were mixed in a vortex mixer and then placed simultaneously in a water bath at 75°C, removed after five minutes, cooled in cold water and allowed to stand at room temperature for fifty minutes. Absorbance at 560nm was read in a Unicam S.P.600 Spectrophotometer against the blank.

Extraction and recovery of cholesterol.

In general the methods used for extracting cholesterol from biological specimens will also extract sterols which are precursors in the biosynthetic pathway leading from lanosterol to cholesterol. To determine cholesterol alone it is therefore important to apply procedures which reduce the contribution of the precursors to the cholesterol concentration. This can be achieved firstly by attempting to separate the precursors from the cholesterol, and secondly by applying a colorimetric procedure in which the molar extinction coefficient of the precursors is considerably less than that of cholesterol. i.e. (cholesterol) x absorbance is greater than (precursor) x absorbance.

One method for obtaining a sterol fraction free from other lipid components that might interfere in a colorimetric reaction is to precipitate the unesterified form of the alcohol as the digitonide. This procedure was first used by Windaus (1910), and most 3-β-hydroxylated sterols are precipitated from an acidified aqueous solution of alcohol or acetone. Precipitation of the digitonide of cholesterol requires that the 3-β-hydroxy group be unesterified and therefore to determine the total cholesterol the esterified fraction must first be hydrolysed.

The application of a suitable colorimetric reaction to the precipitated complex could further reduce the contribution of the non-cholesterol fractions. Franey

and Amador (1968) showed that in a reaction employing ferric chloride and sulphuric acid, the molar absorption of pregnenolone, lanosterol, 7-dehydrocholesterol, cholestanol and cholic acid were low when compared to that for cholesterol. As the blood concentrations of these constituents were also low, then the contribution of these constituents to the absorbance of the final reaction mixture could be ignored.

Silicic acid chromotography of the sample extract to obtain the free and esterified fractions of the sterols was considered (Hirsch and Ahrens, 1968; Horning et al. 1960). Although this method shows excellent results for relatively large scale operations, adaption to a micro scale and use in batch mode could raise difficulties and the method was not seriously considered.

Digitonide precipitation of cholesterol.

The procedure described by Sperry and Webb (1950), was used with a few minor technical modifications.

Reagents

1. a) Acetone : alcohol (1 : 1)
   b) Acetone : ether (1 : 1)
   c) Ether
2. Digitonin solution 0.5% in 50% aqueous alcohol
3. Aqueous solution of potassium hydroxide 33g/100ml
4. Hydrochloric acid 5% v/v
5. Phenolphthalein 1% solution in ethanol

Procedure

The cholesterol was extracted from the specimen with acetone:alcohol (1:1) ensuring that a ratio of at least 20 volumes of solvent to one of specimen was achieved (Boyd, 1962). Extraction was performed at room temperature and allowed to proceed overnight. The protein precipitate was removed by centrifugation and aliquots of the supernatant used for the precipitation of free and total cholesterol. This was carried out as described by Sperry and Webb (1950) with the following modification. Resuspension and washing of the precipitated digitonide was performed using a vortex mixer. Hydrolysis of the esterified cholesterol was performed by addition of 33% aqueous potassium hydroxide followed by incubation at 40°C overnight to ensure complete reaction. Complete hydrolysis can be achieved in two hours for the esters found in normal human serum, but esters with more saturated acids require additional hydrolysis (Boyd, 1962). As the predominant fatty acid of rat serum is arachidonic (Goodman, 1962), hydrolysis should be complete under the conditions used in this study.

Neutralisation of the hydrolysis mixture was carried out by the addition of 5% hydrochloric acid rather than 10% acetic acid, because a non-flocculent precipitate was obtained using acetic acid. This initially raised problems of washing the precipitate free of absorbed radioactivity associated with the un-metabolised mevalonic acid and was overcome by using hydrochloric acid.

Recovery of digitonide.

A spectral scan of the chromogen developed with the Zlatkis reaction from cholesterol in the free form and from the digitonide is shown in Fig. 13. In the figure the

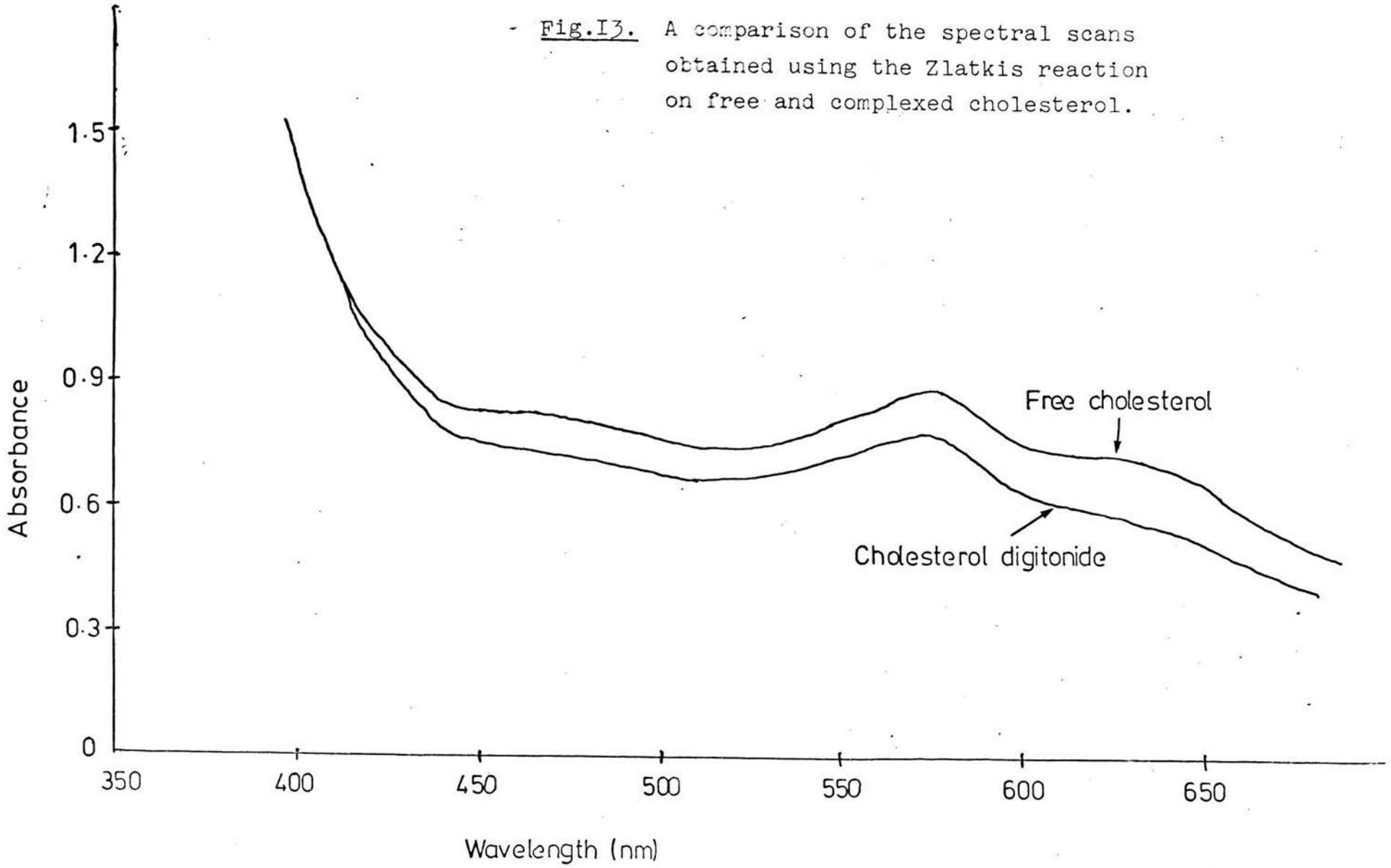


Fig.I3. A comparison of the spectral scans obtained using the Zlatkis reaction on free and complexed cholesterol.

two curves shown do not represent the same concentration of cholesterol but serve to illustrate the similarity of the scan for both the molecular species. Measurement of the absorbance of the digitonide at 560nm would be appropriate, and similar to that for free cholesterol.

A comparison of the absorbance at 560nm of the chromogen formed from two sets of standards, one of which was precipitated as the digitonide is shown in Fig. 14. The two procedures do not yield identical results as shown by the divergence from the equivalence line.

The determination of cholesterol in the experiments in this study was therefore standardised by using five standards at different concentrations and the standards were carried completely through the analytical procedure.

Repeatability of complete quantitation procedure

Pooled specimens of rat plasma and of erythrocytes were divided into 10 equal aliquots and the unesterified and total cholesterol concentration determined. The results are shown in Table 3. There was no significant difference ($p > 0.1$) between the mean values observed for total and unesterified cholesterol in the erythrocytes. This confirms the report of Nelson (1967) that all of the erythrocyte cholesterol is in the unesterified form. Consequently throughout this thesis only the concentration of unesterified cholesterol in the erythrocytes was determined.

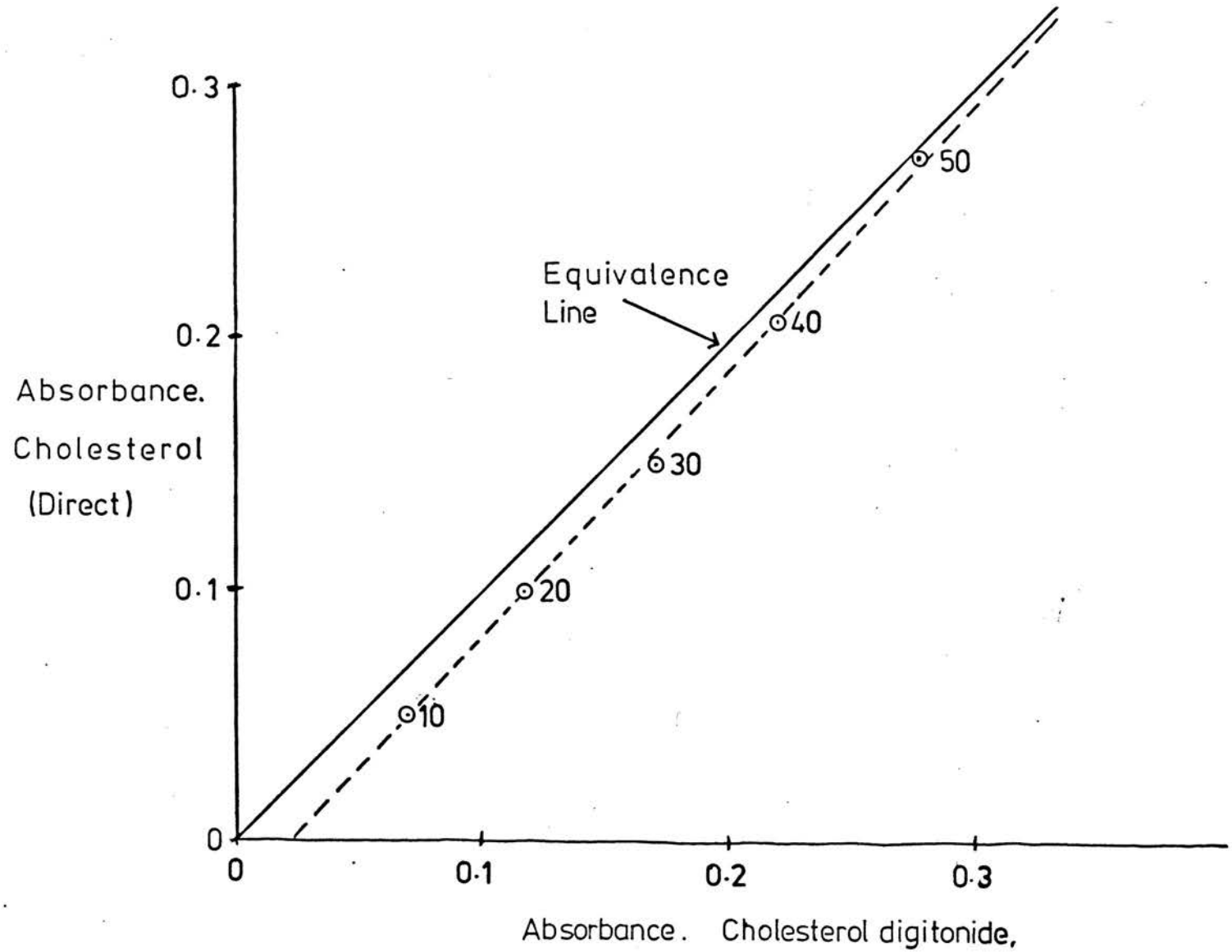


Fig.14. Recovery of cholesterol as digitonide.

	Mean amount of cholesterol in sample (µg)	S.D. (µg)	C.V. (%)
Plasma unesterified	9.41	0.54	5.7
Plasma total	25.96	1.46	5.6
Erythrocyte unesterified	63.27	3.67	5.8
Erythrocyte total	63.14	3.54	5.6

Table 3

Repeatability of the determination of cholesterol in plasma and erythrocytes.

Radioactive contamination of cholesterol digitonide.

During the perfusion experiments a single pulse of (2^{14}C)DL mevalonic acid lactone was used as a cholesterol precursor. It has been shown that up to only 50% of this precursor is metabolised to cholesterol under the conditions used in this study.(Percy-Robb, 1968; Tavormina et al., 1956). The biologically inactive isomer remains in solution. It is therefore necessary that in determining the specific radioactivity of the cholesterol, no radioactivity should be included from the unmetabolised isomer. This was achieved by isolating the cholesterol as the digitonide, and experiments were carried out to verify that radioactive contamination of the digitonide did not take place.

a) A standard solution of cholesterol was treated as if to determine total cholesterol by digitonide precipitation, after addition of a known quantity of radioactivity associated with (2^{14}C)DL mevalonic acid. The counts remaining with the digitonide after separation and washing of the precipitate showed that only 0.08% of the added radioactive mevalonic acid remained with the digitonide precipitate.

b) A specimen of plasma remaining from a perfusion experiment in which (2^{14}C)DL mevalonic acid had been added to the perfusate was extracted from alcohol:acetone and the extract divided into two parts. Additional (2^{14}C)DL mevalonic acid was added to one part and the digitonide of cholesterol was precipitated from both parts and washed as previously described. The digitonides were then taken up in the methanol and applied to a Silica gel thin-layer chromotography plate. A standard of unesterified cholesterol and a small quantity of labelled (2^{14}C)DL mevalonic acid were also independently spotted onto the plate. The digitonide prepared from the unadulterated extract was

hydrolysed by the addition of pyridine, the cholesterol extracted into ether and spotted onto the plate.

The chromatograms were developed in chloroform:acetone (90:10) and examined for the presence of sterol and radioactivity. The results are illustrated in Table 4 and show that no radioactivity other than that found in cholesterol was associated with the digitonide prepared from the perfusion specimen.

Cholesterol standard

25g of cholesterol (B.D.H. Reagent) were recrystallised from hot 100% methanol and dried at 55°C *in vacuo* overnight. This preparation was tested for purity by thin-layer chromatography on silica gel plates. (Silica Gel G Antec T.L.C. plates SGF 20/20 0.250mm). A solution of cholesterol in ethanol containing about 50µg was spotted onto the plate and the sterol was located in the developed chromatogram by spraying with 2% phosphomolybdic acid in ethanol and heating at 120°C for 5min (Stahl, 1965).

Solvent System	Rf found	Rf reported (Stahl, 1965)
Benzene:chloroform (40:60)	0.11	0.13
Chloroform:acetone (90:10)	0.70	0.65

No other material was located and the prepared cholesterol was considered to be sufficiently pure to use for standardisation purposes. A standard containing 100µg/ml methanol was prepared. 500ml of this standard was stored in sealed 10ml aliquots for use throughout this study.

Fraction	Colori-metric Rf	Radio-active Rf
1. Digitonide before addition of(2^{14}C)DL mevalonic acid	0	0
2. Digitonide after addition of (2^{14}C)DL mevalonic acid	0	0 and 0.27
3. Cholesterol standard	0.7	0
4. Mevalonic acid	Not detected	0.27
5. Cholesterol derived from 1. above	0.7	0.73

Table 4.

Chromatographic verification of the purity of the digitonide of cholesterol, illustrating that radioactive contamination of labelled cholesterol was not detectable.

Confirmation of molecular species

A recrystallisation experiment was performed to ascertain whether the substance being examined for specific radioactivity values was in fact cholesterol. A specimen of blood obtained from a perfusion experiment was extracted with acetone:alcohol and the presumed radioactive cholesterol was diluted with non-radioactive cholesterol as carrier and repeated recrystallisations carried out from either ethanol/water or acetone. The specific radioactivity of the cholesterol was determined between each re-crystallisation. Table 5. shows the results of this experiment.

Since the specific radioactivity is not significantly different after five recrystallisations it is probable that the only radioactive substance present is that of the carrier molecule, i.e. cholesterol.

2. Radioactive counting.

Two radioactive isotopes were used in this study, namely carbon - 14 and tritium. The carbon - 14 was incorporated in mevalonic acid and the tritium in sodium acetate.

All counting procedures were performed on a Packard Tri-Carb liquid scintillation spectrometer. Model 574.

Samples were prepared for scintillation counting by adding 10ml of scintillator (see Appendix) to the digitonide of cholesterol dissolved in methanol and counts determined for three 10min periods on each batch. This method of counting was to ensure that any instrumental changes during counting would be distributed throughout all the samples counted and not confined to a particular segment of the sample batch. A blank containing pure

Recrystallisation	Solvent	Specific radioactivity of cholesterol nCi/mg
1.	Ethanol/water	3109
2.	Ethanol/water	3069
3.	Ethanol/water	3241
4.	Acetone	3141
5.	Acetone	3090

Table 5.

The repeated recrystallisation of extracted cholesterol showed that the molecular species originally extracted was cholesterol.

methanol was used in every batch to determine background radioactivity.

Suitable instrumental conditions were established by plotting integral bias curves for both isotopes used in this study. Standards were prepared from (^{3}H) hexadecane and (^{14}C) hexadecane and 20 x 10min counts performed on each standard with different extra high tension (E.H.T.) and pulse-height discriminator settings. A plot of the different integral discriminator bias curves is shown in Fig. 15. The E.H.T. settings on the instrument used are represented by a 'gain' setting and are shown on the x axis. The numbers on each of the curves in Fig. 15. indicate the upper settings of the pulse-height discriminator with the lower limit being 50 for all the curves illustrated.

When counting carbon-14 only, the discriminator bias set on the potentiometers was from 50 - 1000 with a gain of 9%. This gave counting efficiencies of 81 - 85%. The efficiency of the counting procedure was checked at intervals during this study. In later experiments both carbon-14 and tritium were counted in the same sample and settings were as follows.

^{14}C	Gain = 6%	Discriminator bias	50 - 530
		Counting efficiency	^{14}C = 55%
			^{3}H = 9%
^{3}H	Gain = 24%	Discriminator bias	50 - 200
		Counting efficiency	^{14}C = 9%
			^{3}H = 29%

Since a discriminator ratio method (Okita et al., 1957) was used to correct the observed count it was necessary

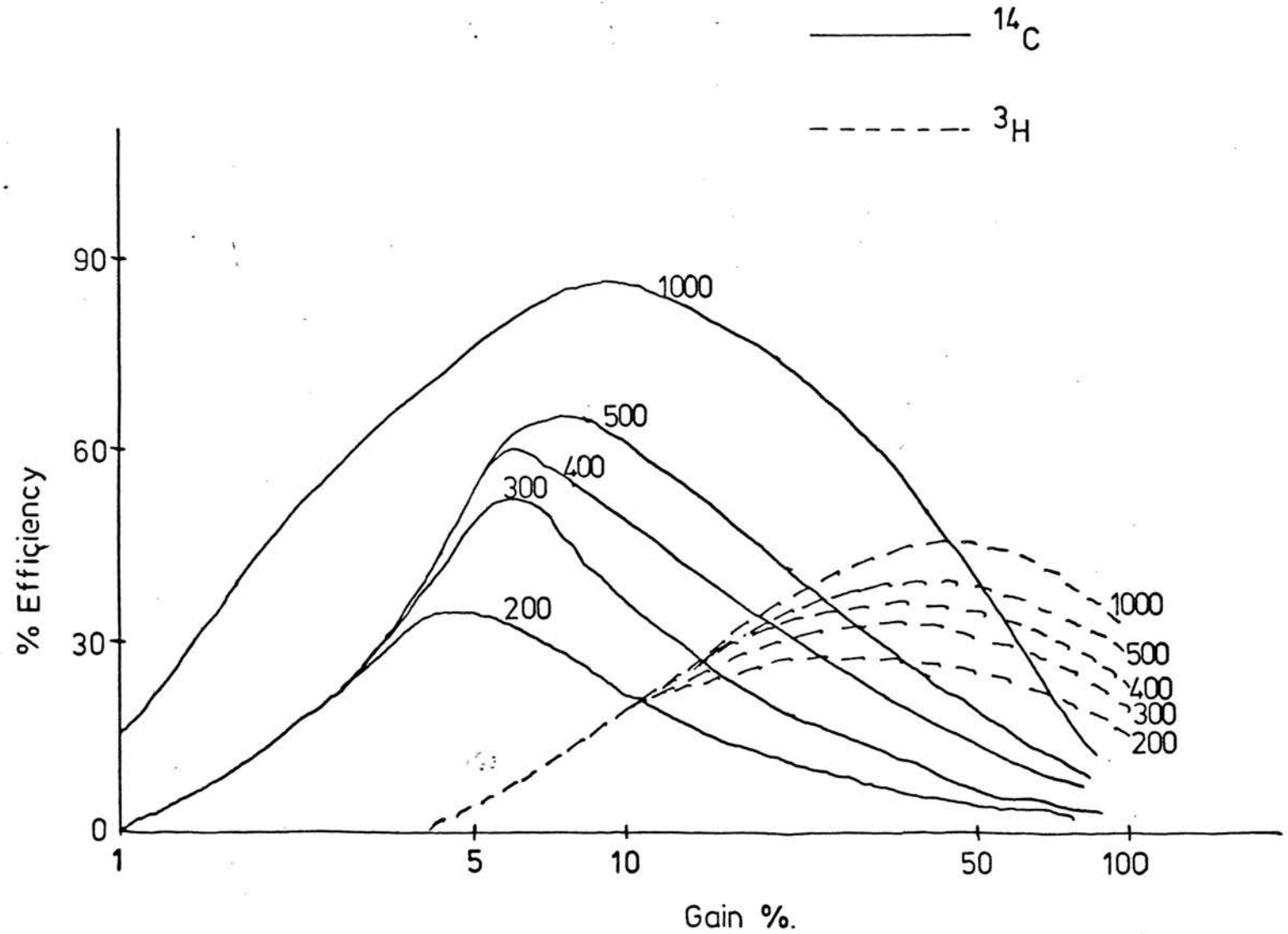


Fig.15. Calibration curves for scintillation spectrometer.

that both tritium and carbon-I4 pulses could be detected in both channels to obtain a ratio value. A gain setting of 6% in the carbon-I4 channel accomplished this. The discriminator bias settings used in the carbon-I4 channel were 50-530 because at these settings the efficiency was at a maximum and would therefore be less susceptible to instrumental fluctuations. The choice of settings for the tritium channel was governed by the need to obtain the maximum discrimination between carbon-I4 and tritium, as well as to maintain a high efficiency in counting. This was apparent with a discriminator range of 50-200 and a gain of 24% as can be seen from Fig.I5.

Having established optimum conditions for counting, the discriminator-ratio method described by Okita et al. (I957) was used to derive the disintegrations per minute (d.p.m.) for each isotope and the method checked by counting a mixed standard of carbon-I4 and tritium.

Carbon-I4	d.p.m added= I823	d.p.m. found = I870 % error = 2.6
Tritium	d.p.m. added = 950	d.p.m. found = 995 % error = 4.7

The efficiency of the counting procedures and of the differential separation of the two isotopes was checked at intervals during the course of this work.

3. Sub-cellular fractionation.

The procedure described by Dounce et al. (1955) was used as a basis for fractionation into four components, namely, nuclei, mitochondria, microsomes and the soluble fraction. The effectiveness of the procedure was tested by determining the relative enzyme activity in the separated cellular components of enzymes known to be selectively located (de Duve et al., 1955). Additionally the preparations were examined by microscopy to verify visually the separation of the sub-cellular organelles.

Fractionation procedure.

The liver was homogenised in 0.44M aqueous sucrose at 4°C in a Potter type rotating homogeniser which applied a shearing stress to the cell. Prolonged homogenisation results in destruction of the nuclei (Dounce et al., 1955) and experiments were performed using different homogenisation and centrifugation periods, and different gravitational fields during centrifugation, in order to obtain a reasonable harvest of nuclei, as well as to obtain good separation between the fractions. At each stage of these experiments the enzyme distribution between fractions was determined.

The following scheme was finally adopted:-

1. After removal from the animal, the liver was perfused by way of the portal vein with cold 0.44M sucrose solution and then pulped by pressing through a coarse steel mesh inserted into the bottom of a modified 20ml syringe. The pulp was placed into a tared homogeniser and the weight determined by difference.

2. 20ml ice-cold 0.44M aqueous sucrose solution was added and the pulp homogenised by twice pushing the rotating plunger to the bottom of the homogeniser tube.

3. The crude homogenate was filtered through four layers of surgical gauze to remove fibrous connective tissue and the filtrate made up to 25ml with sucrose solution.

4. This crude homogenate was further homogenised by 30 passes of the plunger without applying a rotational stress. The whole slurry was then centrifuged at 380g for I0min.

5. The centrifuged deposit was resuspended and re-centrifuged as follows:-

a) 7ml sucrose solution.....250g for I0min.
b) 7ml sucrose solution.....200g for I0min.
c) I0ml I% Gum Arabic.....I85g for I0min.
d) I0ml I% Gum Arabic.....I60g for I0min.

steps a) and b) were used to remove contaminating mitochondria and steps c) and d) effectively haemolyse any erythrocytes present and allow the erythrocyte "ghosts" to be removed with the supernatant.

6. The residue from the last wash in Step 5 was suspended in Iml of water and labelled NUCLEAR.

7. The 380g supernatant from Step 4 and the supernatant wash fluid from Step 5(a & b) were mixed and centrifuged at I3000g for 7 min.

8. The centrifuged deposit from Step 7 was washed three times by resuspension in 7ml sucrose solution and centrifuged at I0000g for 7min.

9. The deposit from Step 8 was resuspended in 1ml water and labelled MITOCHONDRIAL.

10. The supernatant from Step 7 and the first wash supernatant from Step 8 were combined and centrifuged at 95000g for 60min.

11. This deposit was resuspended in 1ml of water and labelled MICROSOMAL.

12. The remaining supernatant was labelled SOLUBLE. Repeated enzyme checks during the derivation of the procedure showed that three washings of the mitochondrial fraction were necessary to reduce the microsomal contamination.

Enzyme markers.

de Duve et al. (1955) described the subcellular localisation of a number of enzymes in rat liver and from their data two enzymes were selected which, when quantitated, would provide an index of the degree of separation of the subcellular components. These enzymes were Cytochrome c :O_2 oxidoreductase, E.C. 1.9.3.1. (cytochrome oxidase) and D-glucose-6-phosphate phosphohydrolase, E.C. 3.1.3.9. (glucose-6-phosphatase). The distribution of these enzymes is shown in Table 6 (from de Duve et al., 1955).

The methods for the assay of the cytochrome oxidase and glucose-6-phosphatase activity are described in the Appendix. The distribution of enzyme activity in the fractions prepared as described above is shown in Table 7 and was considered to be acceptable in relation to those shown in Table 6 (from de Duve et al., 1955)

Enzyme	Nuclei	Mitochondria	Microsomes	Soluble
Cytochrome oxidase	10	74	4	0
Glucose-6-phosphatase	7	9	74	3

Table 6

Subcellular distribution of enzymes % (from de Duve et al., 1955)

Enzyme	Nuclei	Mitochondria	Microsomes	Soluble
Cytochrome oxidase	7.6	83	9.4	0
Glucose-6-phosphatase	7	14	79	0

Table 7

Subcellular distribution of enzymes found. %

Microscopy.

An additional check on the separative procedure was made by examining each of the fractions under the microscope. Figs. 16 and 17 show photomicrographs (x500) of the crude homogenate (Step 3) and the purified nuclear fraction. A well marked degree of nuclear enrichment can be seen. Photomicrographs of the mitochondrial and microsomal preparations showed no distinguishing features at this magnification, but did provide evidence of the complete absence of any nuclei.

The fractionation procedure described in this section was considered to be sufficiently discriminatory to allow kinetic studies of the movement of radioactive cholesterol between subcellular organelles. Repeated checks that the performance of the procedure had not deteriorated were made during the course of this part of the study.

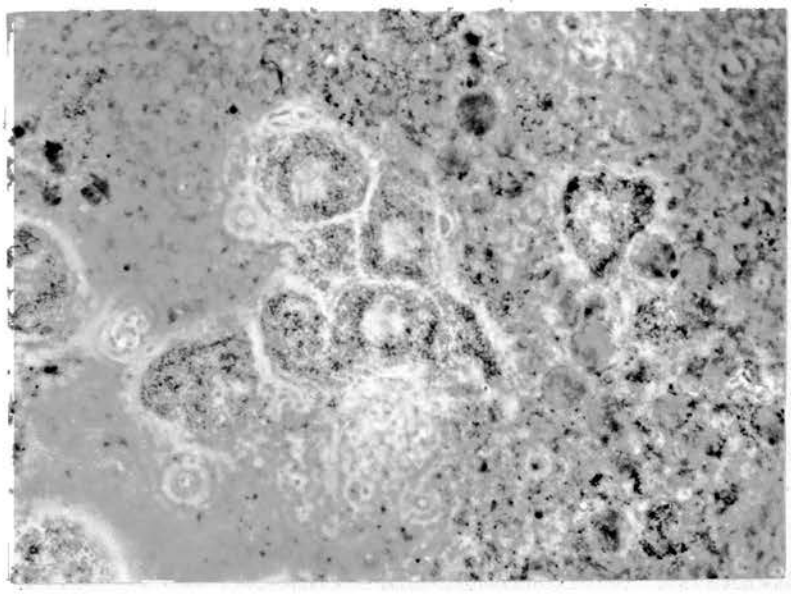


Fig.I6. Photomicrograph (x500) of the crude liver homogenate.

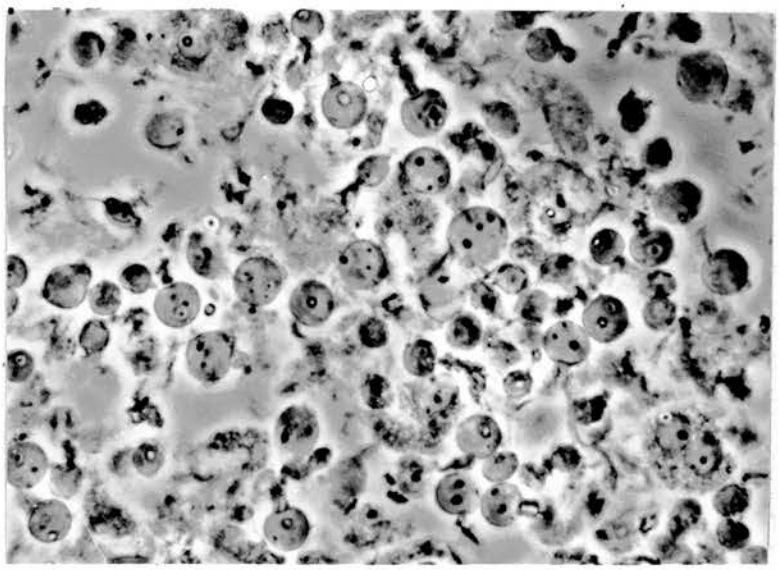


Fig.I7. Photomicrograph (x500) of the prepared nuclear fraction.

4. Surgical and related techniques.

Liver perfusion

The operative technique for the removal of the liver for perfusion was similar to that described by Brauer et al. (1951). The donor was maintained under light anaesthesia during surgery. A long mid-line incision was made through the anterior abdominal wall and the flaps retracted with haemostats. The oesophagus was transected and ligated near to the stomach to assist in the final removal of the liver. The liver was then mobilised as far as possible, leaving only its vascular and bile duct attachments. Two loose sutures were placed around both the bile duct and the portal vein. The bile duct distal to the liver was then ligated and traction applied to demonstrate the position of the duct.

A small incision was made in the duct with a 25 gauge hypodermic needle and a polythene cannula (Portex Poly 45, external diameter 0.030", internal diameter 0.024") inserted and secured in place by a second suture. Before proceeding further it was confirmed that there was a free flow of bile in the cannula. The insertion of the cannula was expedited by holding it inside an 18 gauge needle with the end of the cannula projecting about 5mm.

The portal vein suture furthest from the liver was then tied and traction applied to the suture to demonstrate the position of the vein. An incision was made with an 18 gauge needle and the portal vein cannula (glass), prefilled with perfusate, was inserted and secured in place. This cannula was directly connected with the blood reservoir in the perfusion apparatus by a narrow plastic tube. The vena cava was cut as it passed through the diaphragm, immediately after inserting the portal vein cannula, and

this allowed immediate perfusion of the liver *in situ*. Some perfusate was inevitably lost into the thorax of the donor rat. During the *in situ* perfusion the liver was quickly excised and carefully placed into the perfusion apparatus. Removal of the liver was simplified by prior extraction of the stomach and intestines using traction on the suture tied to the abdominal end of the oesophagus.

Two points need emphasis, firstly none of the donor animals had heparin prior to, or during surgery and this is important because heparin activates the lipoprotein lipase of adipose and muscle tissue which catalyses the hydrolysis of triglycerides in lipoproteins. If heparin had been used the normal disposition of lipoproteins in relationship to their transfer into the liver of the donor animal might have been disturbed prior to liver donation, and this could have had an effect on the exchanges subsequently taking place during the *in vitro* experiments. Secondly, the period of hepatic anoxia was limited to about one minute on average. This was considered desirable because of the adverse effects of anoxia on any respiring system.

Other authors (Miller *et al.*, 1951; Morris and French, 1957; Ostashever, 1960), have also cannulated the vena cava at the diaphragm but this procedure only increased the surgical time and served no useful purpose in the present study.

There are some reports of perfusing the liver in the reverse direction, i.e. through the hepatic vein, (Trowell, 1942; Heimberg *et al.*, 1958) in an effort to overcome some of the problems associated with the supply of oxygen to the liver. In this study the question of poor oxygenation did not arise and it was felt that the normal anatomical and haemodynamic relationships should be retained as far as possible.

Formation of a bile fistula.

The rat was maintained under light anaesthesia during surgery. A mid-line incision from the xiphisternum to a point 2 - 3cm below the xiphisternum was made in the anterior abdominal wall. The bile duct was isolated and cannulated as described previously, and the cannula taken through the anterior abdominal wall in such a way as to avoid occlusion of its lumen. The abdominal muscles were then reconstituted, followed by the skin, using linen sutures. Care was taken not to ligate the cannula during the procedure.

The animal was placed in a restraining cage which allowed free movement of the head and forward part of the body. Bile was collected through the cannula which passed through the floor of the cage. The ambient temperature was kept at 70°F and the animal allowed free access to its normal diet and to a drinking fluid of sodium chloride solution (170mmol/l) containing 5mmol/l potassium chloride.

Bile was collected at hourly intervals over a period of 44h using an automatic fraction collector. During this period the rats lost an average of 7.3% of their body weight, which compares satisfactorily with the figures of 7.6% reported by Percy-Robb (1967), and 5% by Myant and Eder (1961).

Thyroidectomy.

Seventy young rats with a mean body weight of 190g were divided into two groups, one of sixty rats (test group) and one of 10 rats (control group). All animals had their heart rates measured with a Mingograph portable electrocardiograph using needle electrodes. The test group were then subjected to surgical thyroidectomy.

A mid-line incision about 2cm long was made in the loose skin at the throat of the animal. The pre-tracheal fascia and muscle were parted by blunt dissection and the thyroid gland on the anterior surface of the trachea exposed. The gland was carefully dissected free of the trachea using an 18 gauge needle as the cutting instrument. Care was taken not to cut the laryngeal nerves. The muscle and skin layers were reconstituted with continuous sutures and the animal allowed to recover from the anaesthetic.

In order to confirm that the thyroidectomy was successful, the weight of each of the rats was determined at intervals over a ten week period. During this time the animals were fed on their normal soft diet. Fig.18 shows how the body weights of the two groups changed with time and it is clear that the thyroidectomised group shows a smaller weight gain than the control group. An unpaired 't' test on the mean values for each group at the end of the experiment shows the groups to be significantly different. ($p = < 0.001$).

The heart rate of the test and control groups was determined at the beginning and end of the experiment. The mean heart rate of the control group at ten weeks was 390 beats/min and that of the test group 362 beats/min. These rates were significantly different ($p = < 0.05$).

Rats showing the least weight gain and smallest increase of heart rate were used as liver donors; the remainder of the test group were used as blood donors, i.e. to provide the perfusate for the perfusion of livers from thyroidectomised rats.

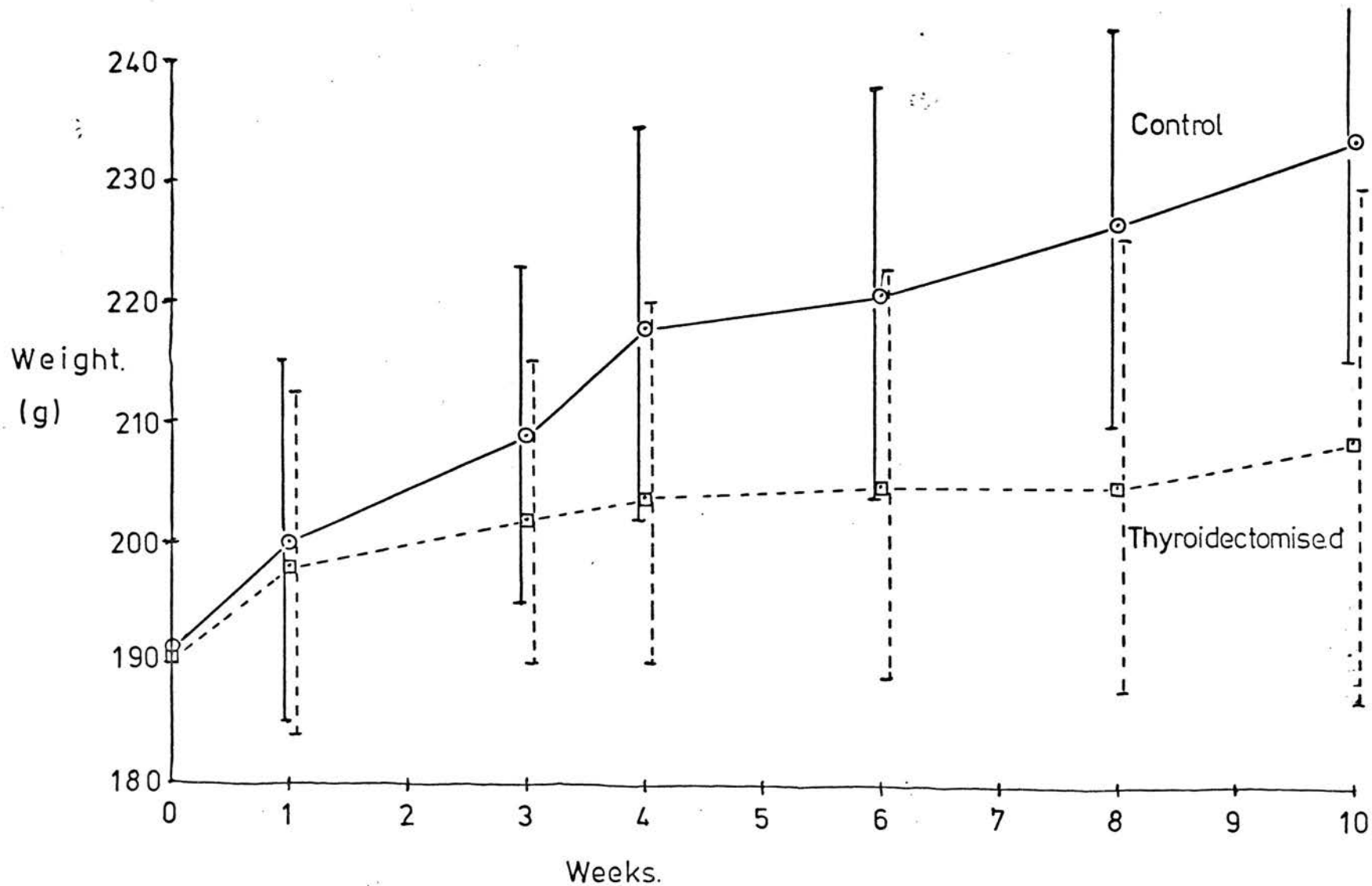


Fig.18. Weight change in thyroidectomised and control rats

5. The perfusion technique.

Introduction

Liver perfusion has been used by a number of investigators as a means of studying various aspects of liver metabolism and function (Trowell, 1942; Miller *et al.*, 1951; Heimberg *et al.*, 1960; Ryoo and Tarver, 1968).

A number of criteria can be laid down to delineate the conditions in order that the liver should be maintained in an environment as similar as possible to that existing *in vivo*.

a) The perfusate should be undiluted anticoagulated blood obtained from the same species as the liver donor.

b) The blood volume should approximate to that of the intact animal (15 - 20ml in a rat weighing between 250 and 300g).

c) The perfusate should provide an adequate supply of oxygen to the liver.

d) In the rat the perfusion pressure should be 15 - 16cm water pressure (Kunkel and Eisenmunger, 1949).

e) The temperature of organ and perfusate should be 37°C.

The apparatus for perfusion should be designed so that:-

i) Haemolysis due to mechanical disruption of the erythrocytes during passage through the circulation system is kept to a minimum.

ii) The initial placement of the cannula into the portal vein is permanent and provides a continuous connection from the blood reservoir to the liver.

iii) The perfusion pressure can oscillate about a mean pressure to simulate 'heart beat'.

iv) Adjustments to the pumping and oxygenation functions are available without disturbing the environmental temperature of the liver.

Experimental

A. Gas-lift pump.

Two major functions are required of the perfusion apparatus, namely pumping and oxygenation. In all previous perfusion systems the pumping function has been provided by reciprocating piston type pumps or by peristaltic pumping. Oxygenation of the perfusing fluid has been obtained by various adaptions of a thin-film oxygenator. There would be obvious advantages in having the two functions combined. A gas-lift pump has the dual characteristics required and is shown in diagrammatic form in Fig. I9.

When a gas is pumped into a liquid lying in the bottom of a narrow glass tube, menisci are continuously formed at the surface of the fluid and rise up the tube as the passage of gas into the apparatus continues. If the tube is bent into a horizontal position at the upper end then the menisci burst on reaching the open end and the fluid droplets may be collected. In effect the fluid has been moved through a vertical distance.

Experiments aimed at setting up a working gas-lift system to pump blood were started and a critical examination of the factors influencing stable meniscus formation in the area X (Fig. I9) was conducted. Experiments with different tube sizes and different modes of injection of gas and blood showed that the most stable meniscus was obtained using the design shown in Fig. 20. Different bore sizes of the tube Y showed that with internal diameters above I.2cm the menisci were unstable and if the internal diameter fell below 0.5cm then the blood flow

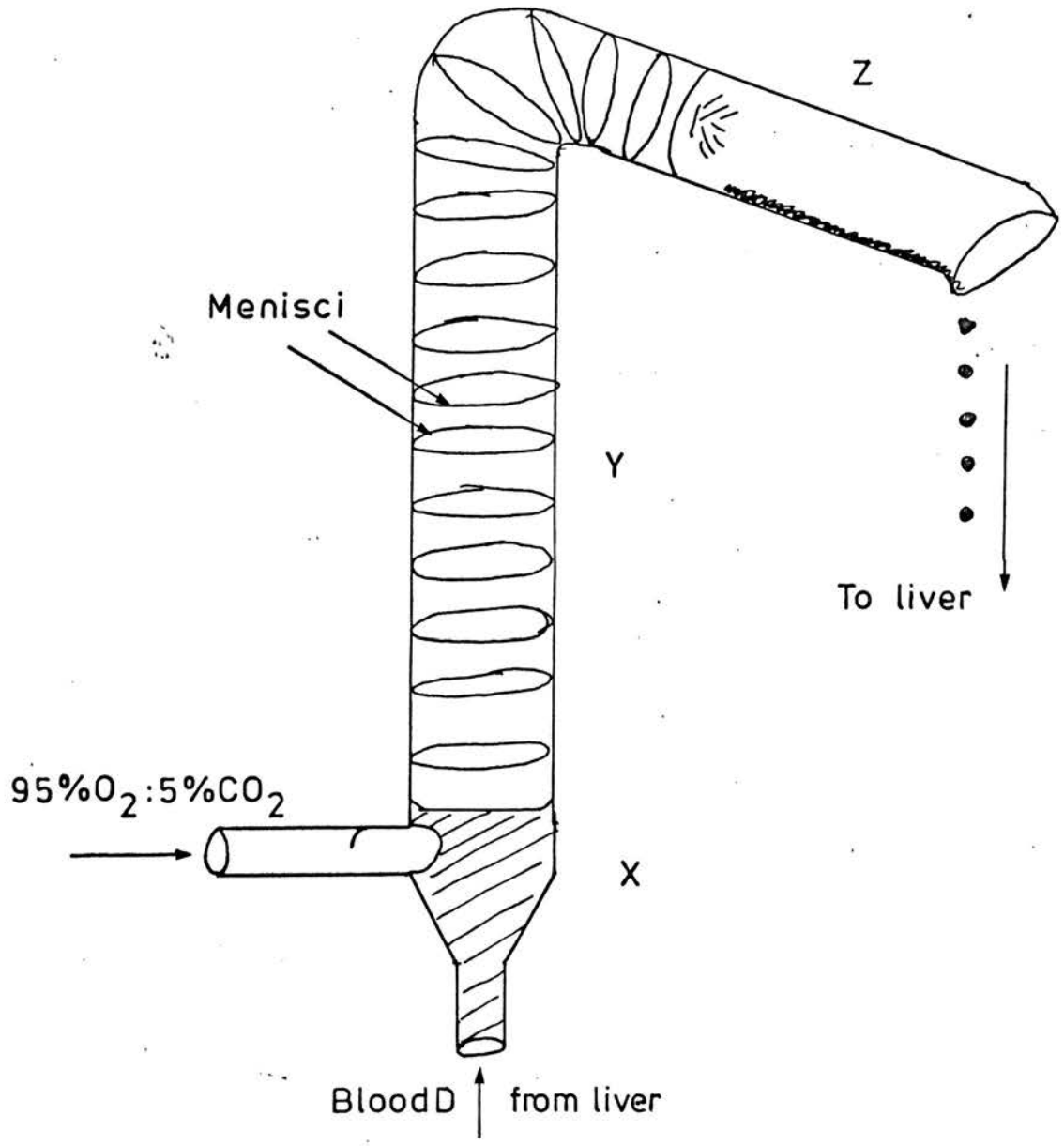


Fig.I9. Gas lift pump showing the principle whereby gas pressure allows a working fluid (blood) to be raised through a vertical distance.

rates were low and the percentage oxygenation of the blood was less than 90%. In addition it was found that there was a critical rate of gas injection (100 - 150ml/min) below which meniscus formation was irregular and unstable. Several methods of disruption of the menisci in the outlet tube (Z) of the pump were investigated, the most successful being a very fine, high pressure jet of oxygen which impinged upon the meniscus as it reached the end of the outlet (Z). This consistently disrupted the meniscus.

The blood pumping rate was adjusted by controlling the blood flow into the injection point. Using this system blood flow rates up to 20ml/min could be obtained. These were considered adequate for the liver perfusion judged by the reports of other investigators (Morris and French, 1958; Ryoo and Tarver, 1968).

When deoxygenated blood was pumped through the mechanism there was complete saturation of the haemoglobin with oxygen on one cycle through the pump. There was an appreciable amount of haemolysis present in the blood after recycling the blood for 4h. This averaged 4.2% in five different experiments. (See Appendix for the method of determination of the amount of haemolysis).
However the use of the gas-lift pump was abandoned because:-

1) changes in blood flow were mediated by the rate of addition of blood at X(Fig. 19), and this meant that blood committed to the lift tube Y would have to pass over before the change in flow rate at Z became apparent. Consequently rapid adjustments to the 'demand' by the liver would be difficult to achieve.

2) there was evidence of protein denaturation brought about by the mechanical disruption of the menisci. This led to the accumulation of small clots or thrombi in the blood perfusate which would cause rapid occlusion of

smaller capillaries in the liver.

3) it was difficult to fit an adequate filtration device into the circulatory system.

In view of these difficulties, other pump/oxygenator combinations were tried and the performance of these compared with that of the gas-lift pump.

B Blood oxygenators.

Two types of thin-film oxygenator were tried, a rotating and a non-rotating type.

In the first instance (Fig. 21) the walls of the rotating vessel provided a thin, continuously changing blood surface to allow haemoglobin to equilibrate with ambient gas (95% O_2, 5% CO_2). In the second case the blood was distributed around the top edge of a vertical tube and descended under gravity, exchanging oxygen with a counter-current of humidified gas (95% O_2, 5% CO_2). A curtain of cotton gauze was used to distribute the blood evenly around the internal surface of the tube.

C.Peristaltic pumps.

Two pumps were investigated, firstly a standard AutoAnalyzer Mark 1 peristaltic pump (Technicon Instrument Co. Ltd.), and secondly, a pump (delta) constructed by the author by moulding Perspex acrylic resin. (See section on equipment design, to follow).

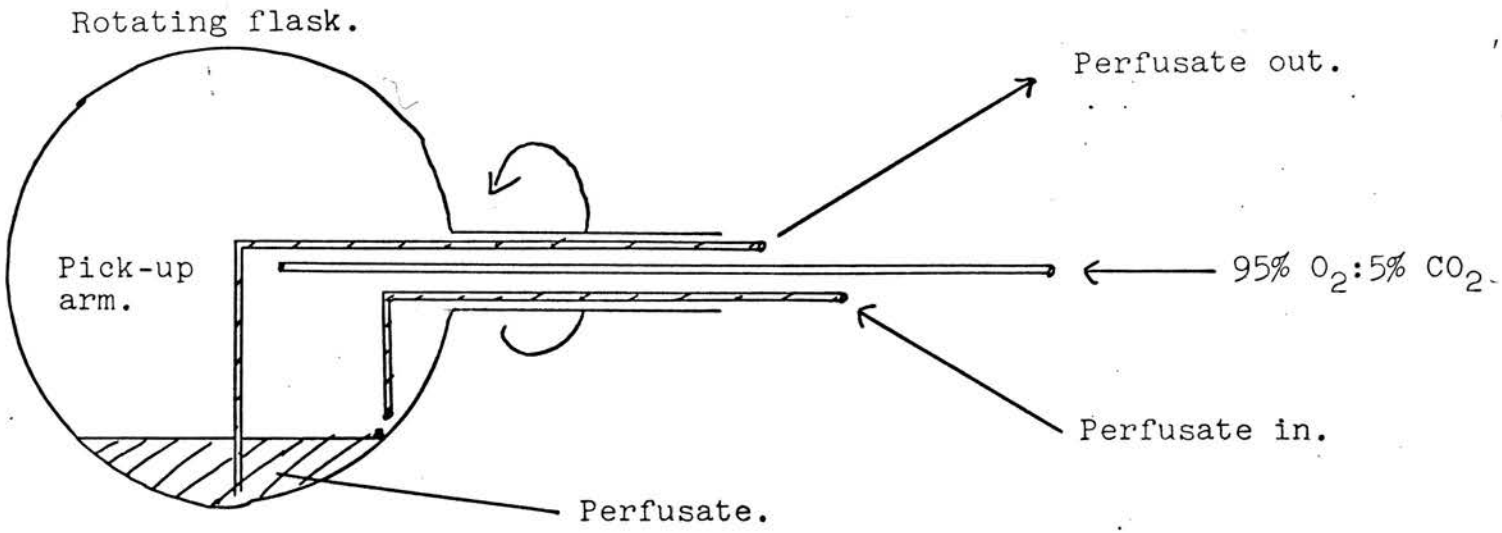


Fig.2I. Rotating thin film oxygenator.

The relative performance of combinations of these oxygenators and pumps was assessed in terms of their ability to produce maximum oxygenation on one cycle, and to minimise the amount of haemolysis. The results are summarised in Table 8.

These data show that the least haemolysis was produced by the 'delta' pump used in conjunction with the stationary thin-film oxygenator. Against this must be set the lower oxygen saturation obtained.

According to Trowell (1942) the oxygen requirement of rat liver is approximately 2ml/g/h. Assuming a liver weight of 8.0g this means that the perfusate must supply 16ml oxygen/h, or 0.266ml/min. If the blood flow rate is 15ml/min and the haemoglobin concentration 12g/100ml then the amount of haemoglobin passing through the liver will be 1.8g/min. At 100% saturation 1.34ml oxygen will combine with 1.0g haemoglobin. Therefore the liver can be supplied with 2.41ml oxygen/min if the haemoglobin is fully saturated, or with 1.93ml oxygen/min if the haemoglobin is 80% saturated. This is obviously in excess of requirements and the small differences in oxygen saturation noted in Table 8 can be ignored.

As a result of these experiments it was decided to adopt the 'delta' pump/stationary thin-film oxygenator combination in these studies.

D. Equipment design.

The perfusion apparatus used is shown in outline in Fig. 22 and was constructed by the author. The cabinet was designed to allow access to the liver through ports which obviate the need to open the main compartment doors. The pump and oxygenating mechanisms were isolated from the liver compartment so that any necessary adjustments

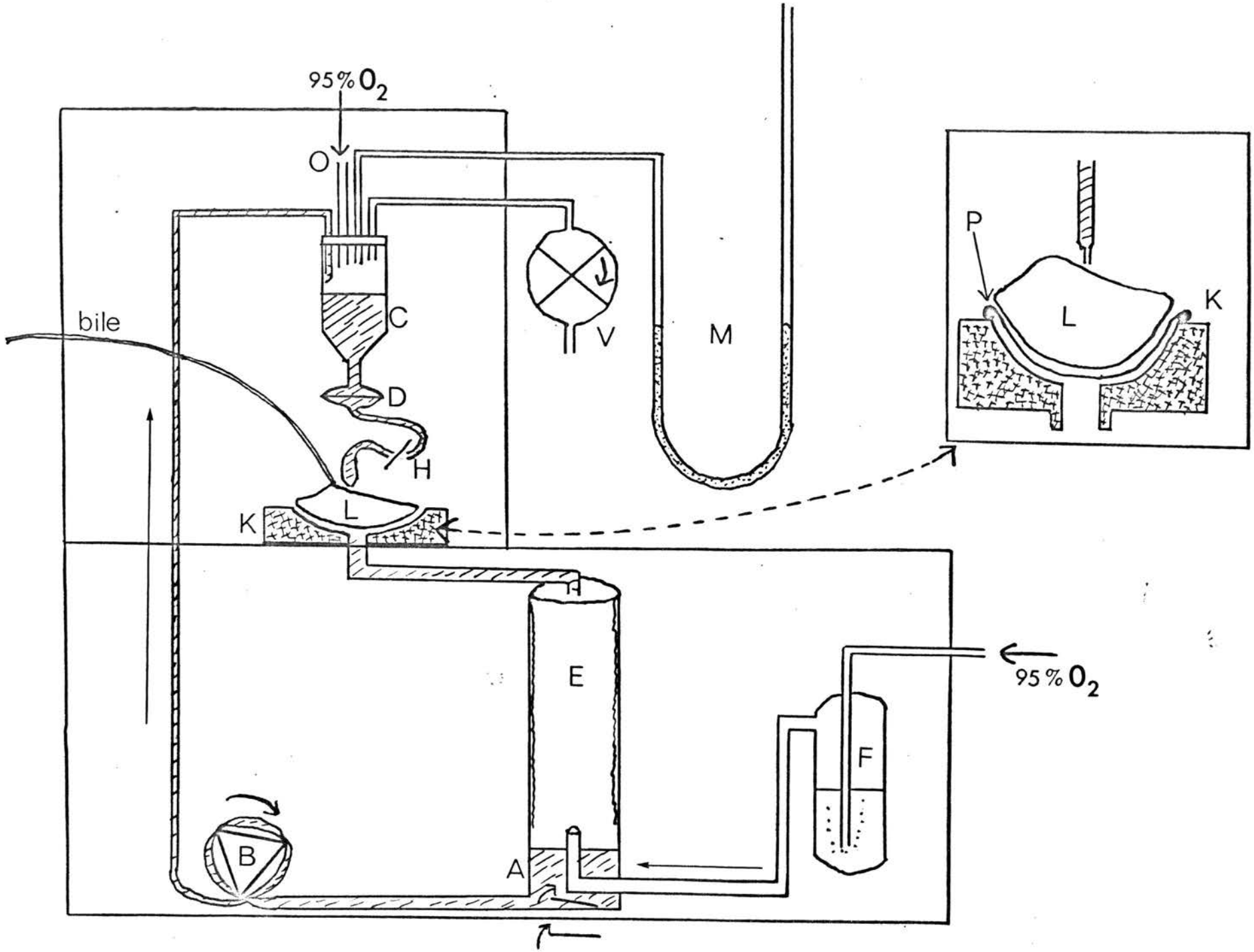
Pump	Oxygenator	% haemolysis in 4h	% oxygenation (one cycle)
Gas lift	Gas lift	4.2	100
Auto-Analyzer	Stationary T.F.O.*	2.1	85
Auto-Analyzer	Rotating T.F.O.	1.8	96
Delta	Stationary T.F.O.	1.6	87
Delta	Rotating T.F.O.	2.2	95

* Thin-film oxygenator

Table 8

Percentage haemolysis and oxygenation obtained using using different pump and oxygenator combinations.

Fig.22. Outline of the perfusion apparatus used in this study. The various items constituting the equipment are delineated in the text.


95% O_2
O
C
V
M
bile
D
H
L
K
P
L
K
E
F
95% O_2
B
A

to them could be made without disturbing the environmental temperature of the liver.

The pump rotor was made of moulded Perspex in a triangular form. A silicone rubber tube was wrapped around the apices of this rotor. Permanent lubrication of the silicone rubber tube was obtained by passing each of the apices through the oil bath as rotation occurred (Fig. 23). The tension in the pump tubing could be regulated to produce 'pumping' contact between the inner walls of the tube. It seemed likely that this would minimise the compression and mechanical disruption of erythrocytes suspended in the perfusate.

The cabinet was heated by resistance tape controlled with a transformer and toroidal resistance. Heat distribution was fan assisted in each compartment and frequent checks that the temperature was being maintained could be made on two thermometers. Wherever possible in the apparatus glass tubing was used because of reports of the adverse effects of substances leached from plastic tubing upon the organ being perfused.(Boyd and Pathak, 1964; Duke and Vane, 1968). All of the glassware used in the apparatus was siliconised by immersion in polymethylsiloxane, followed by baking in a hot air oven at 120^{o}C.

The course of the perfusate through the system was as follows (See Fig. 22). The blood was placed initially in reservoir A, which had a magnetic stirrer, and collected there by draining down the sides of the oxygenator (E) placed immediately above. From this reservoir the blood passed via the 'delta' pump (B) to reservoir C, and subsequently under pressure to filter D. This consisted of a Swinnex 25mm diameter filter holder (Millipore Ltd.) supporting a 40 nylon mesh (Pharmacia Ltd.). After filtration the blood entered a 40cm length of Tygon plastic

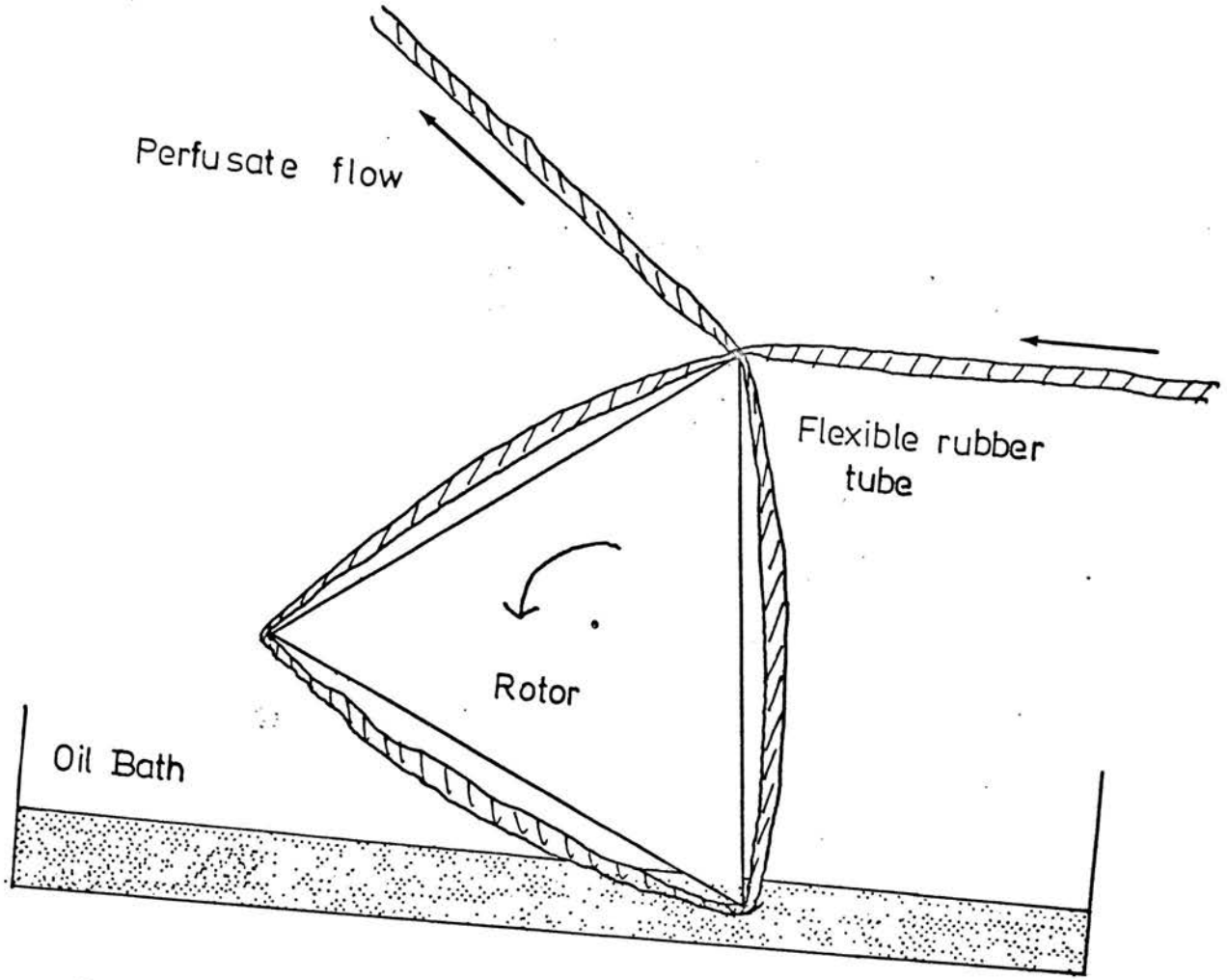


Fig.23. Design of perfusate pump.

tubing (i.d. 0.065") at the end of which was fitted the portal vein cannula. The blood passing through the liver was collected in a Perspex dish (K) and allowed to fall by gravity into the oxygenator (E) where it equilibrated with a counter-current stream of humidified oxygen provided from F.

Thus it can be seen that the apparatus allowed the blood to be recycled through the liver.

The pressure exerted in the reservoir C was checked on a manometer M and was provided by a constant pressure gas supply entering reservoir C through tube O. Excessive build up of pressure was prevented by allowing the valve system V to vent to atmosphere at frequent and controlled intervals of time. This produced an oscillating pressure which was applied to the liver. The 'pulse' rate was set at about 60/min. The mean perfusate pressure was 15cm water, oscillating from 13 - 17cm. If the 'pulse' rate had been increased to simulate the rat heart rate of 350/min, then because of the damping effect of the long path length between reservoir C and the liver, the resultant pulse wave would have been so smoothed as to be meaningless.

The portal vein cannula was made of glass with the tip bevelled and fire-polished. This was fitted to the end of a length of plastic tubing (H) which led from the reservoir C. The portal vein cannula could be taken from the perfusion cabinet to allow cannulation of the liver prior to removal from the donor.

A direct and continuous supply of blood was therefore immediately available to the liver shortly after cannulation. The liver was placed onto a stainless steel mesh support (P) moulded to correspond to the in vivo shape of the rat diaphragm. This support lay on a similarly shaped Perspex block (K). Blood passing from

the liver fell freely through the mesh into this collecting receptacle, and thence to the oxygenator. The bile duct was led outside the cabinet and bile collected at intervals of one hour. The liver and its supports were covered with Parafilm to prevent drying of the liver surface

A charge of between 45 and 55ml of blood was necessary at the start of each perfusion, due allowance being made for the samples of blood to be withdrawn during perfusion and for the loss of perfusate during cannulation of the portal vein. Prior to perfusion the cabinet was heated to 37°C. After the initial equilibration the temperature remained constant at 37°C ± 1.5°C. The blood perfusate was then circulated without the liver being in place until it was fully oxygenated.

E. Non-cyclic perfusion.

The perfusion apparatus described above is of the recycling type, but some of the experiments performed in this thesis required a assessment of the very early exchanges occurring in the blood and liver system. These latter experiments were performed by allowing the blood perfusate to exit from the liver and not to be recycled. This required some modification to the perfusion apparatus. An additional reservoir was added to the compartment of the perfusion cabinet which contained the liver. This contained about 100ml of heparinised rat blood + 10μCi (2^{14}C)DL mevalonic acid and was connected to the main reservoir C at a three way tap inserted between the filter D and reservoir C. The main reservoir contained 40ml blood and was used in the normal manner to establish a liver perfusion by the recycling method. Once the perfusion was running the reservoir tap was operated. This

stopped the flow of blood from the main reservoir and allowed the additional reservoir to deliver blood to the liver without recycling.

6. Physiological and metabolic status of the liver during perfusion.

Bile flow rate.

Rats (5) with an external bile fistula had bile flow rates as shown in Fig. 24.

There was a steady fall in flow rate for the first 16 - 18h and then a return to a relatively constant value below the original flow rate. The first 3h drainage in these in vivo experiments gave a mean flow rate of 0.47g bile /h. The bile flow rates found in the perfusion studies using normal rat livers averaged 0.31g/h over a 3h period. Direct comparison of these results is difficult because of the isolated status of the liver in the perfusion system, contrasting with the intact feed-back mechanism to bile secretion in the drained animal.

Blood flow rate.

The blood flow rate in six perfusions using normal rat livers was established by determining the time required to collect a 1ml sample at each time point during the perfusion. This collection time was always less than 5sec (12ml/min) except towards the end of a perfusion when flow rates may have dropped to 8ml/min. All perfusions were terminated at flow rates of less than 8ml/min.

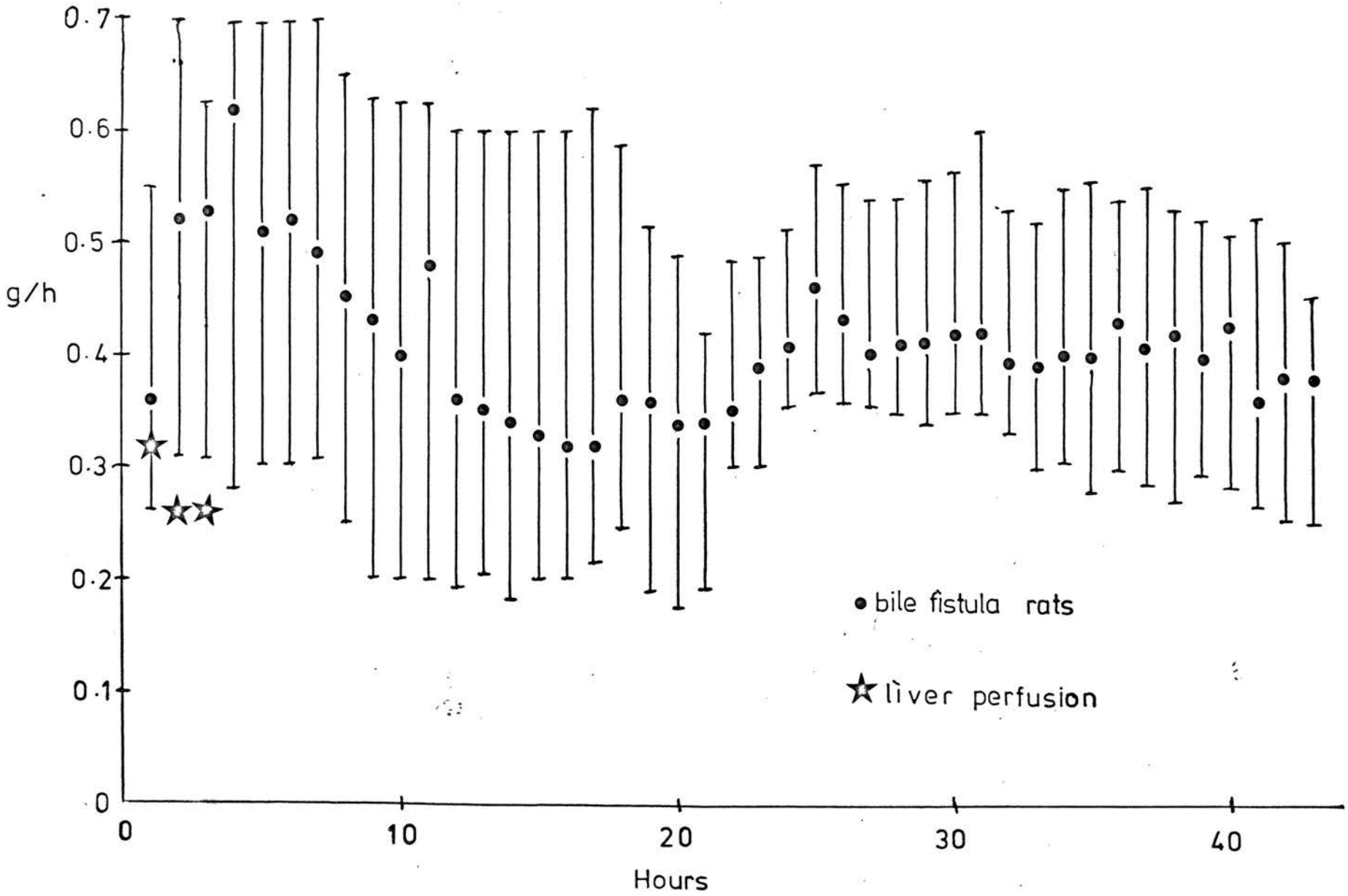


Fig.24. Bile flow rates in fistula rats and during isolated liver perfusion.

Macroscopic appearance of the liver.

At intervals of approximately 1h the macroscopic appearance of the liver was examined by gently lifting the Parafilm cover. Widely distributed areas which were necrotic in appearance were a sign that perfusion should be terminated. Occasional localised necrotic areas at the periphery of the lobes were ignored.

Synthesis of glucose and urea.

The metabolic status of the liver was tested by examining the capacity of the liver to maintain the synthesis of both urea and glucose during the course of a liver perfusion. Plasma samples obtained from five perfusions were analysed to determine the concentration of both glucose and urea.

A control experiment was performed where the whole blood perfusate was circulated in the perfusion equipment for 4h and 37°C without the presence of a liver in the system. Samples were taken at 30min intervals and the concentrations of urea, glucose and cholesterol were determined. The results of the test and control experiments are shown in Figs. 25 and 26.

The liver was clearly active in terms of its capacity to synthesise both urea and glucose when compared with the control experiments.

The cholesterol concentration of the perfusate increased with time. This was probably due to a haemoconcentration effect because the perfusion system allowed evaporation of water from the perfusate. The increase in cholesterol concentration was linear and could be defined by an equation of the type

$$y = mx + c \quad (m = 0.2142)$$

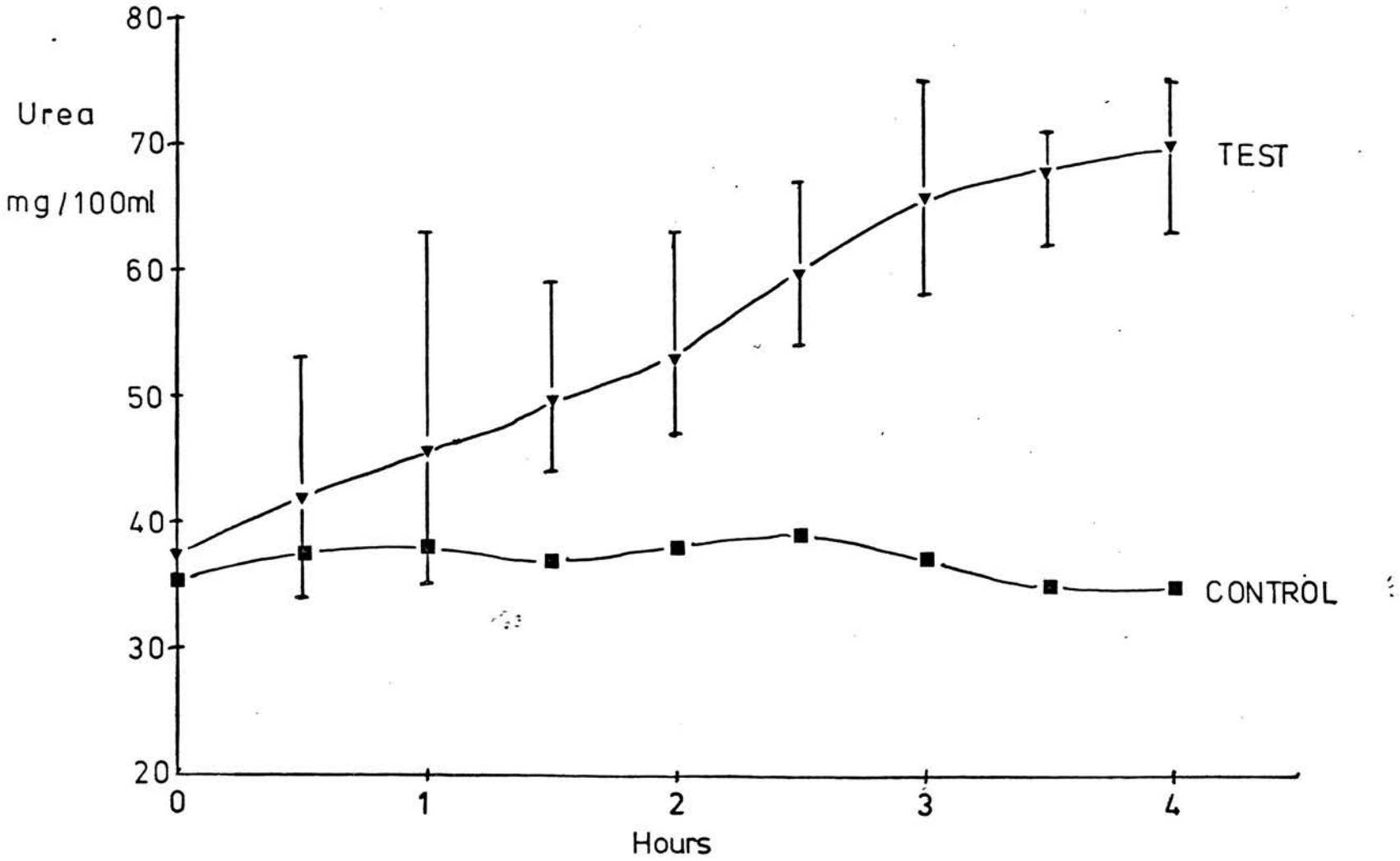


Fig.25. Concentration of urea in perfusate during perfusion

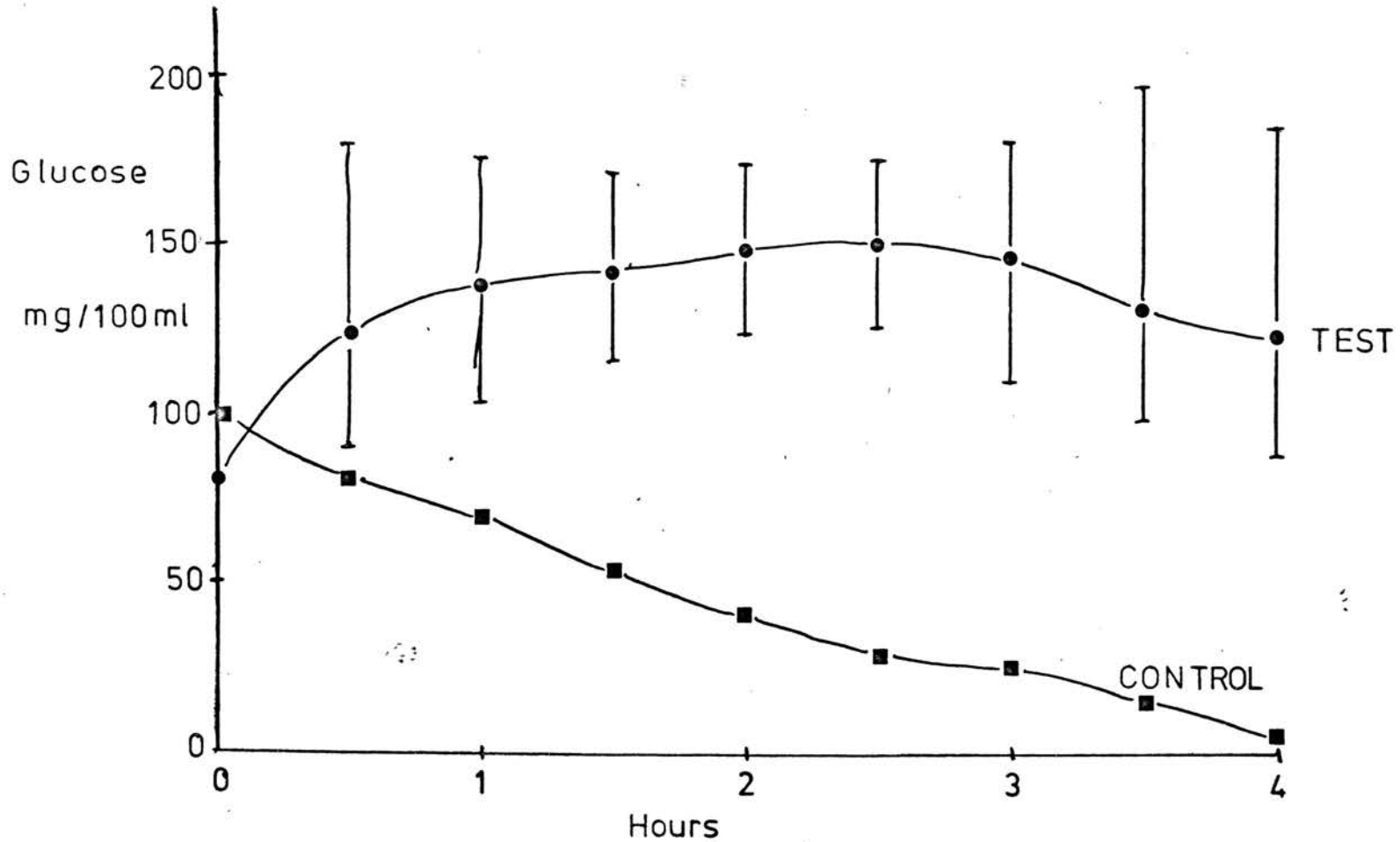


Fig.26. Concentration of glucose in perfusate during perfusion

7. Experimental details of the method used to examine the exchange of cholesterol between plasma and erythrocytes.

The estimates of the rate constants describing movement of cholesterol between the compartments shown in Fig. 8 were derived by first determining the rate constants in the exchange of cholesterol between plasma and erythrocytes. This was performed by allowing radioactive cholesterol in erythrocytes to exchange with unlabelled cholesterol in plasma and vice versa. The specific radioactivity of the cholesterol in each compartment was determined at increasing time intervals.

Method.

Radioactive cholesterol in plasma and erythrocytes was prepared by giving three rats an intra-peritoneal injection of 5μCi of (2^{14}C)DL mevalonic acid lactone in acetone:saline (1/1,v/v). One hour later the rats were bled by cardiac puncture and the three blood specimens combined. Bleeding the animal hour hour after injection has been shown to produce the highest in vivo specific radioactivity values for plasma cholesterol (See p99); the values fall after one hour. A similar blood collection was made from three untreated rats.

Each blood pool was centrifuged for 10min at 1000g and the plasma separated. At this point an estimate of the haematocrit (See Appendix) was made. The erythrocytes were then washed twice with isotonic saline and in the final wash the top few millimetres of cells were removed and discarded. The labelled fractions were then mixed with their unlabelled counterparts to give haematocrit values as near as possible to those existing in vivo.

Because it had been shown previously that the blood glucose concentration fell steadily over a period of three hours when whole blood was incubated at 37°C (Fig.26) then 1.0ml of 1.0g% (w/v) glucose was added at the start of incubation to maintain cellular respiration.

The mixed specimens were then incubated at 37°C with continuous gentle shaking and 1.0ml samples taken at 0, 5, 10, 15, 20, 30, 40, 60, 90, 120 and 180min.

The specific radioactivities of unesterified and esterified cholesterol in the plasma and erythrocytes were then measured as described previously.

8. The Analogue Computer.

The computer used in this study was a PACE TR 48 instrument (Electronic Associated Ltd.) which is a solid state, general purpose, analogue computer. This particular model had thirty operational amplifiers; thirty manually operated, co-efficient setting attenuators; four dual integrators; one log x diode function generator (DFG); one x^2 DFG; two bipolar multipliers; two comparator units with five function switches and one variable diode function generator. The control and monitoring circuits included and electronic digital voltmeter, a multirange voltmeter and push-button selector systems for readout and computer mode control. An overload display unit gave visual indication of over-load conditions on each operational amplifier.

An x - y recorder, Type PRO - 12 (Yokogawa Electric Works Ltd.) and a dual channel trace oscilloscope (Tektronix, Guernsey Ltd.) with photgraphic recording facilities, were permanently wired to the output stages.

The biochemical models.

Two experimental situations were simulated in this study. These were the exchange of cholesterol between the plasma and erythrocyte compartments and the exchange of cholesterol between blood and liver in the perfused preparation. It is an implicit assumption of these simulations that the transfer of cholesterol between each of the compartments is governed by first order kinetics and is not subject to an active transfer process in any individual instance. In addition it was assumed that the tracer material, i.e. ^{14}C labelled cholesterol was treated in exactly the same fashion as the carrier material.

If this was the case then the rate of change of the amount of substance in any compartment in the steady state is given by:-

$$\dot{Q}_j = R_j - k_o Q_j$$

where Q_j = quantity of substance in compartment j, R_j = rate of entry into the compartment and k_o is the rate constant for the removal of Q from compartment j.

Cholesterol exchange between plasma and erythrocytes.

This process is represented by the model shown in Fig. 27. The rate of change of the amount of cholesterol in each compartment is given by:-

$$\dot{Q}_2 = Q_5 k_{25} + Q_3 k_{23} - Q_2(k_{52} + k_{32})$$

$$\dot{Q}_5 = Q_2 k_{52} - Q_5 k_{25}$$

$$\dot{Q}_3 = Q_2 k_{32} - Q_3 k_{23}$$

($\dot{Q}_j$ is a notational form of $\frac{dQ_j}{dt}$)

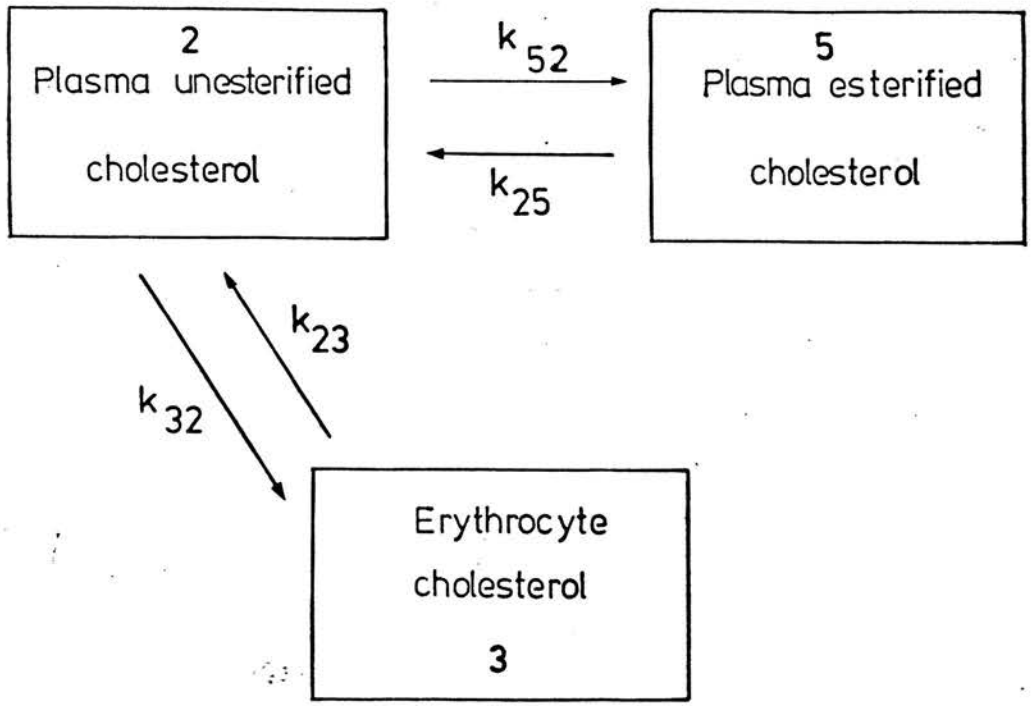


Fig.27. Compartmented model representing the exchange of cholesterol between erthrocytes and plasma.

In the general case the rate of change of the amount of substance in compartment j is governed by its relationship with other compartments j - 1 to j - n. Specifically for example, the rate of change of the amount of cholesterol in compartment 3 is equal to (quantity of cholesterol in compartment 2 multiplied by rate constant k_{23}) minus (quantity of cholesterol in compartment 3 multiplied by rate constant k_{32}). In this manner differential equations may be constructed to describe changes in all of the compartments of any particular model. Thus in compartment 2 the rate of change is governed by addition to the compartment from two sources and a concomitant loss from the compartment to two recipient compartments. In the steady state, $\dot{Q}_j = 0$, i.e. the amount of material in the compartment remains the same even though an active exchange process is occurring.

The differential equations can be transposed directly into a program representing this three compartmental model and this is shown in Fig. 28. The units illustrated as in the diagram represent the compartments containing cholesterol etc. The output from compartment 3 (via potentiometer k_{23}) is split, one part is made negative and fed back into itself and the other part is led to the input side of compartment 2. At the same time the output from 2 (via potentiometer k_{32}) is led to the input of compartment 3. Thus the differential equation

$$\dot{Q}_3 = Q_2 k_{32} - Q_3 k_{23}$$

is satisfied. Following this logic to its conclusion it can be seen that the three physical compartments (Fig. 27) can be represented by three differential equations, which in turn can be transposed into an analogue program of the form shown in Fig. 28.

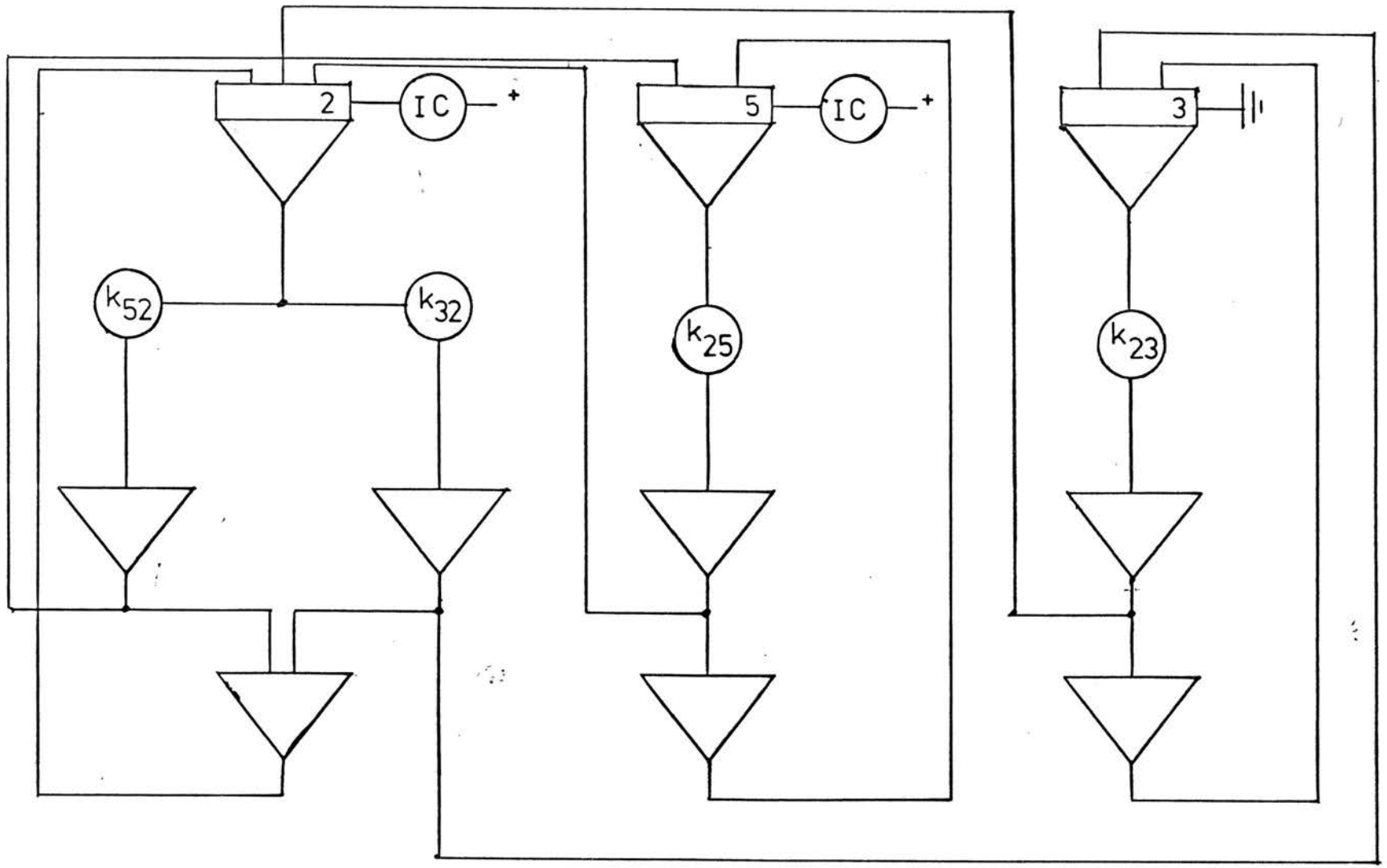


Fig.28. Three compartment analogue program representing cholesterol exchanges in whole blood. (Corresponds to fig.27)

Where the tracer material is initially in the plasma compartment the amount of tracer, known from the experimental observations on the real system, can be represented by a voltage in compartments 2 and 5 and is called the initial condition (I.C.). When the program is operated, differentiation of this representational voltage occurs under the control of the potentiometers representing the rate constants. The voltage, representing radioactive tracer, is dispersed between compartments 2, 5 and 3, and this change can be followed on the digital voltmeter of the computer. Alternatively it can be plotted using an x - y recorder.

When the radioactive tracer is entirely associated with the erythrocytes (compartment and integrator 3) then integrators 2 and 5 are earthed to represent the absence of tracer at zero time.

A series of curves can be obtained, simulating the behaviour of tracer in the real system, and the rate constants (potentiometers) manipulated to obtain correspondence to the experimentally observed curves.

Cholesterol exchange during isolated liver perfusion.

The minimum number of discrete cholesterol compartments that may be expected in the blood and liver system is shown in Fig. 29. The mathematical model appropriate to this system is given by:-

$$Q_1 = Ae^{-k_1 t} + Be^{-k_2 t} + Ce^{-k_3 t} + De^{-k_4 t} + Ee^{-k_5 t}$$

and in terms of differential equations:-

$$\dot{Q}_1 = Q_4 k_{14} + Q_2 k_{12} - Q_1(k_{21} + k_{41} + k_{61})$$

$$\dot{Q}_2 = Q_5 k_{25} + Q_1 k_{21} + Q_3 k_{23} - Q_2(k_{12} + k_{52} + k_{32})$$

$$\dot{Q}_3 = Q_2 k_{32} - Q_3 k_{23}$$

$$\dot{Q}_4 = Q_1 k_{41} + Q_5 k_{45} - Q_4(k_{14} + k_{54})$$

$$\dot{Q}_5 = Q_2 k_{52} + Q_4 k_{45} - Q_5(k_{25} + k_{45})$$

$$\dot{Q}_6 = Q_1 k_{61}$$

These were constructed in a similar manner to that described on p69.

These equations can be translated into the analogue program shown in Fig. 30, in a similar manner to that described previously. In this basic program the I.C. potentiometer on compartment 1 allows all of the tracer to be in this compartment at t = 0. The results of the experiments using isolated liver perfusion showed that the

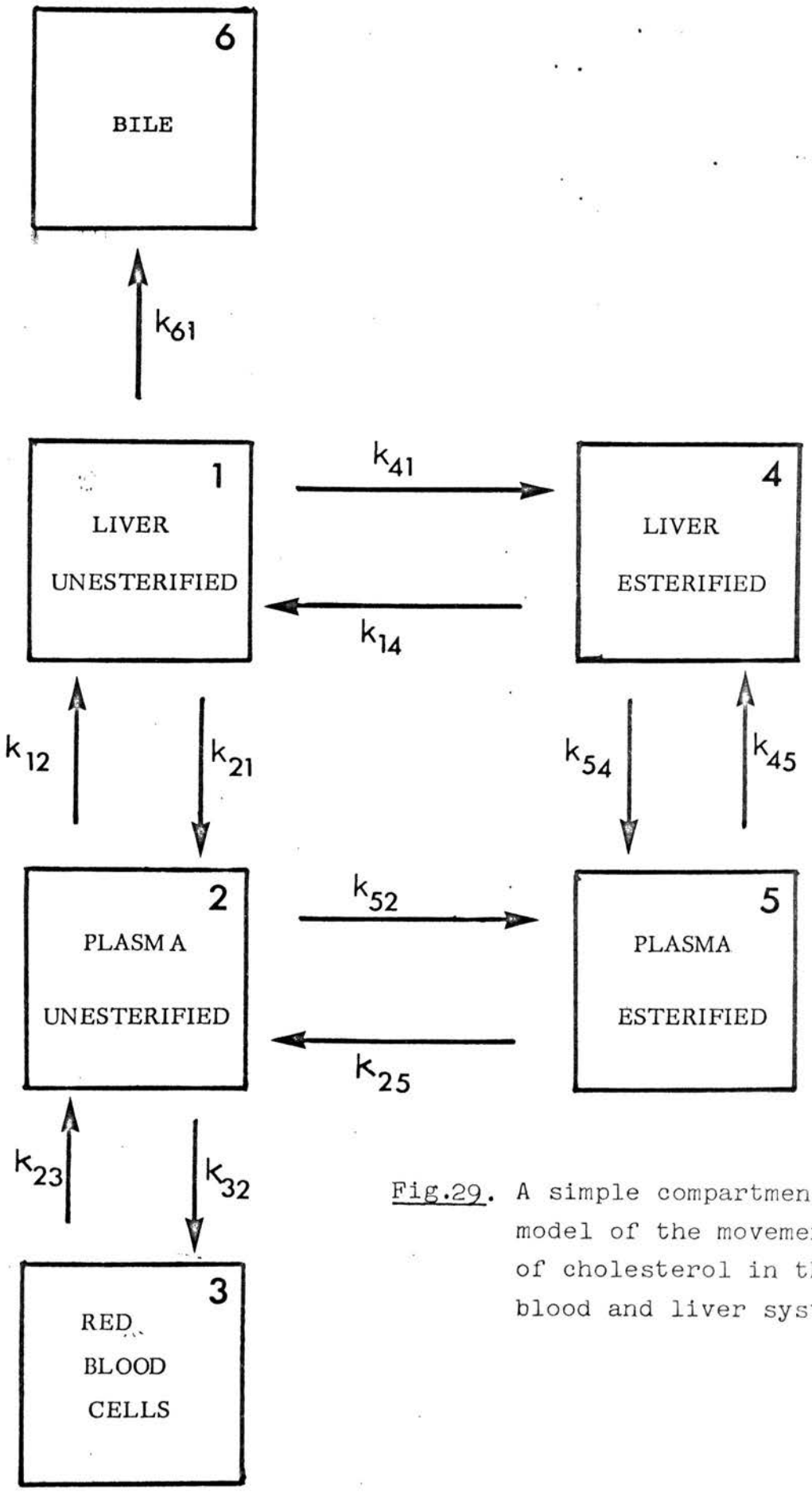


Fig.29. A simple compartmented model of the movement of cholesterol in the blood and liver system.

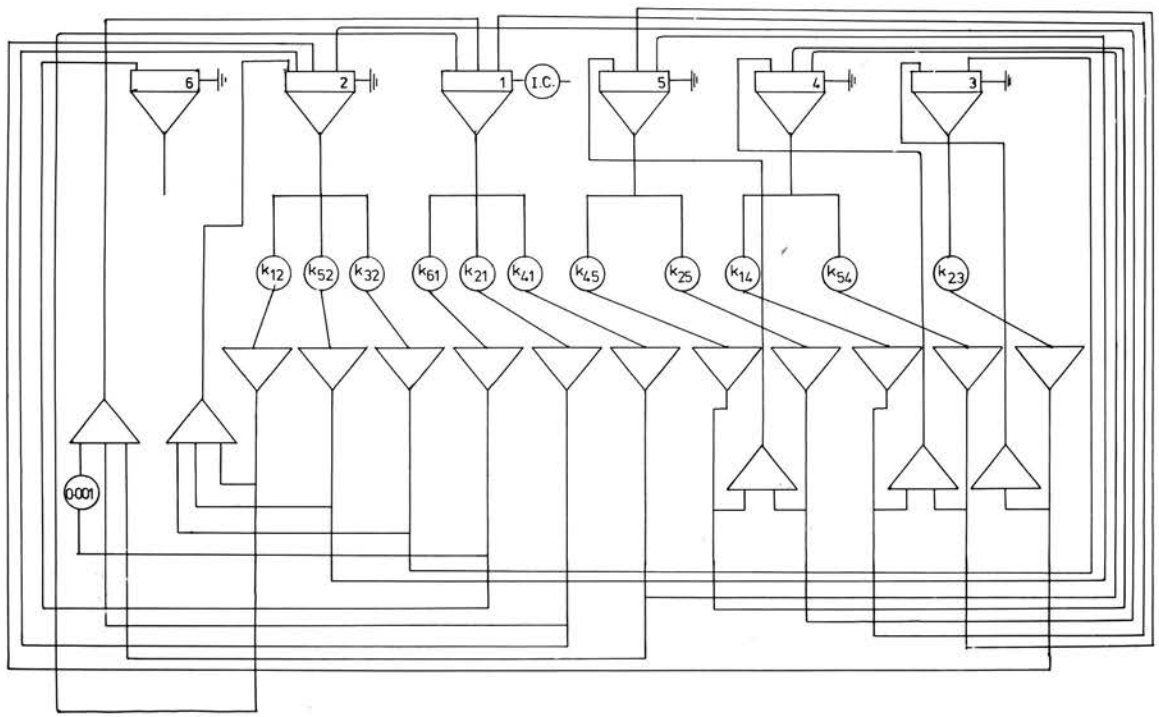


Fig.30. The analogue computer program (corresponding to Fig.29) representing the exchange and transfer of cholesterol within the isolated perfused rat liver preparation.

amount of tracer which transferred into the bile compartment (6) was very small relative to the movement of tracer between the other five compartments. It was likely therefore that there was a difference of several orders of magnitude between rate constant k_{61} and the other rate constants in the model. This was accomodated in the analogue program by the use of a feed-back potentiometer which effectively reduced the value of k_{61} by a factor of 1000. The machine equations for both programs were time scaled to allow fast time solutions on the computer (Taylor, 1971; Charlesworth and Fletcher, 1970). 72sec computer time was equivalent to 3h problem time and the output matched on an appropriate time ramp, which generated a time function by differentiating a constant. The program and time function output were respectively led to the x and y axes of an x - y recorder.

RESULTS

Studies on normal rats.

In the blood and liver system at least six compartments of cholesterol should be considered. These are shown in Fig. 8. When developing a biological model as a first approximation the simplest model should be adopted. As all of the compartments shown in Fig. 8 are known to exist, and can be examined, then this was considered to be the simplest biological model available.

In order to obtain values for the rate constants in the model as a whole the blood and liver system was 'fragmented' and exchanges occurring within the blood considered initially. This was considered necessary because simulation of complex experimental models is time consuming and the process of deriving a full kinetic model can be reduced by fragmenting the major problem into a number of smaller units, ultimately combining these units to represent the entire model.

The first section of the results therefore deals with the determination of the rate constants for the three compartment model shown in Fig. 31.

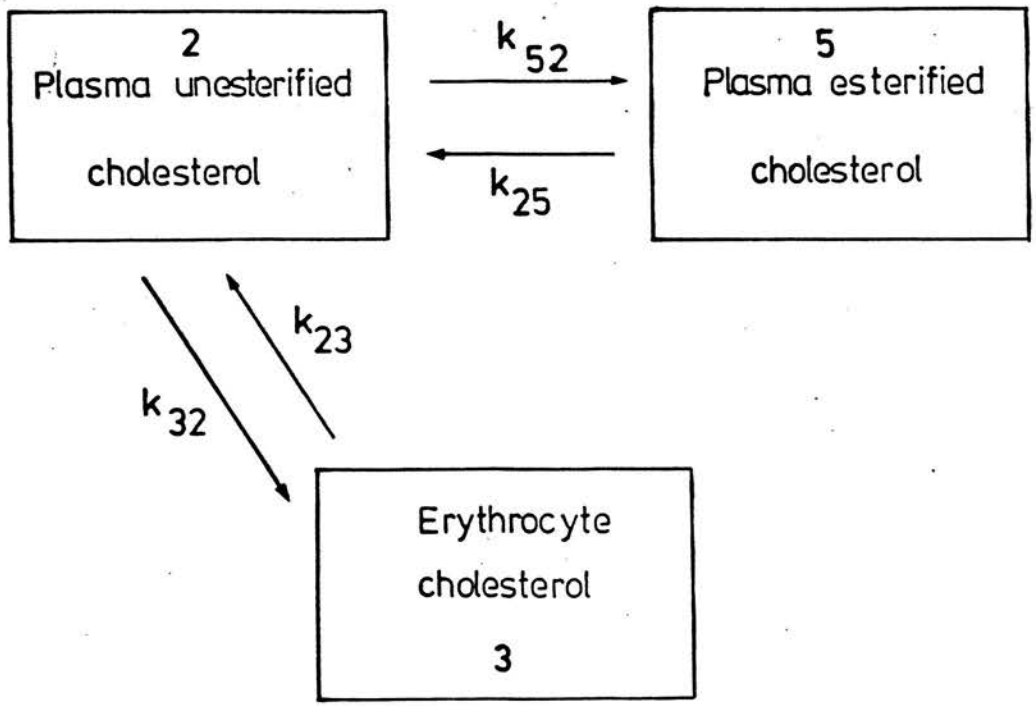


Fig.3I. Compartmented model representing the exchange of cholesterol between erthrocytes and plasma.

Kinetics of cholesterol exchange between plasma and erythrocytes.

The exchange of cholesterol between plasma and erythrocytes in blood from normal rats (Fig.31) was studied in a series of five experiments. The experimental details of this part of the study are described in the section on methodology and in principle allowed radioactive cholesterol in plasma to exchange with unlabelled cholesterol in plasma and vice versa.

An example of the change in specific radioactivity of cholesterol associated with the plasma unesterified, plasma esterified and erythrocyte compartments for the transfer of radioactive cholesterol from plasma to erythrocytes is shown in Fig. 32. At zero time all of the radioactive tracer in the form of (^{14}C) cholesterol was in the plasma ester and unesterified cholesterol. As can be seen there was a rapid fall in the amount of radioactivity in the plasma unesterified cholesterol with an associated rise in the amount of radioactivity in the erythrocytes. This rise was not as rapid as the fall in the plasma unesterified cholesterol compartment because of the unequal size of the compartments, e.g. the erythrocyte cholesterol compartment was about six times the size of the plasma unesterified cholesterol compartment. Equilibrium was achieved between the plasma unesterified compartment and the erythrocyte compartment after approximately 240min. Little change was noted in the plasma esterified compartment during these experiments.

Although it was considered that this exchange could be represented by a three compartmented system this was verified by analysing the curve showing changes in the unesterified cholesterol compartment. This could be achieved in two ways:-

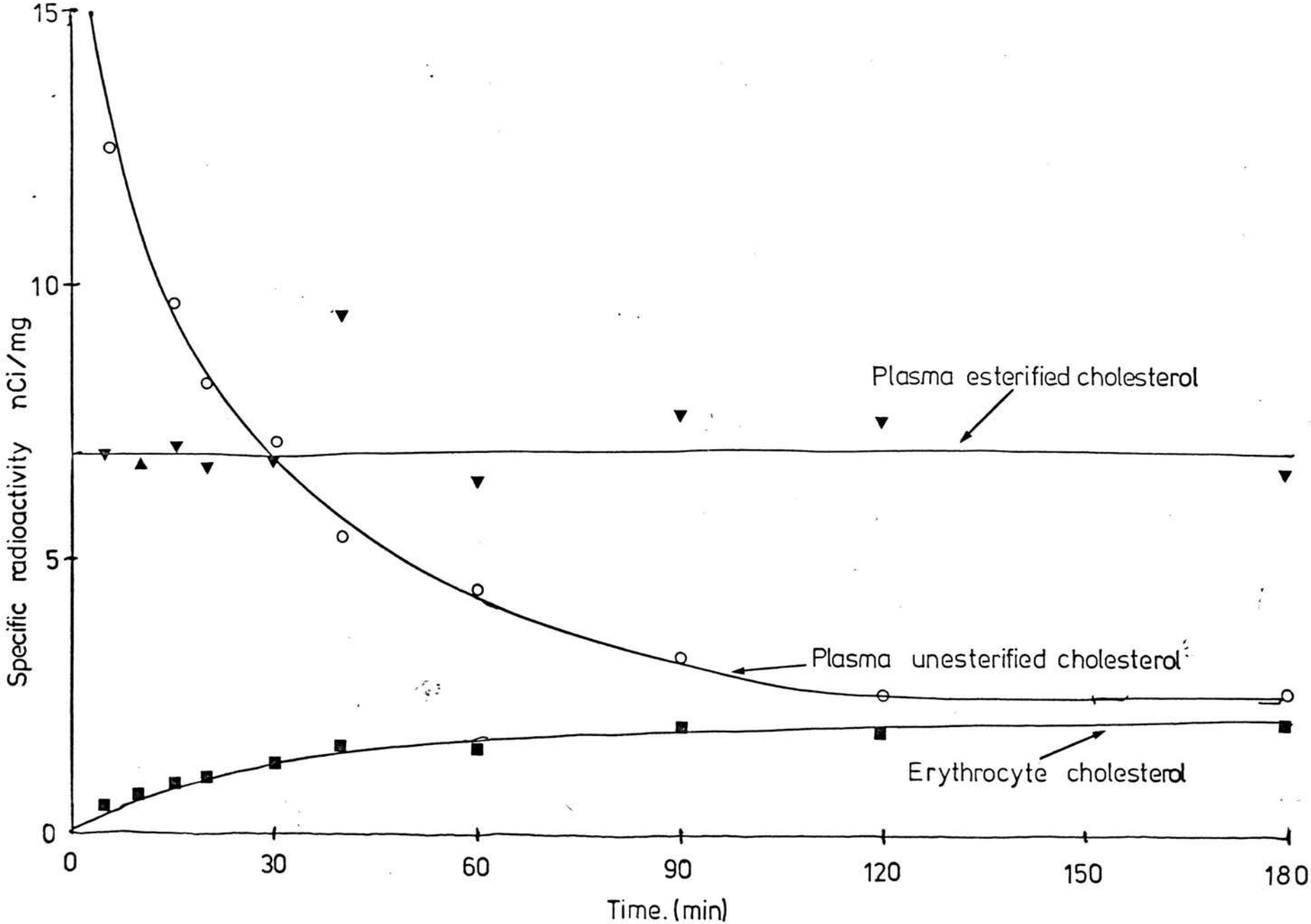


Fig. 32. Equilibration of radioactive cholesterol between plasma and erythrocytes

i) Graphical analysis

This technique has been used extensively to determine the number of exponential terms describing a curve. The technique has been described by a number of authors, Veall and Vetter (1965), Atkins (1970), Ottaway (1970) and is not considered here. Application of this technique to the data for the specific radioactivity of unesterified plasma cholesterol shown in Fig. 32 gave a result shown in Fig.33. The graph of the change in specific radioactivity of the plasma unesterified cholesterol was decomposed into three exponential terms which when summated yielded an equation:-

$$x \text{ (specific radioactivity)} = 13.6e^{-0.0857t} + 4.9e^{-0.0108t} + 2.8$$

giving an amplitude ratio of 2.8 and
an exponent ratio of 7.94

Ackerman et al. (1967) indicate some of the difficulties associated with using this technique in a reliable manner.

ii) Minimisation of the function

$$\sum \left(a_{jt} - \sum_{i=1}^{i=m} X_i e^{-\lambda_i t} \right)^2$$

where a_{jt} is the observed specific radioactivity of compartment j at time t and

$$\sum_{i=1}^{i=m} X_i e^{-\lambda_i t}$$

is a function made up of the sum of m exponential terms at time t.

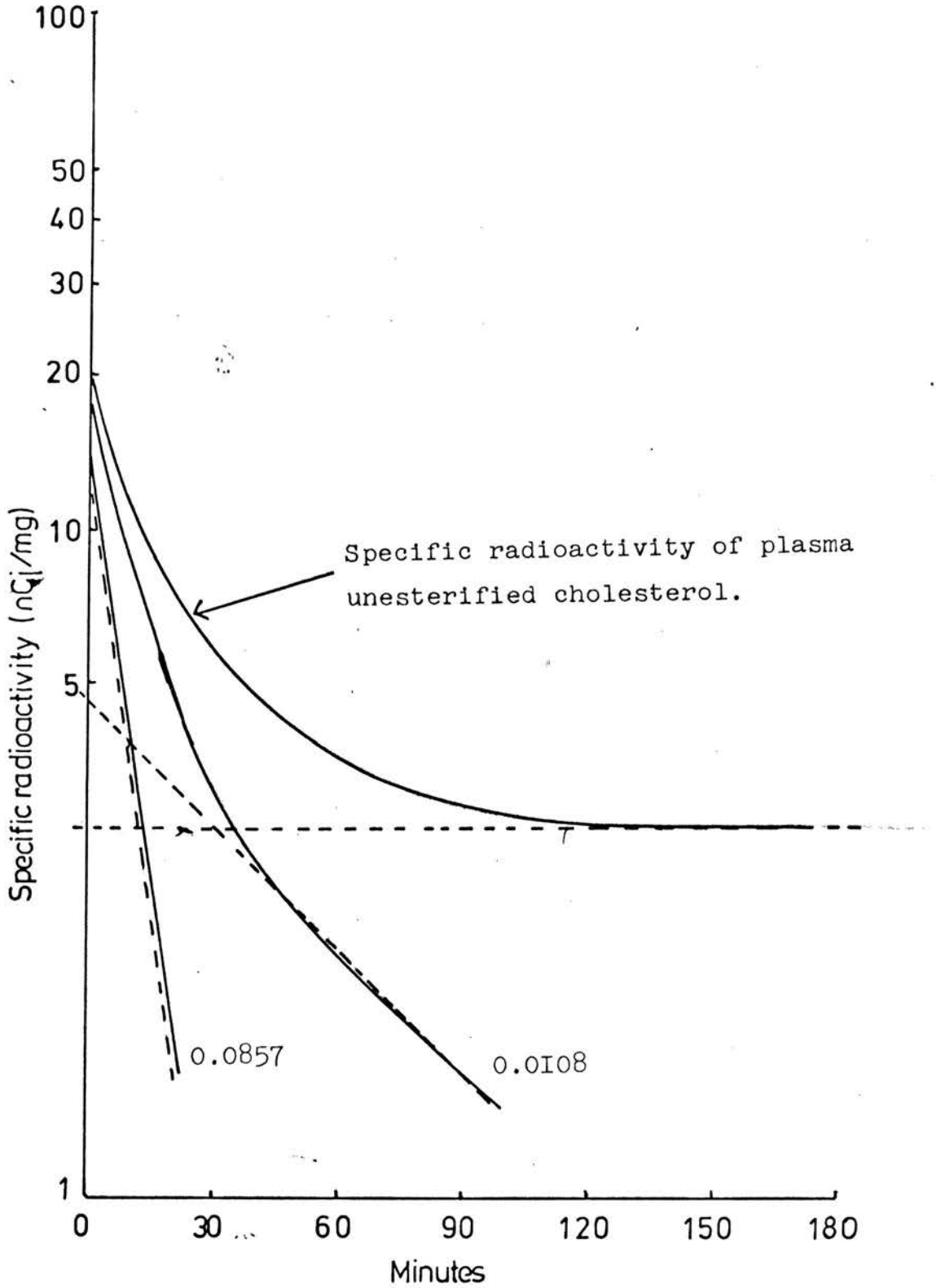


Fig.33. Decomposition of the specific radioactivity curve of plasma unesterified cholesterol into three terms.

By suitable selection of X_i and λ_i a curve giving the best fit to a_j at all time points can be constructed.

A computer program developed by Atkins (1971) based upon this principle, was used to fit the specific radioactivity curve of the plasma unesterified cholesterol (Fig. 32). This gave an equation:-

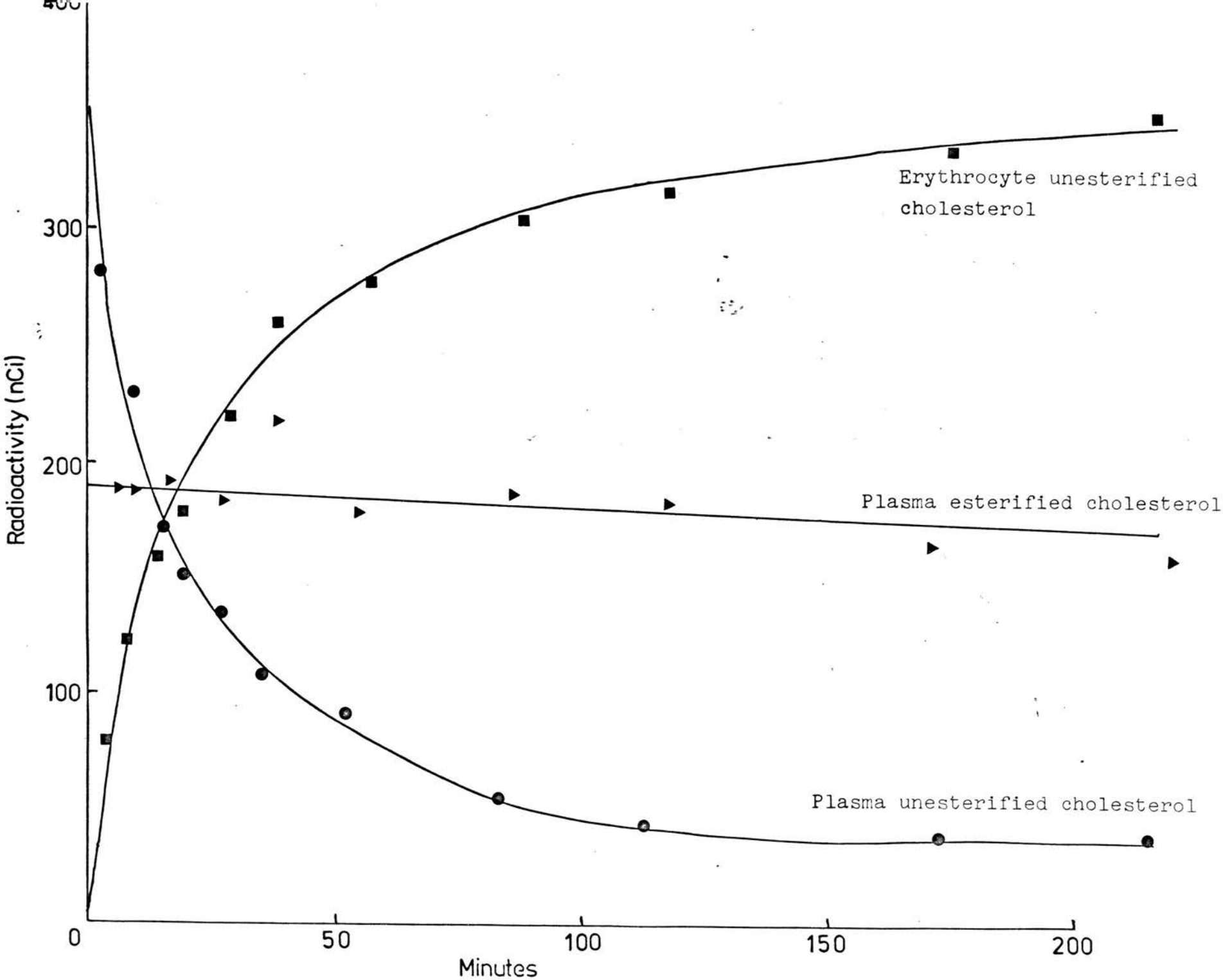
$$\underset{\text{(specific radioactivity)}}{x} = 17.4e^{-0.1t} + 3.8e^{-0.008t} + 1.6$$

with an exponent ratio of 12.5 and with an amplitude ratio of 4.6. These values supported the presence of three compartments in the system when twelve datum points were used (Glass and Garretta, 1971), although the parameter errors would be high, probably of the order of 100%.

The observed specific radioactivity data (Fig. 32) were then transformed to show the amount of radioactivity (nCi) in each compartment as equilibration occurred and is illustrated in Fig. 34. This transformation was obtained by multiplying the observed cholesterol pool size for each compartment by the appropriate specific radioactivity. The specific radioactivity data were reduced to this form to remove any need for computer scaling thus allowing easier manipulation in the simulated model.

A check upon the reliability of this experiment was obtained by summating the amount of radioactivity in all compartments at any point in time. These sums should be equal at all time points if the accuracy of the analytical procedures was good. The recovery in this experiment showed a mean radioactivity of 26.24nCi $\pm$ 2.17nCi (n = 12). Because of the large variance the data were normalised to a mean recovered radioactivity of 22.42nCi before plotting

Fig.34. Observed changes in the amount of radioactivity in cholesterol compartments in the blood during equilibration of labelled plasma cholesterol with unlabelled cholesterol in the erythrocytes.


Radioactivity (nCi)
300
200
100
0
50
100
150
200
Minutes
Erythrocyte unesterified cholesterol
Plasma esterified cholesterol
Plasma unesterified cholesterol

graphically (Fig.34). After normalisation the plasma unesterified cholesterol at zero time contained 17.03nCi and the plasma ester cholesterol contained 9.21nCi.

These values, scaled by a factor of two, were programmed as the initial conditions in the analogue program described in the methods section, (Fig. 28). Limits for the rate constants in this model, Fig. 31, were set as shown in Table 9. These ranges were chosen because they encompass the exponents derived by the digital and graphical fitting program (see p75). The computer generated curves for these ranges of rate constants are shown in Fig. 35.

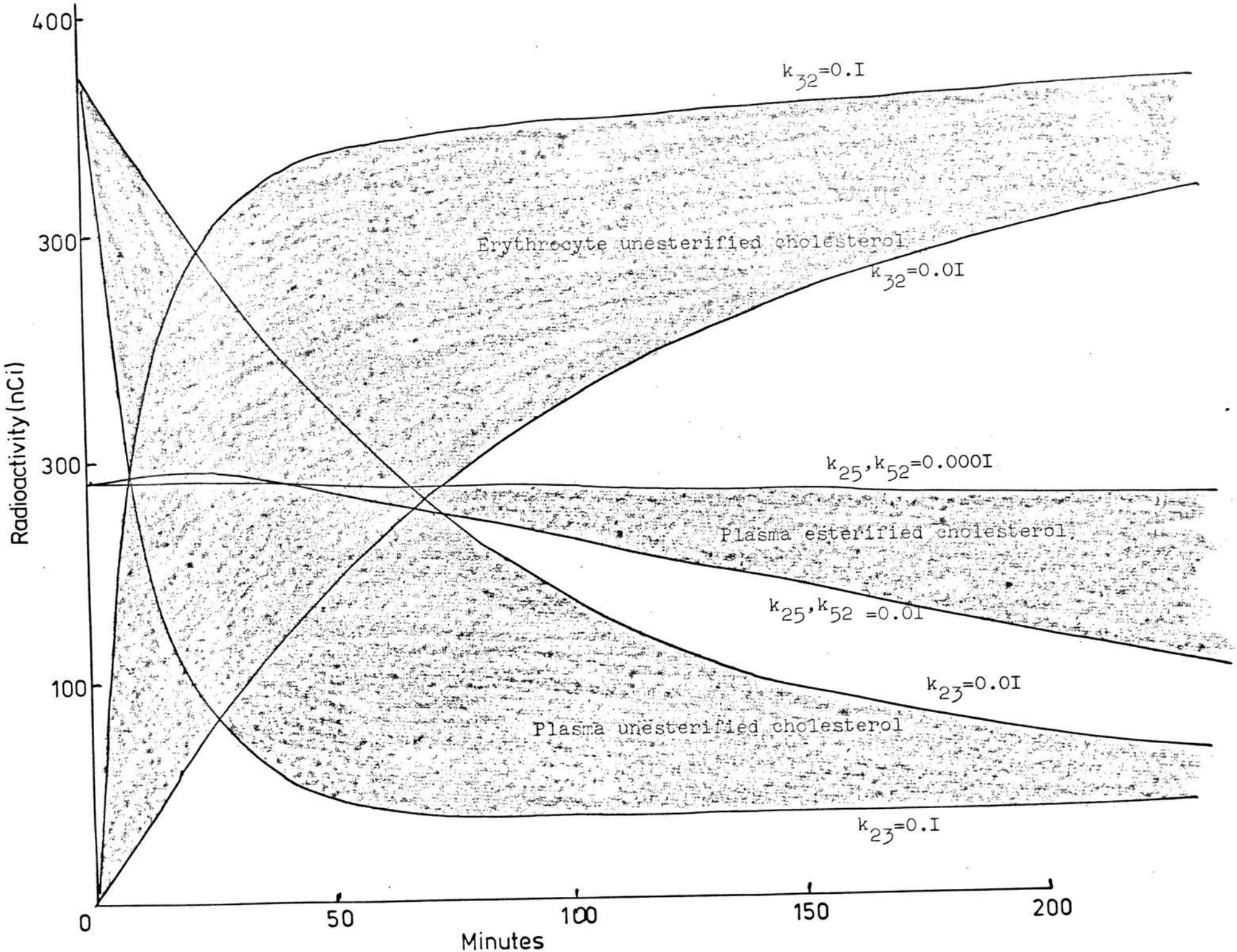
It was apparent that simulated curves examining the behaviour of radioactivity movements between erythrocyte and plasma unesterified cholesterol compartments, were little affected by changes in the rate constants k_{52} and k_{25} in the ranges used. These rate constants were therefore set at a constant value and rate constants k_{23} and k_{32} changed sequentially until the simulated curves output onto a trace oscilloscope gave an eye-fit to the experimental data. Examples of the oscilloscope output are shown in Fig. 36. Smaller sequential changes in these rate constants were then made and the curves output onto an x - y recorder with time generated on the x axis. Examples of the output for constant k_{32} rate constants and different k_{23} rate constants are shown in Figs. 37 and 38. The values on the y axis were preset from a knowledge of the computer millivolt output in conjunction with the scales available on the recorder, and it was therefore possible to calculate the simulated value for any curve at the desired time point. These values were compared with the experimental data, and a sum of residual squares of the difference between the experimental and simulated data calculated. The sum of residual squares for different values of the rate constants

Rate Constant	Range
k_{52}	0.0001 - 0.010
k_{25}	0.0001 - 0.010
k_{23}	0.01 - 0.1
k_{32}	0.01 - 0.1

Table 9

Range of rate constants used in preliminary experiments studying the exchange of cholesterol between erythrocytes and plasma.

Fig.35. Simulated changes in the amount of radioactivity in cholesterol compartments in the blood during equilibration of labelled plasma cholesterol with unlabelled cholesterol in the erythrocytes. Simulated curves derived using different rate constants.


400
300
300
100
0
50
100
150
200
Minutes
Radioactivity (nCi)
k_{32}=0.I
Erythrocyte unesterified cholesterol
k_{32}=0.0I
k_{25},k_{52}=0.000I
Plasma esterified cholesterol
k_{25},k_{52} =0.0I
k_{23}=0.0I
Plasma unesterified cholesterol
k_{23}=0.I

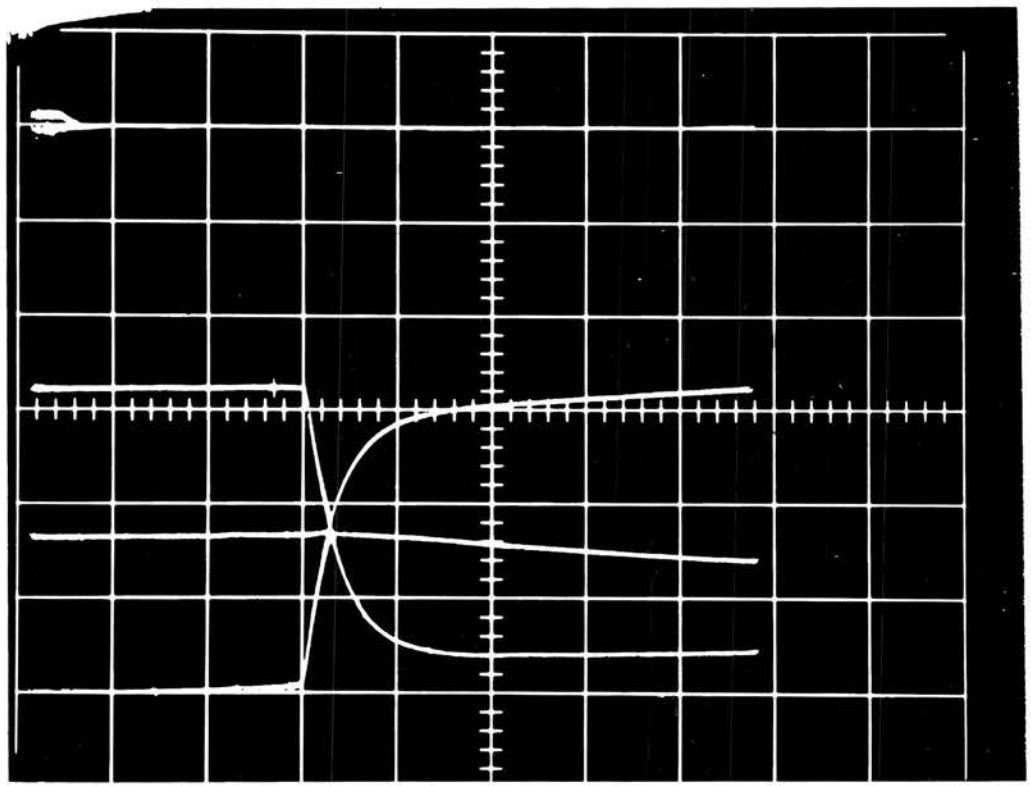


Fig. 36. Oscilloscope recording of the simulated exchange of cholesterol between plasma and erythrocytes. The upper curve represents erythrocyte cholesterol, the middle curve the plasma ester cholesterol and the lower curve the plasma unesterified cholesterol.

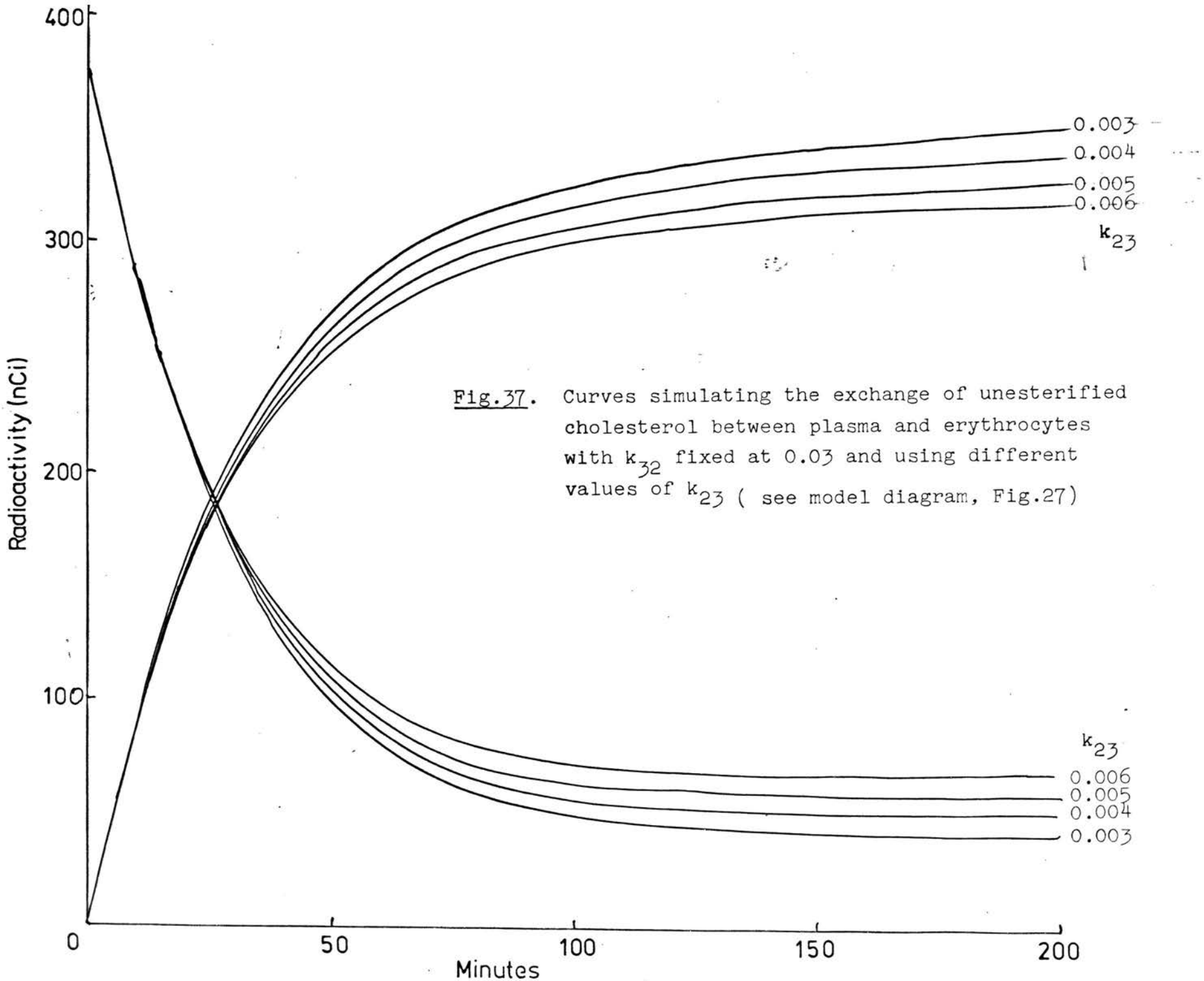


Fig.37. Curves simulating the exchange of unesterified cholesterol between plasma and erythrocytes with k_{32} fixed at 0.03 and using different values of k_{23} (see model diagram, Fig.27)

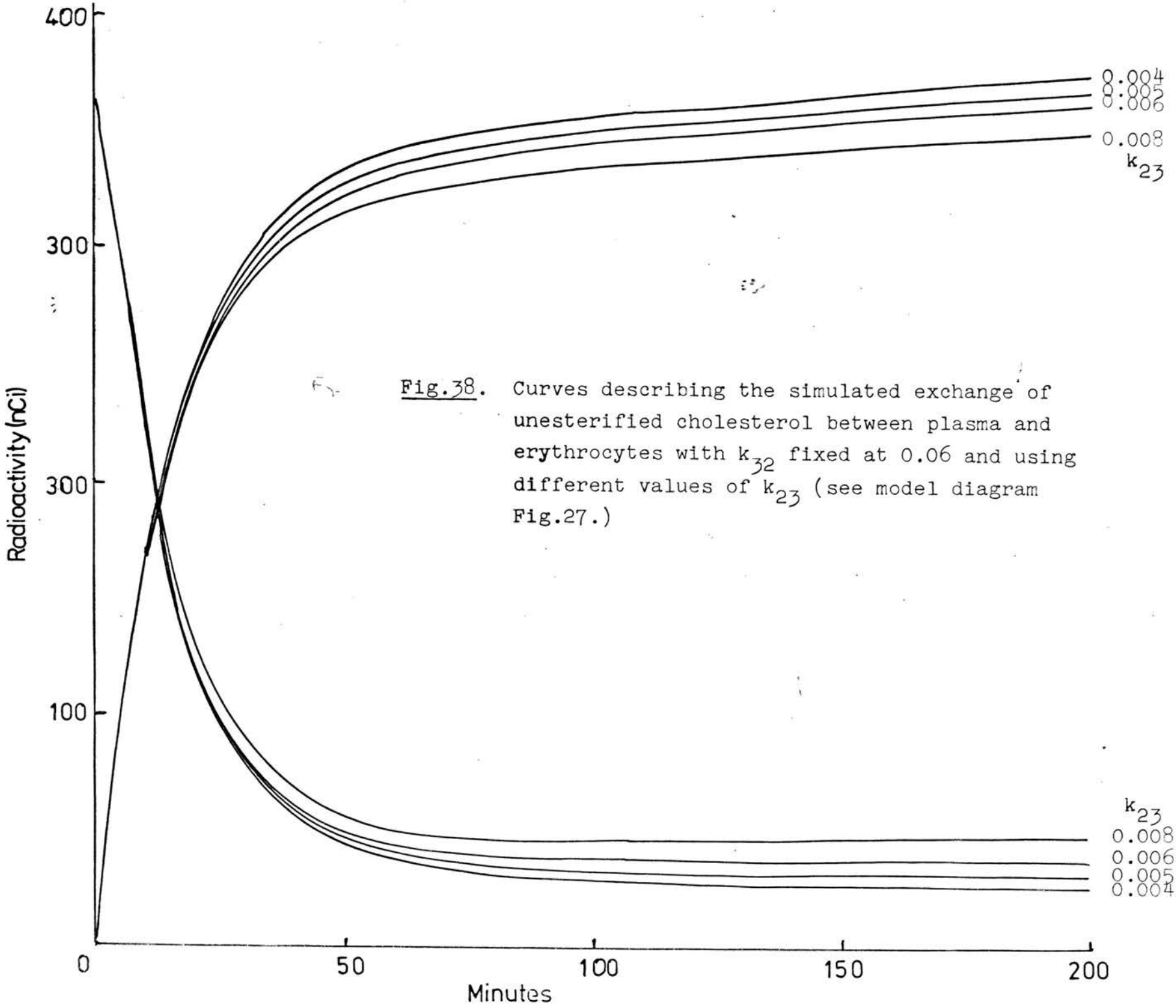


Fig.38. Curves describing the simulated exchange of unesterified cholesterol between plasma and erythrocytes with k_{32} fixed at 0.06 and using different values of k_{23} (see model diagram Fig.27.)

were compared in order to obtain a minimal value.

A plot of the sum of residual squares for different values of rate constants is shown in Fig. 39. This was obtained for the simulation of changes in the plasma unesterified and erythrocyte cholesterol compartments. The best fit of the simulated curve to the experimental data for the change in the plasma unesterified compartment was given by inspection (Fig. 39), showing rate constant $k_{32} = 0.050$ and $k_{23} = 0.006$. For the change in the erythrocyte compartment $k_{32} = 0.040$ and $k_{23} = 0.008$. The rate constant k_{32} was apparently satisfied by two different values, each providing the best fit to curves describing changes in the amount of radioactivity in erythrocyte and plasma unesterified cholesterol respectively. In order to provide the best fit to both curves the values were combined in proportion to the sum of their residual squares.

Erythrocyte $k_{32} = 0.040$, $k_{23} = 0.008$ Residual = 10.9

Plasma unesterified $k_{32} = 0.05$, $k_{23} = 0.006$ Residual = 50.56

Final value $k_{32} = 0.04 + (0.05 - 0.04) \times \frac{50.56}{61.46}$

$= 0.0488$

Final value $k_{23} = 0.008 - (0.008\ 0.006) \times \frac{50.56}{61.46}$

$= 0.0064$

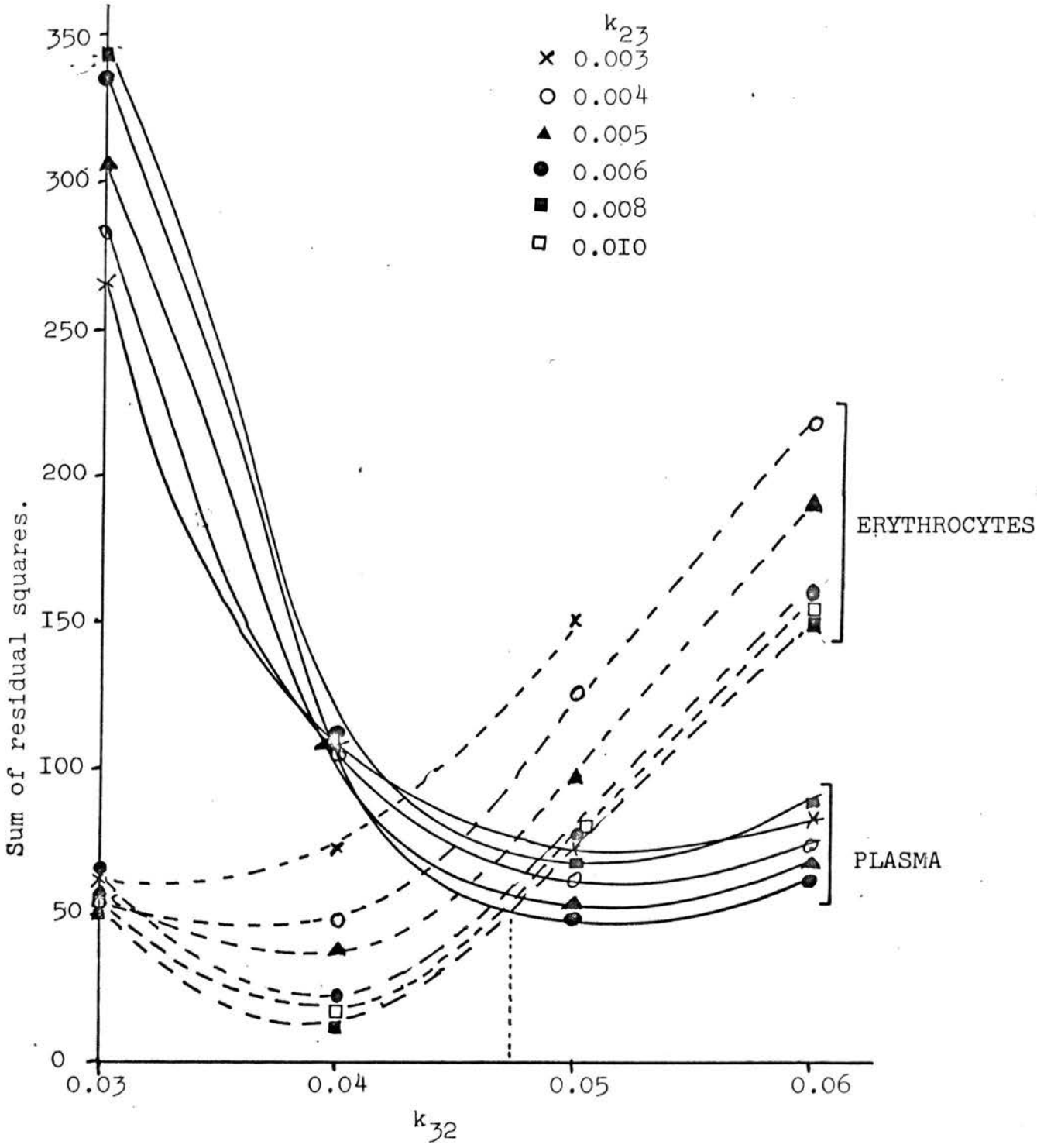


Fig.39. A plot of the sum of residual squares remaining after fitting the simulated curves, for the exchange of cholesterol between erythrocytes and plasma, to the experimental data. The plot is for different values of the rate constants k_{23} and k_{32} in both the plasma and erythrocyte compartments.

Inspection of Fig. 39 shows that these combined figures gives a residual square value of about 60 which represents the intersection of the two sets of curves.

Because little change was noted in the amount of radioactivity associated with the plasma ester cholesterol, it was possible to obtain a good fit to this experimental data by eye. Sums of residual squares were not employed in establishing the rate constants k_{25} and k_{52}. The values given to these rate constants in this experiment were 0.0012 for each constant. Using the four derived rate constants in the analogue program represented by Fig. 28, computer generated curves were plotted and compared to the experimental data. The results are shown in Fig. 40 for this single experiment. It was considered that reasonable agreement between experimental and simulated data had been obtained.

Two similar experiments were performed in which the radioactive cholesterol was initially in the plasma and equilibrated with cholesterol in the erythrocytes. These experiments gave data similar in form to that shown in Fig. 32, but with different amounts of radioactivity in the plasma. These data were treated in a similar manner to that described for the data presented in Fig. 32 and a new set of rate constants determined. The values found for the rate constants in the three experiments are summarised in Table 10.

Two further experiments were carried out in which the radioactive cholesterol was initially in the erythrocytes and subsequently equilibrated with the plasma fraction. Rate constants for these experiments are shown in Table 11.

The mean values for the rate constants describing the exchange of cholesterol between plasma and erythrocytes are shown in Fig. 41 and represent the mean values for five <u>in vitro</u> experiments.

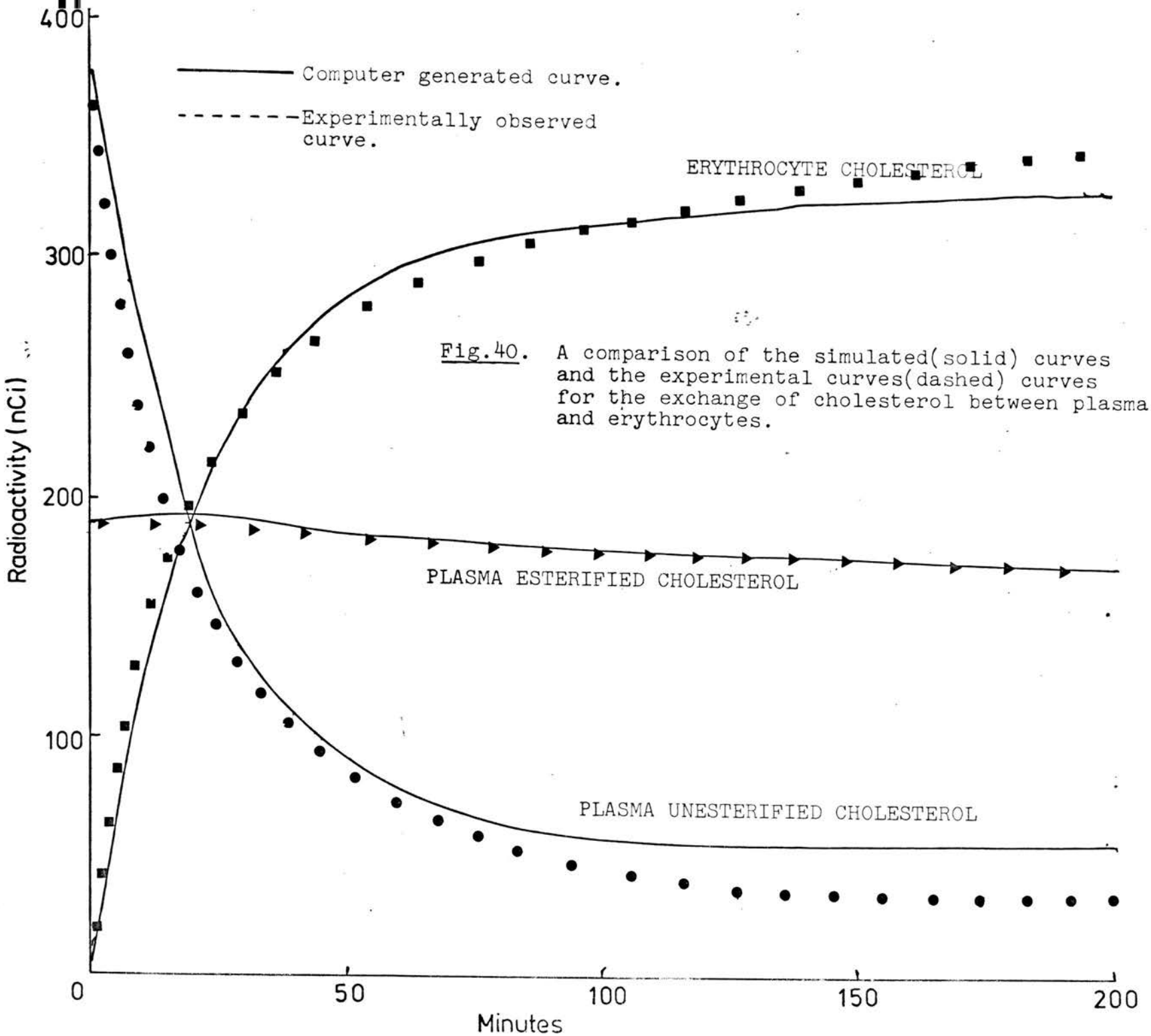


Fig.40. A comparison of the simulated(solid) curves and the experimental curves(dashed) curves for the exchange of cholesterol between plasma and erythrocytes.

Experiment	k_{32}	k_{23}	k_{52}	k_{25}
1.	0.0482	0.006	0.0012	0.0012
2.	0.0252	0.0046	0.0010	0.0010
3.	0.0278	0.0048	0.0008	0.0008

Table 10

Rate constants describing the exchange of cholesterol between erythrocytes and plasma where the label was initially in the plasma cholesterol.

Experiment	k_{32}	k_{23}	k_{52}	k_{25}
1.	0.0305	0.0054	0.0030	0.0030
2.	0.0261	0.0058	0.0025	0.0025

Table 11

Rate constants describing the exchange of cholesterol between erythrocytes and plasma where the label was initially in the erythrocyte cholesterol.

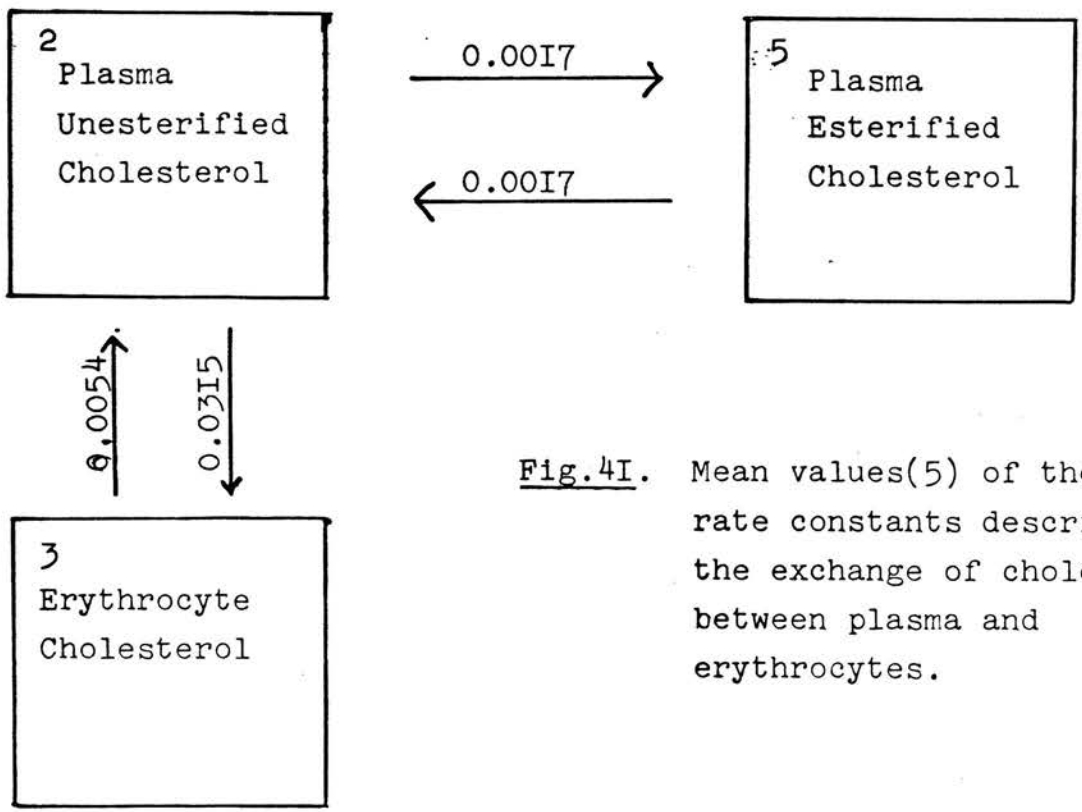


Fig.4I. Mean values(5) of the rate constants describing the exchange of cholesterol between plasma and erythrocytes.

These values only serve as an indication of the relative sizes of the rate constants for this small part (Fig. 31) of the whole model (Fig. 8). They may have to be altered to satisfy the full model when incorporated into the larger six compartment program.

Liver perfusion studies in normal rats.

In the previous section preliminary studies were made of the rate constants describing the exchange of cholesterol between compartments within the blood. Perfusions of livers taken from normal rats were then performed (c.f. methods section) in order to study exchanges occurring between the blood and liver.

After starting each perfusion it was established that the preparation was operating satisfactorily as judged by blood and bile flow and by the appearance of de-oxygenated blood from the hepatic vein. These variables were studied by visual means and it normally took between five and ten minutes for the perfusion to be established.

At this point 4μCi (2^{14}C)DL mevalonic acid lactone in 1ml acetone:saline (1/1) were injected into the perfusate reservoir. In a limited number of later experiments 10μCi of sodium (^{3}H) acetate in saline were also administered in conjunction with the radioactive mevalonate. Prior to the injection of the radioactive material the lower perfusate reservoir (A, p 61) was allowed to fill to its maximum capacity without endangering the blood flow into the liver. In this way the radioactivity was distributed into the maximum volume of the perfusate immediately following injection of the pulse. This reduced the equilibration time for the distribution of the labelled precursor throughout the perfusion system.

Blood samples were removed during perfusion at 5, 10, 15, 30, 60, 90, 120, 150, 180 and 240min after the addition of the radioactive material. The samples (1 - 2ml) were withdrawn by allowing the blood draining from the liver to run directly into a precalibrated centrifuge tube. The blood was centrifuged and the plasma removed. The remaining erythrocytes were washed twice with saline and the top few millimetres removed by suction and discarded. Aliquots of the plasma and erythrocyte fractions were extracted with a known volume of alcohol:acetone (1:1 v/v) as described in the methods section (p 4I). Treatment of the sample up to the stage of extraction with alcohol: acetone was performed as rapidly as possible to reduce the possible exchange of label between erythrocyte, plasma unesterified and plasma esterified compartments.

The weight of the bile collected was determined by difference. The complete bile sample was extracted into alcohol:acetone, and cholesterol concentration and radioactivity determined in aliquots of this extract.

The whole liver was weighed at the end of the perfusion after flushing the vascular bed with 0.9% saline through the portal vein. The liver was then homogenised in 10ml saline at full speed in a Waring blender and the homogenate made up to a known volume. An aliquot of this homogenate was extracted with alcohol:acetone (1:1) and the extract analysed for cholesterol concentration and radioactivity.

Eight perfusion experiments on normal rat liver were performed and the results to follow represent the mean of the eight perfusions. Approximately six other perfusions were carried out but the results are not included here because these perfusions were considered unsatisfactory. For example, the blood flow rate fell markedly after 1 - 2h, or the bile flow stopped or did not appear

at the start of the perfusion. Occasionally the perfusions were discontinued because of failure of some mechanical component of the perfusion apparatus.

Of the eight acceptable perfusions, three were performed in which two radioactive precursors of cholesterol were added to the perfusate, i.e. (2^{14}C)DL mevalonic acid and sodium (^{3}H) acetate.

Perfusate cholesterol concentration.

The mean cholesterol concentration in the perfusate increased during the perfusion (Fig. 42). This suggested a net transfer of cholesterol into the perfusate from the liver. Regression analysis of the data showed that the mean slope of the regression lines of total cholesterol concentration/time was 0.02774, and this was compared to the slope obtained for cholesterol concentration changes in a control experiment when perfusate was circulated in the absence of a perfused liver. The slope for this single control experiment was 0.02142 which was not significantly different from that obtained during perfusions of normal rat livers. It seems likely therefore that the apparent net release of cholesterol from the liver in any quantity is a technical artefact related to the haemo-concentration occurring during perfusion. There was however a significant difference ($p = < 0.001$) between the means of the slopes of the regression lines associated with plasma esterified and unesterified cholesterol. This would imply that unesterified cholesterol is released from the liver in a different manner to the ester cholesterol.

The mean intercept on the x axis (concentration of cholesterol in sample) of Fig. 42 gave a value of 8.2µg for unesterified cholesterol and 23.1µg for total

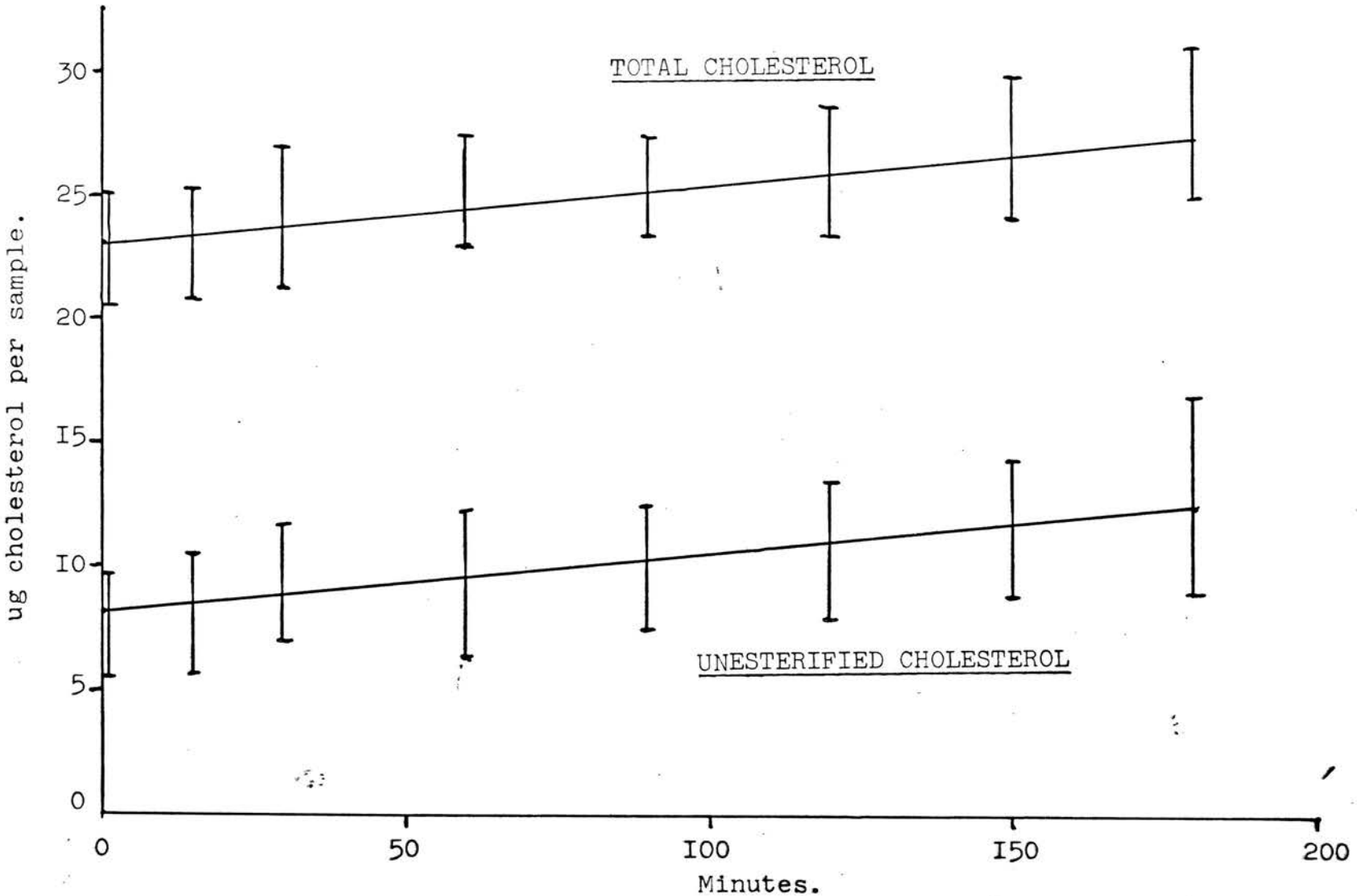


Fig.42. Changes in the concentration of cholesterol in the perfusate during perfusion of livers taken from normal rats.

cholesterol in the plasma. This gave a ratio of 2.82, indicating approximately twice as much esterified cholesterol as unesterified cholesterol in the plasma. This compared well with the ratio of 2.61 found when determining the size of plasma cholesterol compartments *in vivo*.

Recovery of radioactivity

The radioactivity recovered was determined by multiplying the compartment size by the specific radioactivity of the compartment at the termination of the perfusion.

This gave a mean recovery of 47.5% for (^{14}C) labelled cholesterol. Theoretically one would expect 50% of the label to be used if only the natural enantiomer was being utilised. Tavormina *et al.* (1955) have shown that at least 43% of labelled mevalonic acid is incorporated into the cholesterol in liver cell homogenates and Swell and Law (1971) obtained 50% recovery in an isolated perfused liver system. One should theoretically expect that recoveries of radioactivity should be less than calculated because some of the cholesterol is being diverted into other metabolic pathways, principally into bile acids, and in this study no determination of this secondary effect was made. Assuming that the specific radioactivity of the excreted bile acids approximates to that of the biliary cholesterol then the amount of radioactivity needed to correct for the excretion of bile acids is very small. This is because only 125µg/h of bile acids are excreted in this preparation (Percy-Robb and Boyd, 1970) and this represents such a small proportion relative to the total amount of cholesterol in the system (1:80) that the amount of radioactivity associated with bile acids can be ignored for all practical purposes.

For (^{3}H) cholesterol a mean recovery of only 16% was found, allowance being made for the efficiency of the counting equipment. This low recovery is not surprising when one considers the alternative pathways available to 'acetate' in the liver. A large proportion of the tritium label in these experiments is probably being diverted away from the biosynthesis of cholesterol, hence the low recovery.

Specific radioactvity changes during perfusions of livers from normal rats.

The changes in specific radioactivity of (^{14}C) cholesterol in each of the four compartments examined is shown in Fig. 43.

The curves indicate a rapid initial incorporation of radioactivity into each of the compartments sampled and no maxima in the curves were noted for the perfusion times used. If the time of perfusion could be extended then it would be expected that the specific radioactivity of all compartments would eventually reach a common value and then decline very slowly as the radioactive cholesterol is removed from the system in the form of bile acids or cholesterol in the bile. The specific radioactivity of the biliary cholesterol was still rising at the end of a three hour perfusion and had a mean value of 23.28nCi/mg. The specific radioactivity of the biliary cholesterol was always less than that of the unesterified plasma cholesterol over the three hour period studied and no two compartments reached equilibrium.

The curves showing incorporation of (^{3}H) acetate into cholesterol are different in general shape to those using (2^{14}C)DL mevalonate as precursor, Fig. 44. The curves associated with the plasma esterified and unesterified

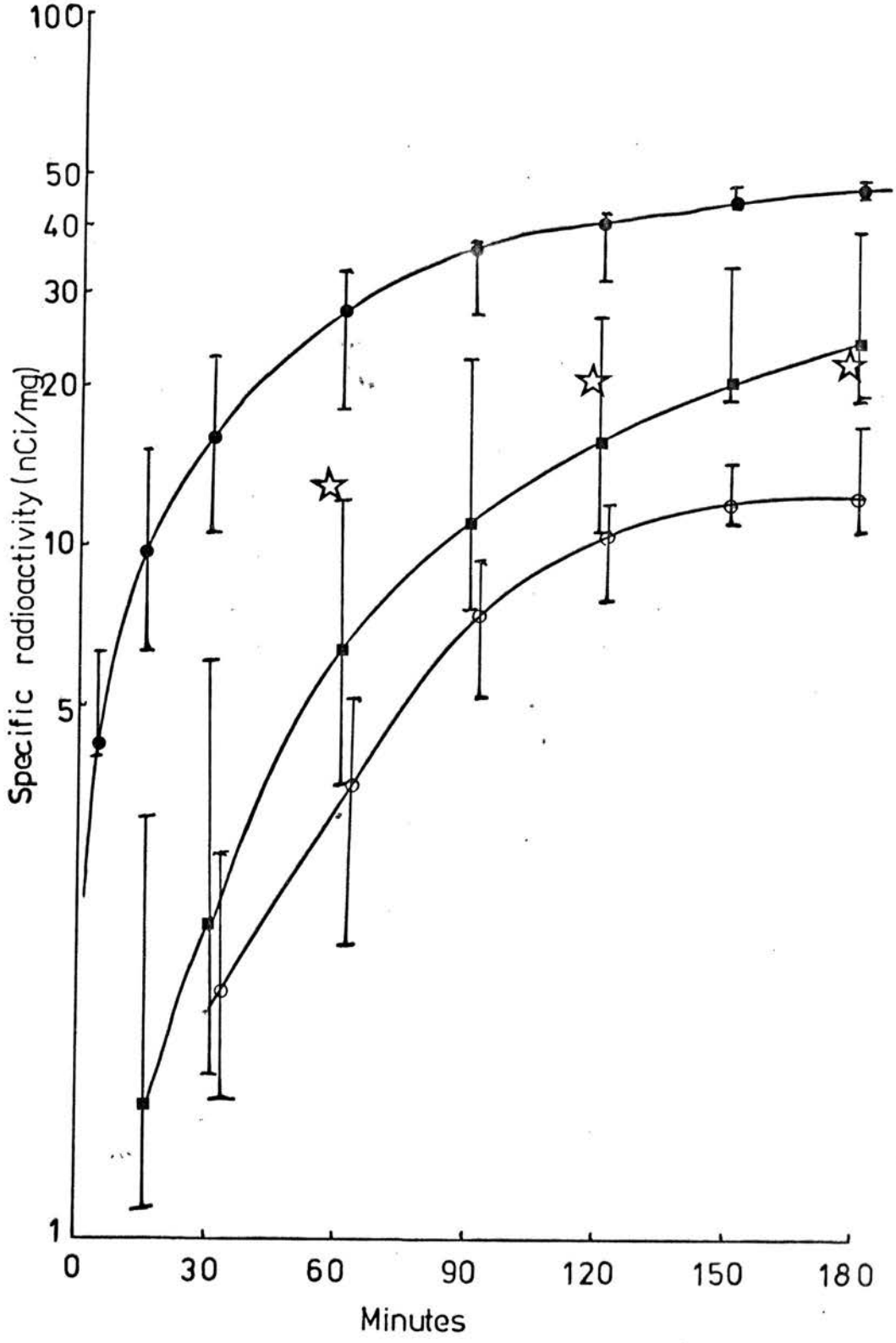


Fig.43. Changes in the specific radioactivity of (^{14}C) cholesterol in the plasma unesterified(●), esterified(○), erythrocyte(■) and biliary compartments(☆) during perfusion of normal rat livers.

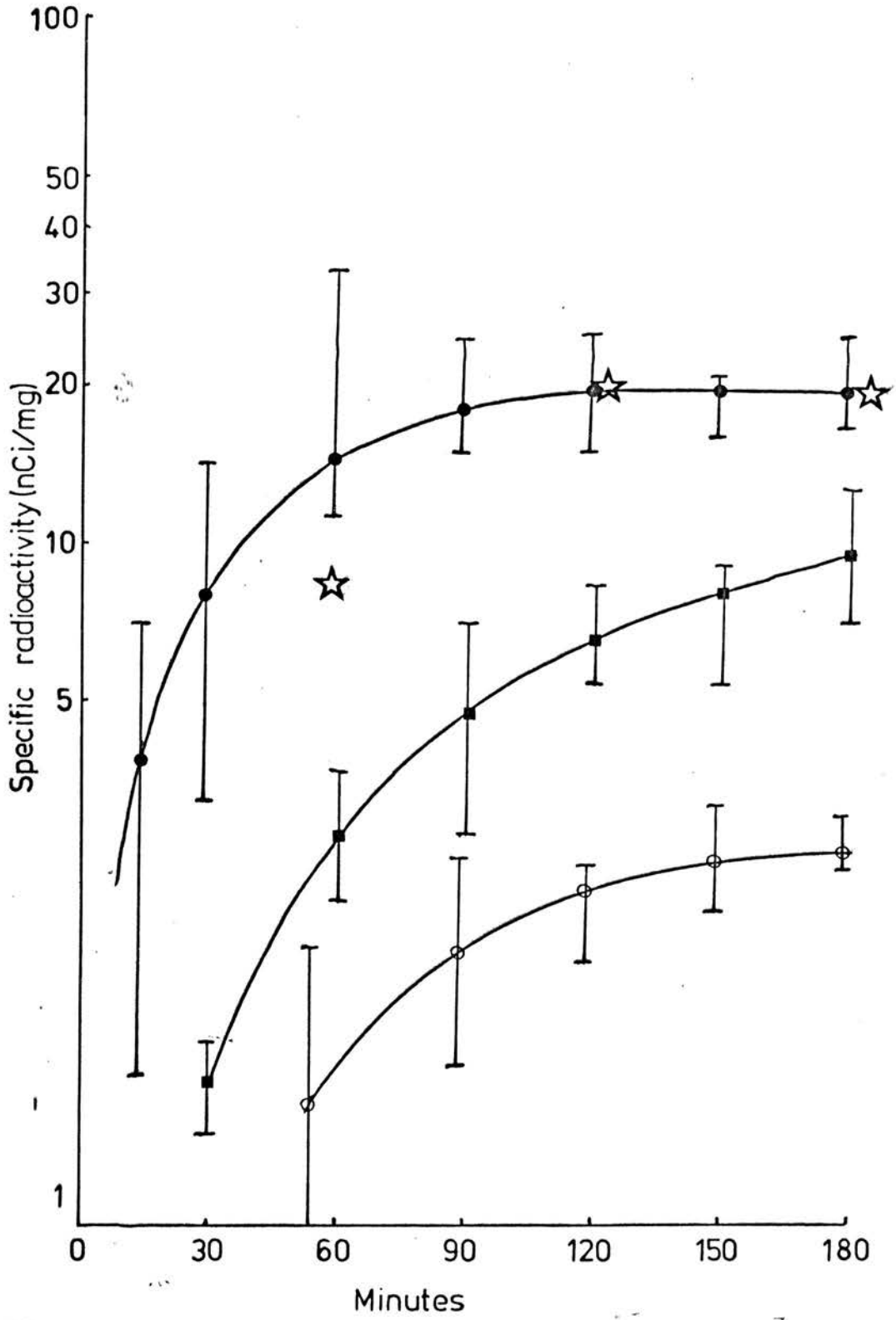


Fig.44. Changes in the specific radioactivity of (^{3}H)cholesterol in the plasma unesterified(●), esterified(○), erythrocyte (■), and biliary(☆) compartments during perfusion of normal rat livers.

compartments show a rapid flattening which is not apparent for the erythrocyte curve.

A direct comparison of the curves obtained from cholesterol labelled with (^{14}C) and (^{3}H) are shown in Figs 45, 46 and 47.

Simulation to fit experimental data.

In order to make an analogue simulation simpler, the specific radioactivity data of Fig. 43 were reduced to the form shown in Fig. 48, i.e. the absolute amount of radioactivity associated with any compartment at any point in time. By this means the analogue programming was made simpler, no account being taken of the relative pool sizes of the compartments considered. The analogue program could have been constituted so as to produce curves simulating the observed changes in specific radioactivity for compartments examined. This would have required scaling of the integrator output representing each compartment in order to allow for the different compartment sizes. The specific radioactivity data were therefore transformed to show the actual amount of radioactivity (nCi) in each compartment.

This procedure had been used in the simulation studies where cholesterol exchanged between plasma and erythrocytes, and in that instance was valid because the system was in a steady state. This does not hold for the exchanges between liver and blood since the compartment size was not constant for the plasma and erythrocyte cholesterol because samples were being withdrawn throughout the time period under study. As the blood flow rate was relatively constant, then the recycling time for any segment of the circulating perfusate was reduced with a consequent decrease in the time for equilibration between the liver and perfusate. This would have given

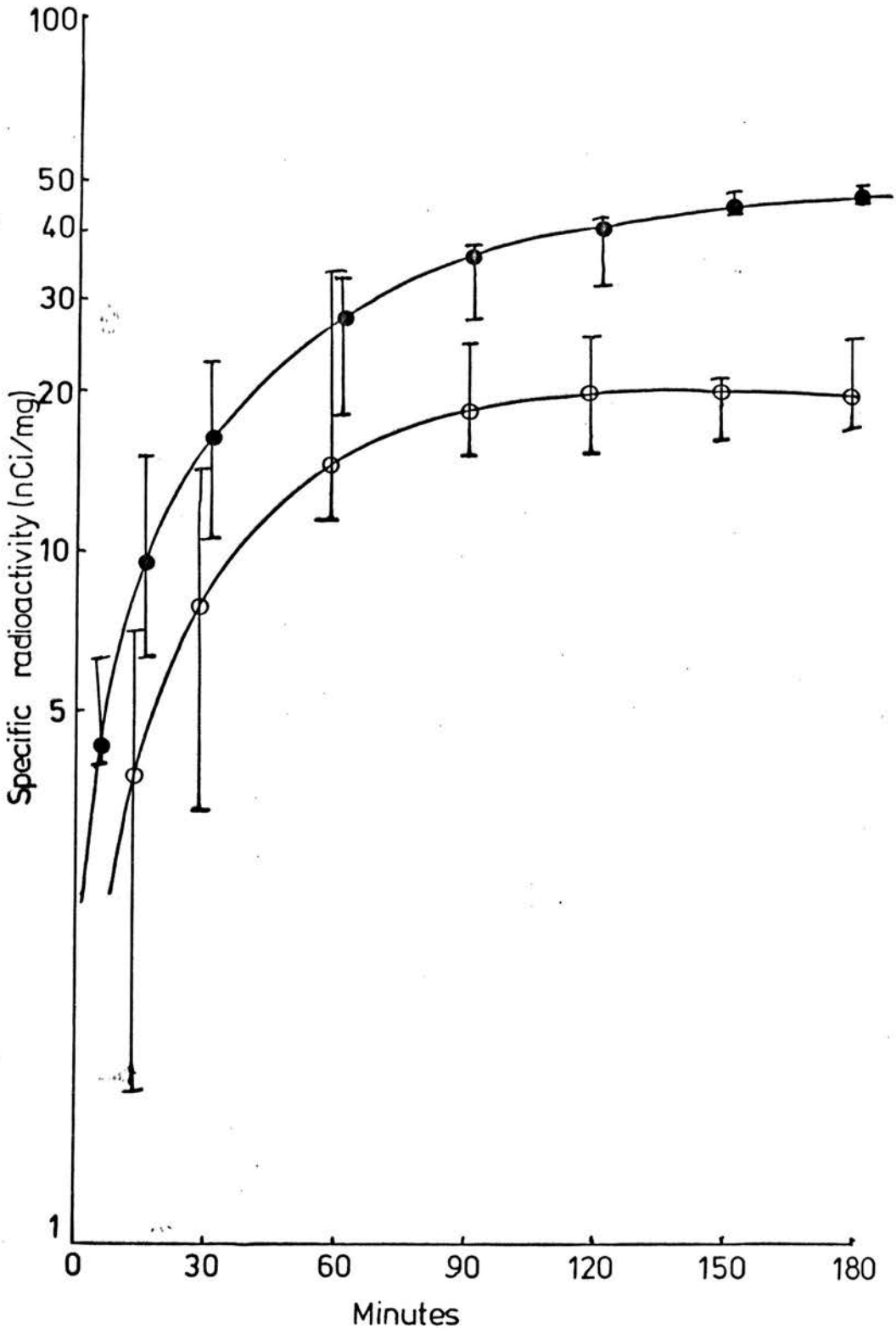


Fig.45. Changes in the specific radioactivity of unesterified plasma cholesterol labelled with carbon-14(●) or tritium(○) during perfusion of livers taken from normal rats.

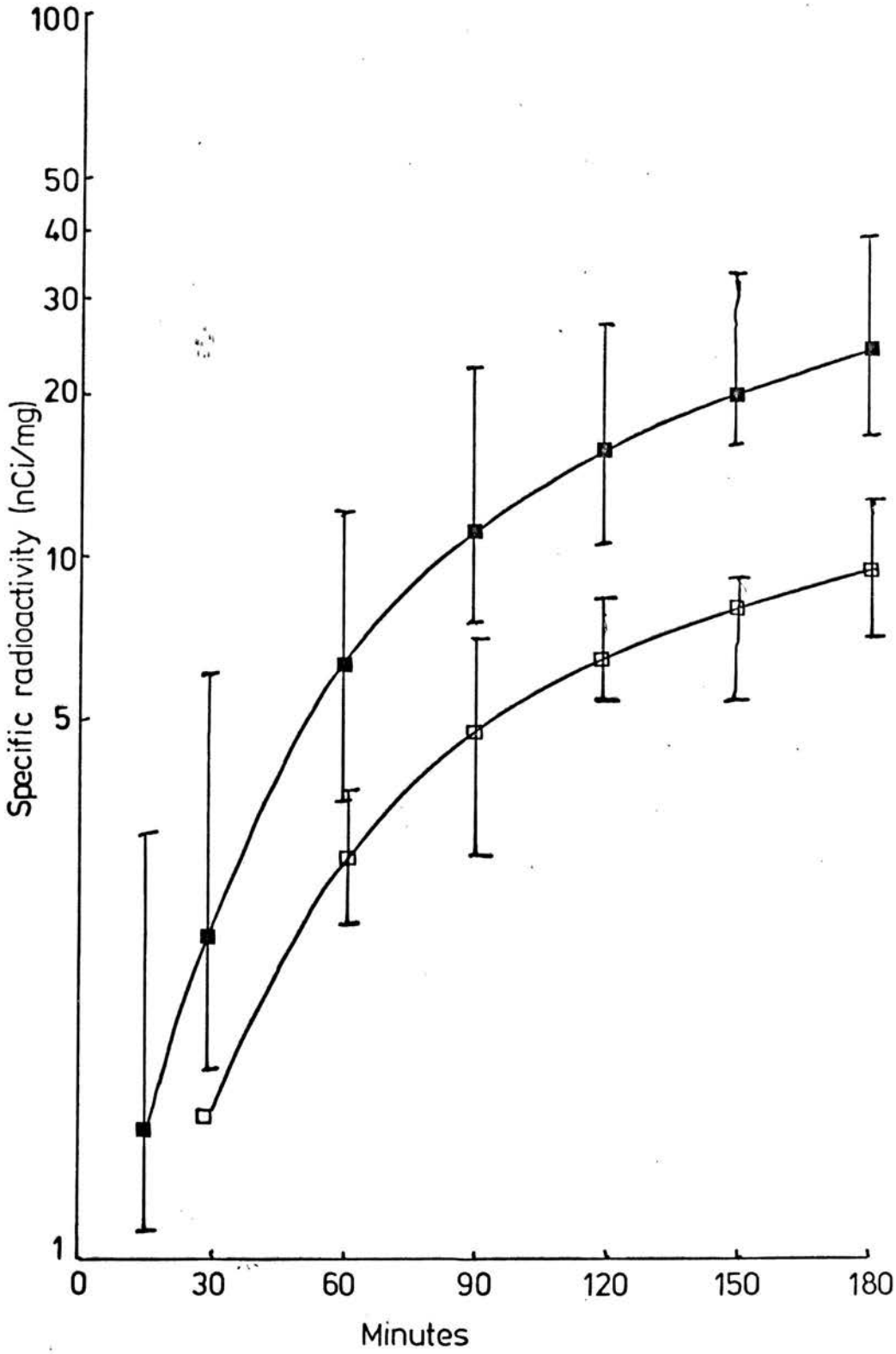


Fig.46. Changes in the specific radioactivity of erythrocyte cholesterol labelled with carbon-I4(■) or tritium (□) during perfusion of livers taken from normal rats.

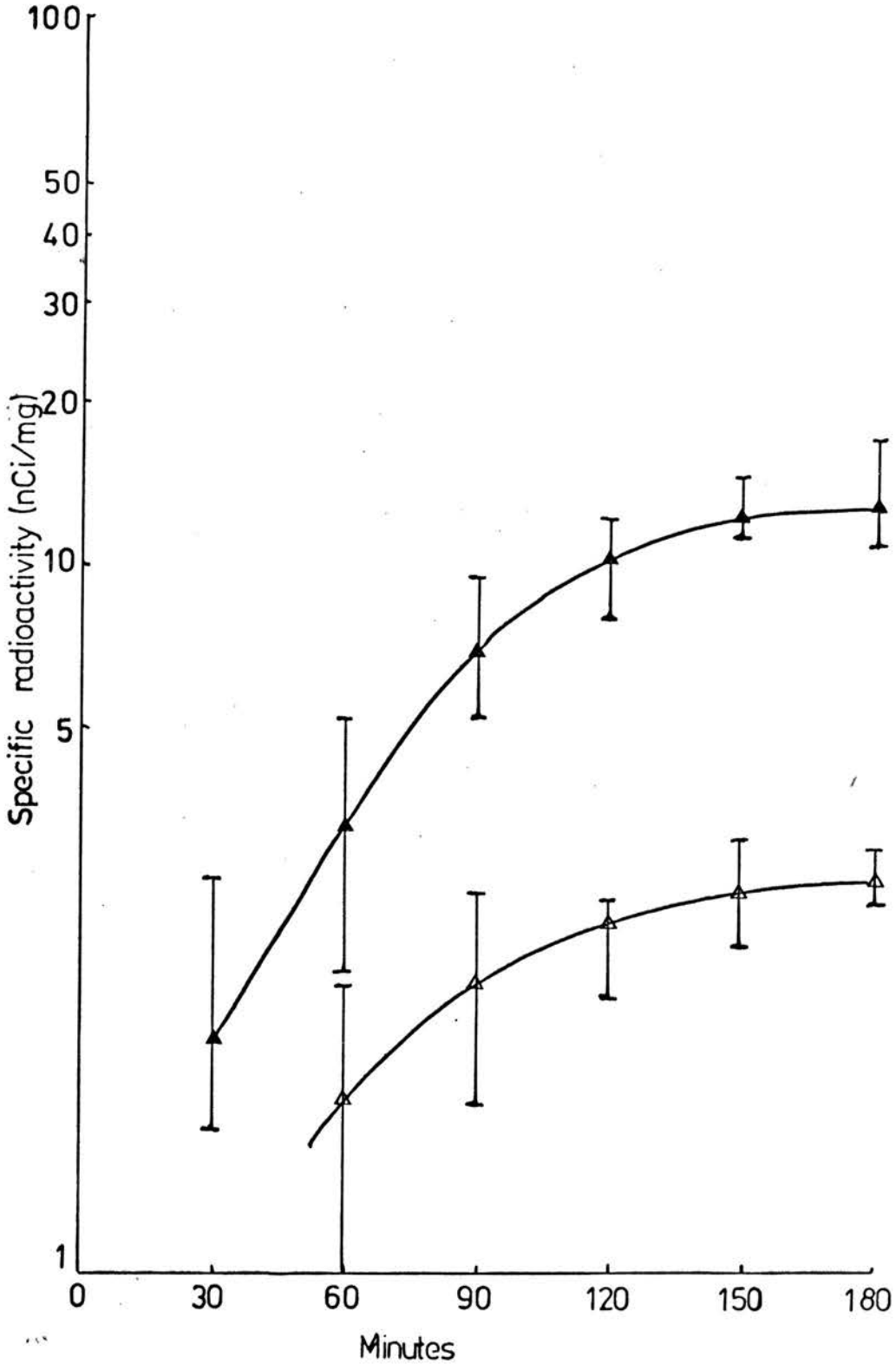


Fig.47. Changes in the specific radioactivity of esterified pla cholesterol labelled with carbon-14(▲) or tritium(△) during perfusion of livers taken from normal rats.

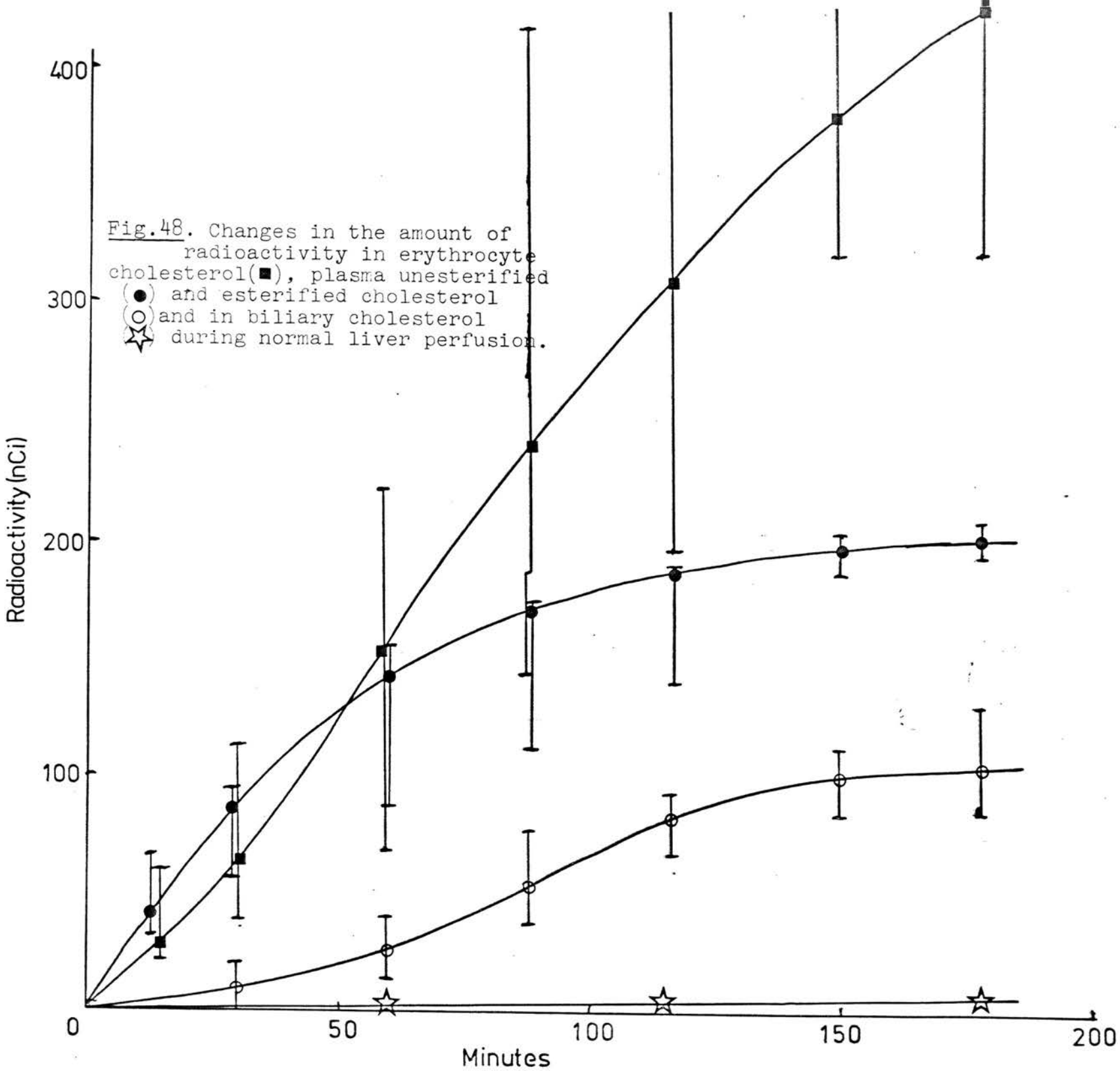


Fig.48. Changes in the amount of radioactivity in erythrocyte cholesterol(■), plasma unesterified (●) and esterified cholesterol (○) and in biliary cholesterol (☆) during normal liver perfusion.

modified curve shapes if compared to the in vivo situation.

In this study it was possible to take account of this criticism because the changes in compartment size could be calculated by making allowance for the sample being withdrawn. For example, with an initial charge of blood perfusate of 50ml and a sample volume of 1.5ml it was possible to calculate the amount of cholesterol remaining in any cholesterol compartment associated with the blood. For 10 - 12 samples this produced a steadily falling amount of cholesterol in the perfusate. The reduced compartment size at each time point was multiplied by the specific radioactivity of the cholesterol in that compartment at that time and these are the data presented in Fig. 48.

With these adjusted data, simulation was performed using the six compartment program described in the methods section (p.71). In the first instance it was assumed that all of the (^{14}C) label entered the liver and was incorporated into the microsomal cholesterol in zero time, and that in effect all of the labelled cholesterol was in the liver unesterified cholesterol compartment at zero time. This was simulated by making the initial condition for the liver unesterified cholesterol compartment = 1 Machine unit. This means that the full voltage of the computer was applied to the liver unesterified cholesterol compartment and was considered to be equivalent to 2uCi of radioactive tracer.

The values for k_{52}, k_{25}, k_{32}, k_{23} (Fig. 41) which had been determined for exchange of cholesterol between plasma and erythrocytes were substituted onto the appropriate potentiometers of the six compartment analogue program (Fig. 30). Initial values for the remaining rate constants were estimated by considering the

relative sizes of the compartments between which the rate constants were unknown. The simulated curves for each of the four compartments sampled were output from the model program onto an x - y recorder with the time axis scaled to give 1min per graph division. The initial output was examined and logical changes made in the estimated rate constants to give a better fit to the experimental data. Examples of the output yielding progressively better 'eye-fit' to the experimental data are shown in Figs. 49, 50 and 51. The final simulated curves accepted are shown in Fig. 52 this being the best 'eye-fit' that could be obtained after approximately forty simulated experiments.

The rate constant values ascribed to the complete model are shown in Fig. 53. Some modification of rate constants k_{52} and k_{25} was necessary to accommodate the model to the data. Simulated curves for the compartments not accessible to experimental sampling, i.e. the liver esterified and unesterified compartments are shown in Fig. 54. The only experimental data points for this plot are the termination of the perfusion and are indicated on the graph.

Using the mean observed pool sizes for each compartment in association with the estimated rate constants the absolute rate of transfer of cholesterol between compartments was calculated (Table 12).

Thus although the rate of transfer of cholesterol from erythrocytes to the plasma unesterified cholesterol was greatest, the most active pool is the plasma unesterified cholesterol which turns over with the erythrocyte compartment every 33min. Conversely, the plasma unesterified compartment viz-a-viz the plasma esterified compartment turns over very slowly, i.e. once in every 1000min.

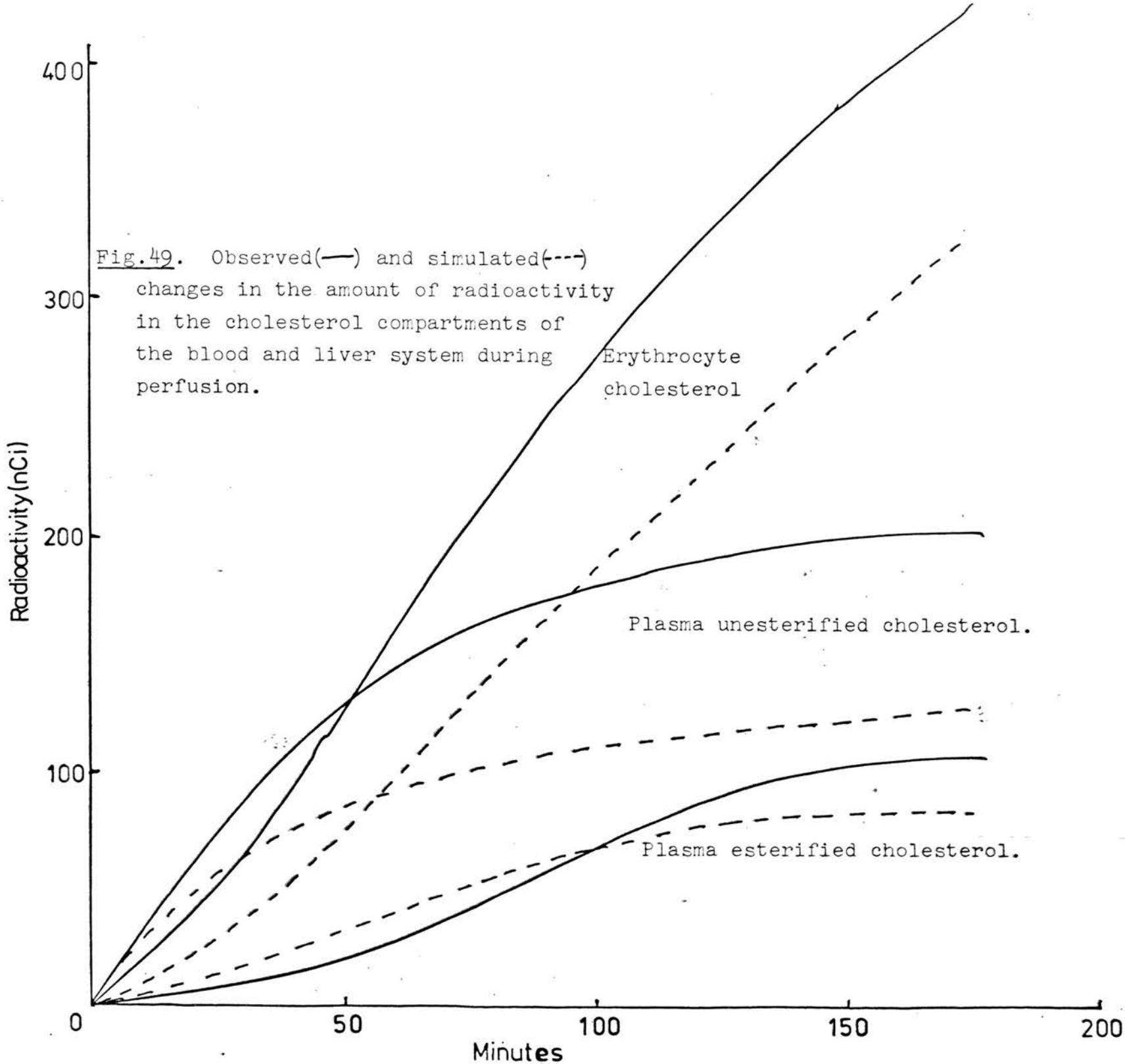


Fig.49. Observed(—) and simulated(---) changes in the amount of radioactivity in the cholesterol compartments of the blood and liver system during perfusion.

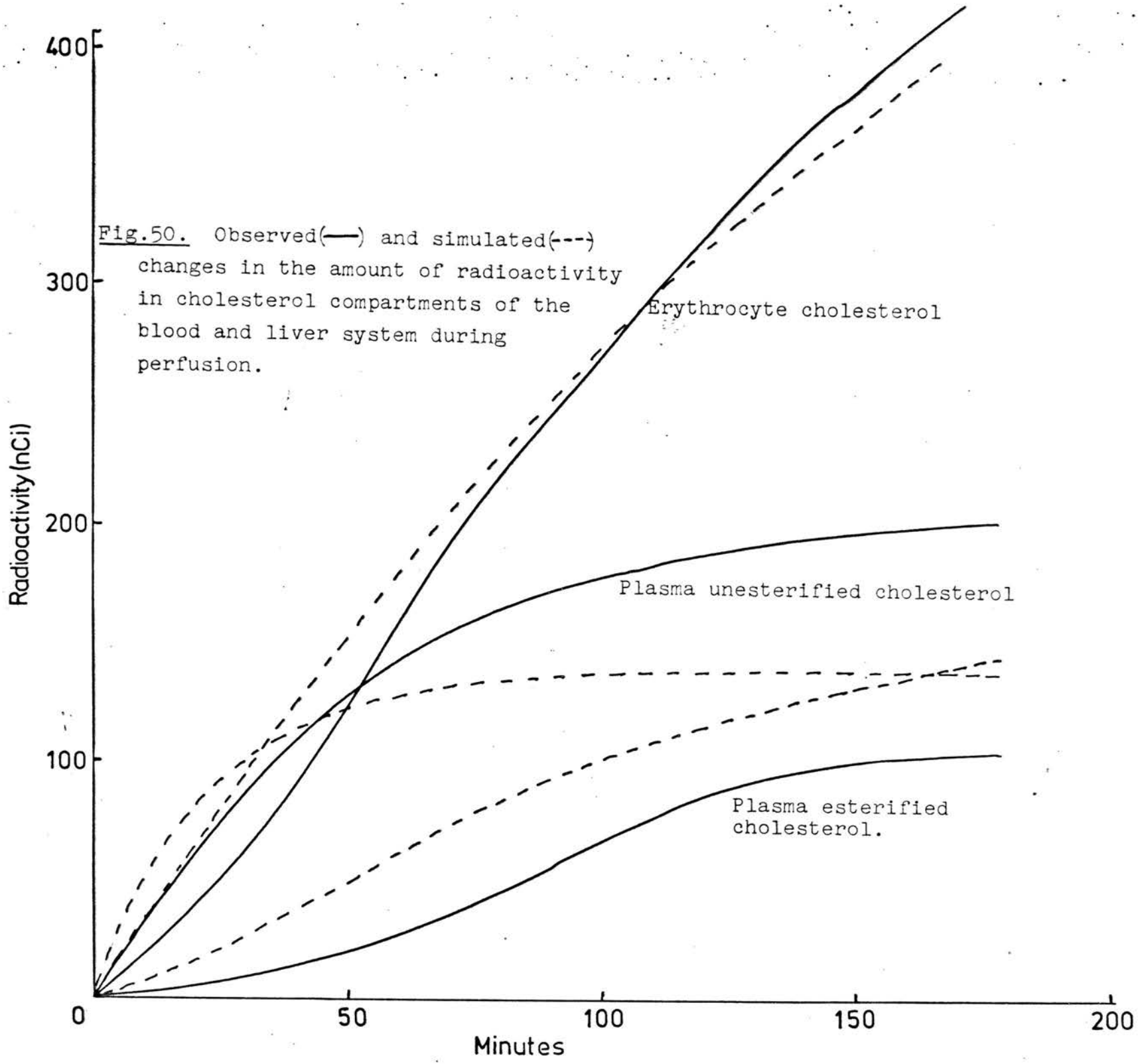


Fig.50. Observed(——) and simulated(---) changes in the amount of radioactivity in cholesterol compartments of the blood and liver system during perfusion.

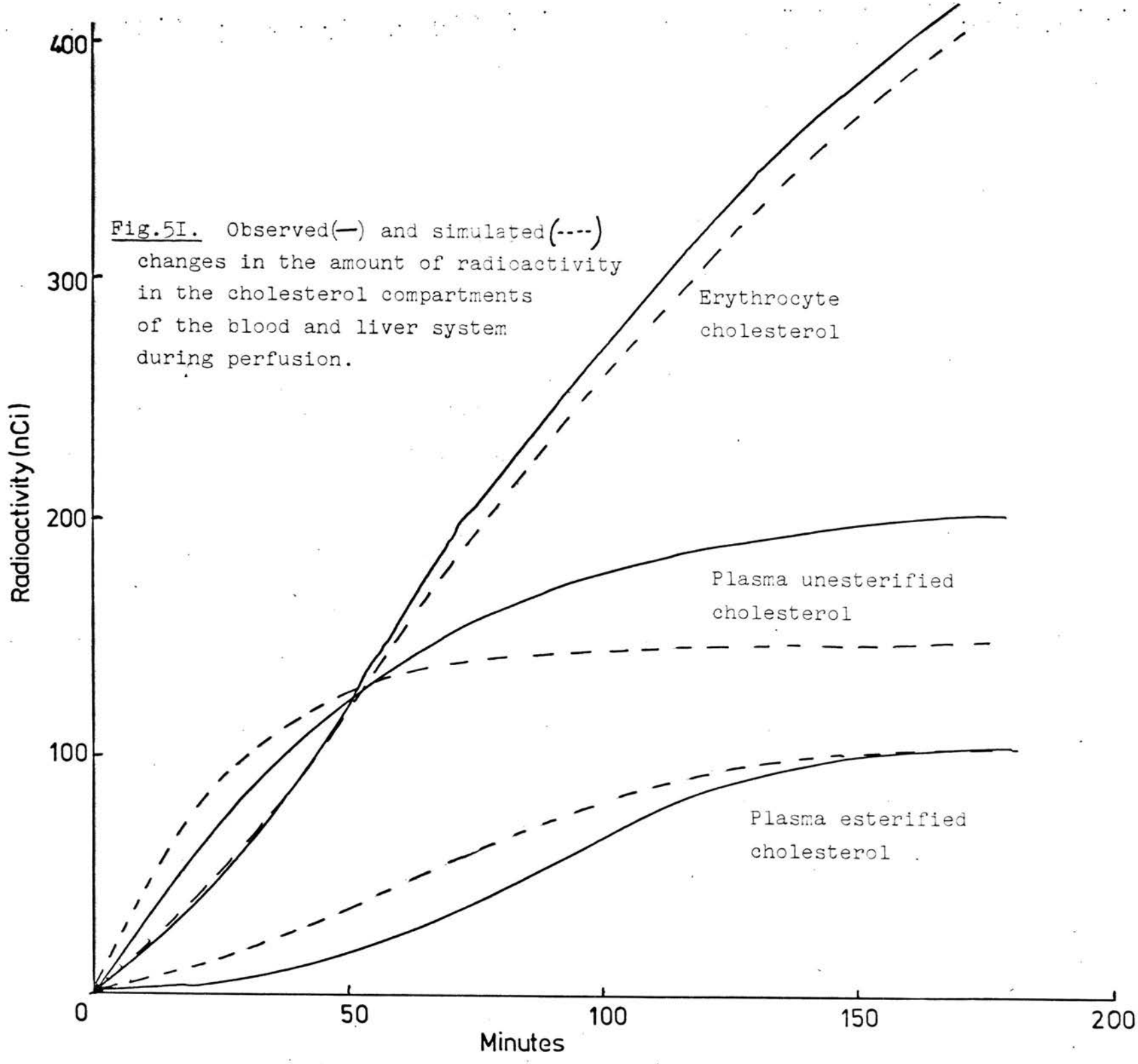


Fig.5I. Observed(—) and simulated(----) changes in the amount of radioactivity in the cholesterol compartments of the blood and liver system during perfusion.

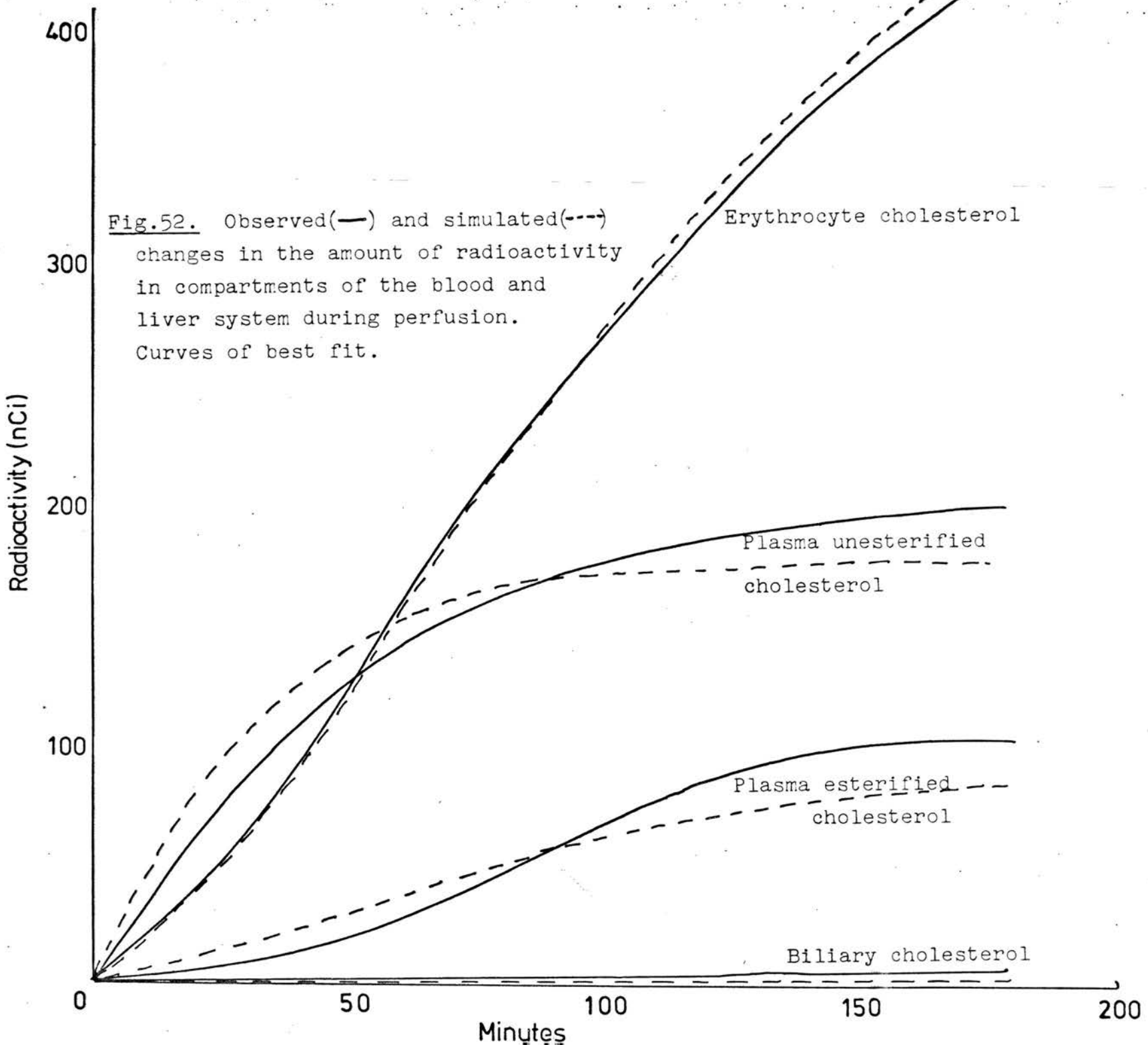


Fig.52. Observed(—) and simulated(---) changes in the amount of radioactivity in compartments of the blood and liver system during perfusion. Curves of best fit.

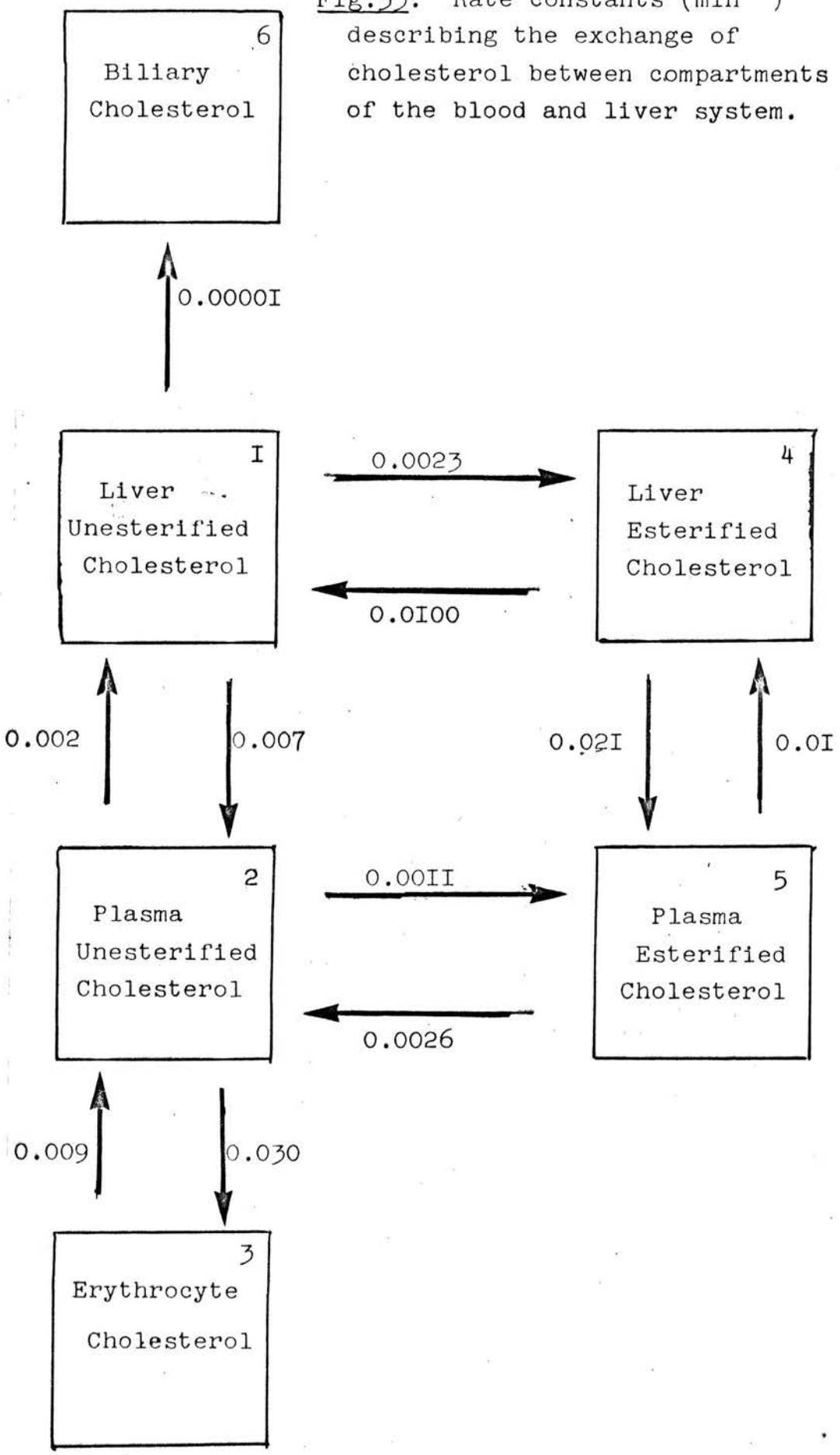


Fig.53. Rate constants (min^{-I}) describing the exchange of cholesterol between compartments of the blood and liver system.

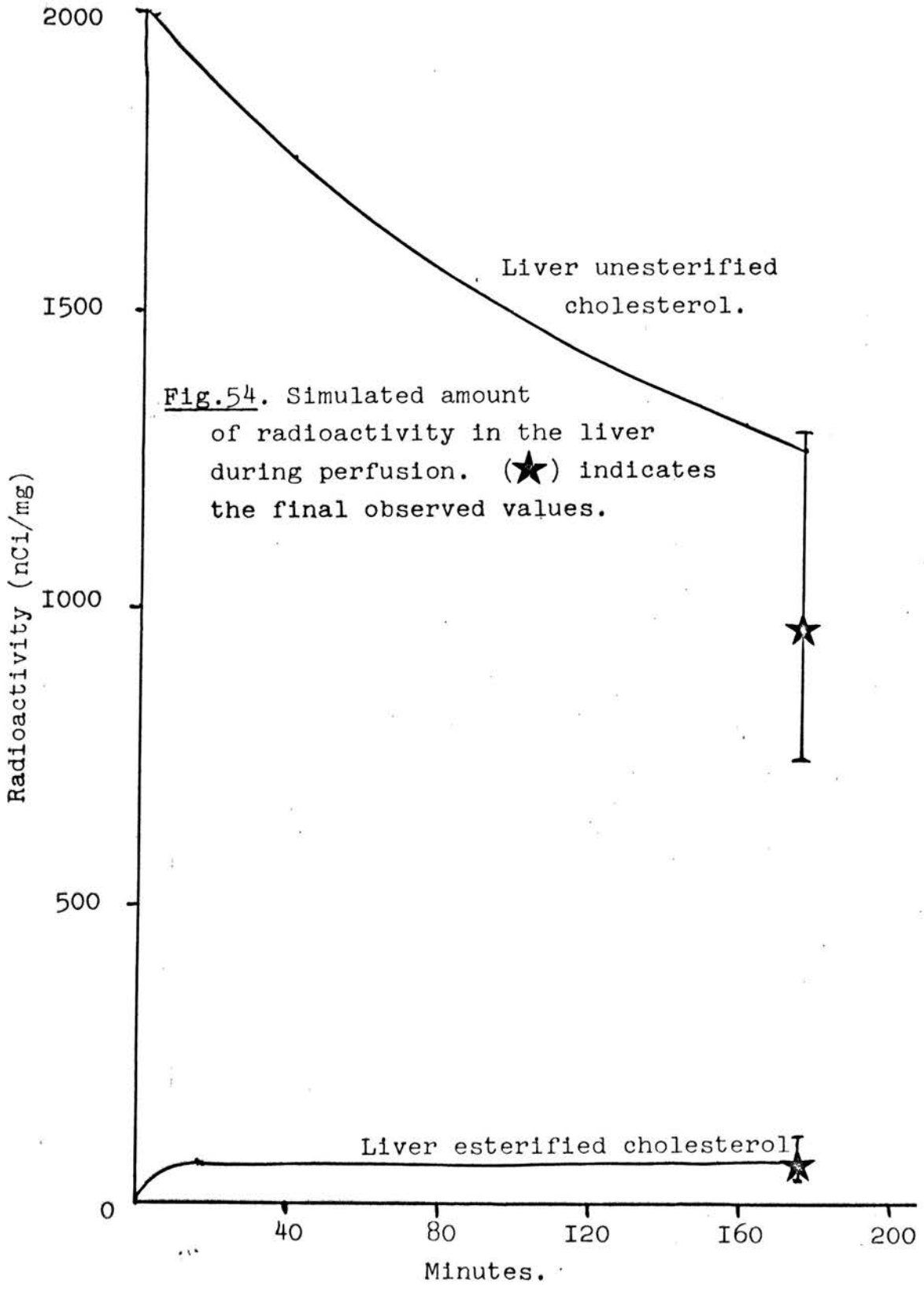


Fig.54. Simulated amount of radioactivity in the liver during perfusion. (★) indicates the final observed values.

k	Rate constant (min^{-m})	Turnover time ($1/min^{-1}$)	Pool size (mg)	Transfer rate mg/min
I2	0.002	500	4.2	0.0084
21	0.007	142	11.1	0.0776
41	0.0027	434	11.1	0.0255
14	0.010	100	1.1	0.0110
54	0.021	47.6	1.1	0.0229
45	0.010	100	7.6	0.0760
52	0.001	1000	4.2	0.0042
25	0.0026	384	7.6	0.0197
23	0.009	111	18.7	0.1685
32	0.030	33	4.2	0.1275
61	0.000001	-	11.1	0.0011

Table 12

The rate constants, turnover times, pool sizes and actual rate of transfer of cholesterol between compartments in the blood and liver system.

Simulation using model.

The model was used to carry out two simulated experiments.

1. A simulation of biliary obstruction was imposed upon the model by removing the integrator circuits associated with the bile compartment (6). In effect this prevented voltage accumulation in compartment 6 and therefore redistributes this voltage throughout the remaining compartments. Thus the model simulates the in vivo situation of biliary obstruction.

This simulation was performed to investigate whether the bile compartment and biliary component of the specific radioactivity curves of the other compartments could be ignored in the model used. This assumption is only valid for the in vivo situation if an obstructive element in liver function would not affect the anatomical relationship between hepatocyte and perfusing blood. The simulated curves are shown in Fig. 55 and it is evident that little change is effected.

2. Several simulated experiments were carried out to investigate the effect of increasing the rate constant k_{61}, i.e. the rate constant controlling transfer from the liver unesterified cholesterol compartment into the bile. The rationale for this simulation was that because the major excretory pathway for cholesterol is by degradation to bile acids, then the rate constant for the elimination of cholesterol alone should be increased to allow for the total excretion of the steroid ring structure in the form of both cholesterol and bile acids. Percy-Robb (1970) showed that the rate of bile acid excretion from perfused rat liver was 125μg/h. The excretory rate for cholesterol is about 45μg/h as judged by the amount of cholesterol

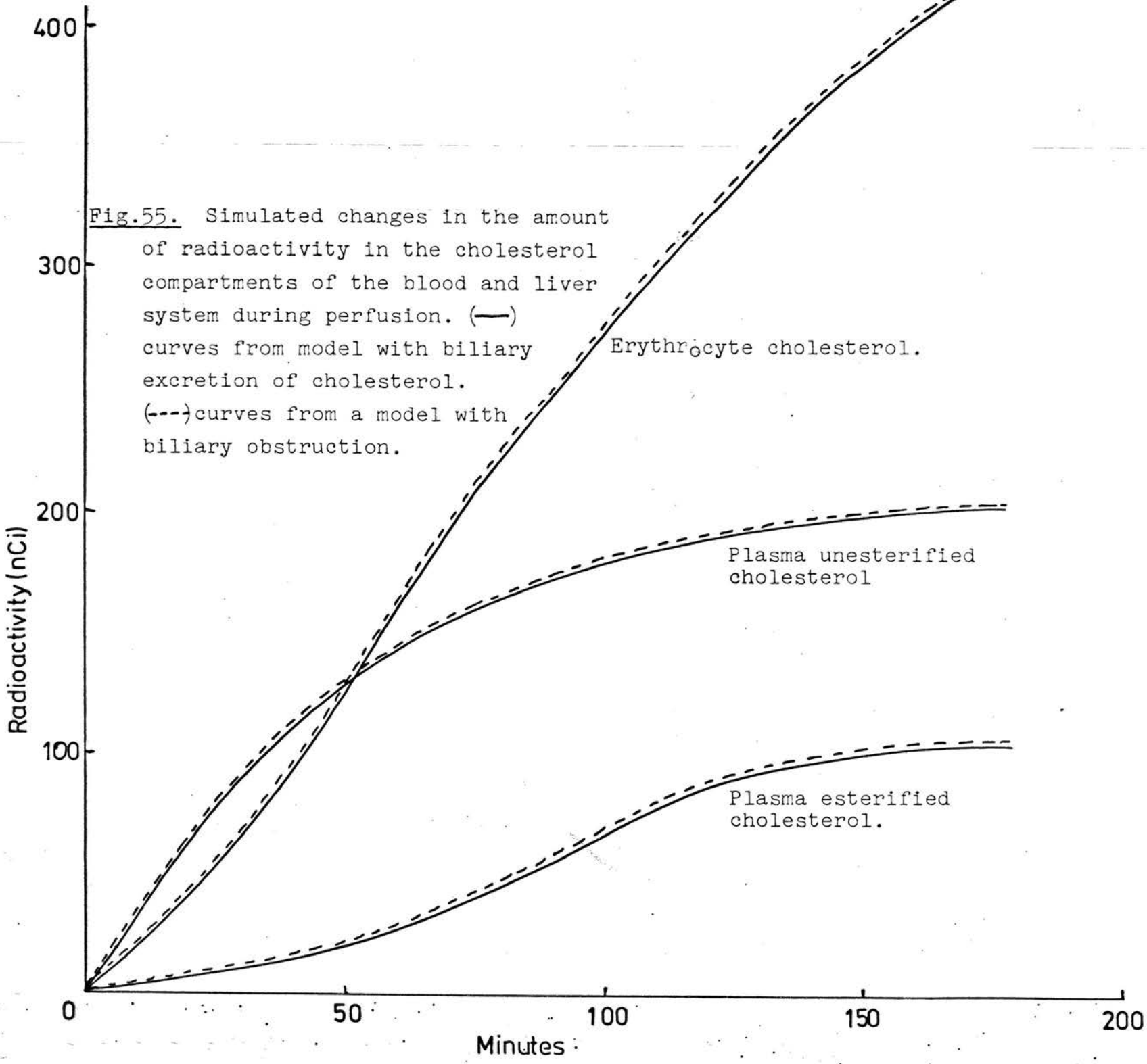


Fig.55. Simulated changes in the amount of radioactivity in the cholesterol compartments of the blood and liver system during perfusion. (——) curves from model with biliary excretion of cholesterol. (---) curves from a model with biliary obstruction.

found in the bile formed during perfusion of livers from normal rats. To allow for the excretion of bile acids the rate constant k_{61} should be increased by a factor of three.

The simulated curves for all of the compartments except the biliary compartment were not significantly changed when the rate constant k_{61} was altered by a factor of three (Fig. 56).

The apparent stability of the curves in this simulation may well be modified if the model took account of the feed-back effect of bile acids on cholesterol synthesis. That is, the increased excretion of cholesterol, as represented by the increased rate constant k_{61}, is really in the form of bile acids and, according to the model of Weiss and Dietschy (1969), this would induce an increased intestinal absorption of cholesterol and hence reduce hepatic cholesterol synthesis. No account of this effect had been taken in this simulation and the curves obtained may not be valid.

Modifications to the model.

In the model simulated above it was assumed that all of the label was in compartment 1 (liver unesterified cholesterol) at zero time. This is an oversimplification because all of the labelled mevalonic acid precursor could not be technically presented to the hepatocyte at zero time. Ellwood and Van Bruggen (1961) showed that all of the biologically active (^{14}C) mevalonic acid was utilised within 30min of an intra-peritoneal injection. Their studies were, however, in whole animals and it seems reasonable to expect a longer time period for

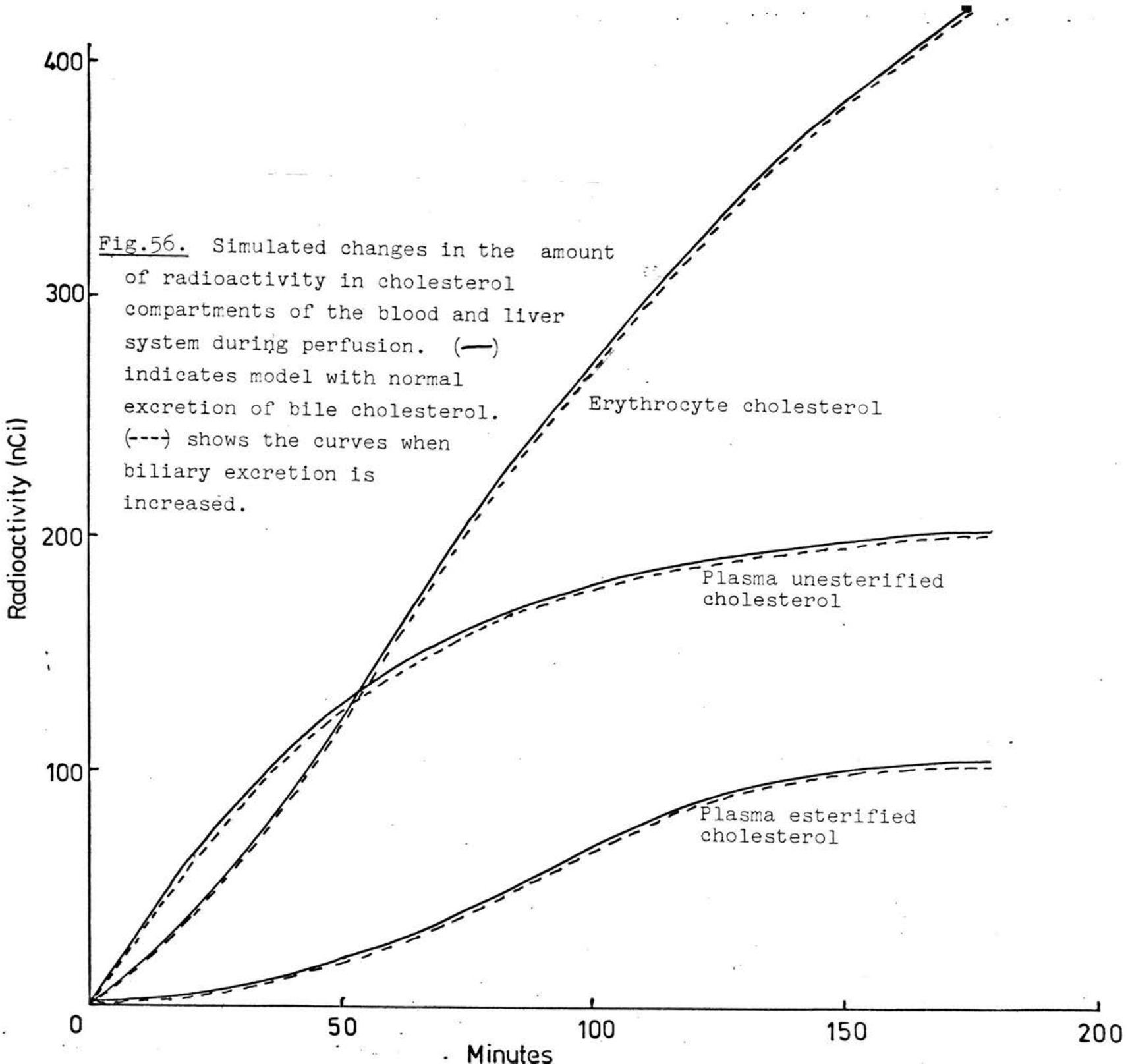


Fig.56. Simulated changes in the amount of radioactivity in cholesterol compartments of the blood and liver system during perfusion. (—) indicates model with normal excretion of bile cholesterol. (---) shows the curves when biliary excretion is increased.

mevalonate utilisation in the isolated liver perfusion. If one assumes that the transfer of mevalonic acid into the hepatocyte is governed by first order kinetics, and is not subject to enhanced uptake by a 'pump' mechanism, then provision can be made in the model for the progressive transfer into compartment 1 of the label from another external compartment representing the blood mevalonic acid pool.

Because of insufficient computational units, this additional compartment was introduced at the expense of simulating a bile compartment, since it has been shown previously (p88) that this would not seriously affect the overall model. The integrator previously representing the bile compartment was substituted into the main analogue program as illustrated in Fig. 57. This additional program sub-unit generated the function $e^{-k_m t}$ and the value of k_m was chosen such that $f(x) \longmapsto 0$ at 180min. The value of k_m was calculated to allow a residual radioactivity of 1% to remain in the blood at 180min and gave a value of 0.255.

Examples of the simulated decay curves in the external mevalonic acid compartment are shown in Fig. 58. Thus a value of 0.25 for k_m allows the label to transfer exponentially from the external mevalonate pool into the liver and therefore simulates a progressive utilisation of mevalonate by the liver. The value of 0.25 was chosen because radioactive recovery was approximately 95% of the tracer in the biologically active precursor. This means that the value of k_m would have to be equal to or greater than 0.25 (Fig. 58).

Retaining the previously determined rate constants and with the initial condition of compartment 1 (liver unesterified cholesterol) set at zero, simulated curves were generated with the additional mevalonate compartment

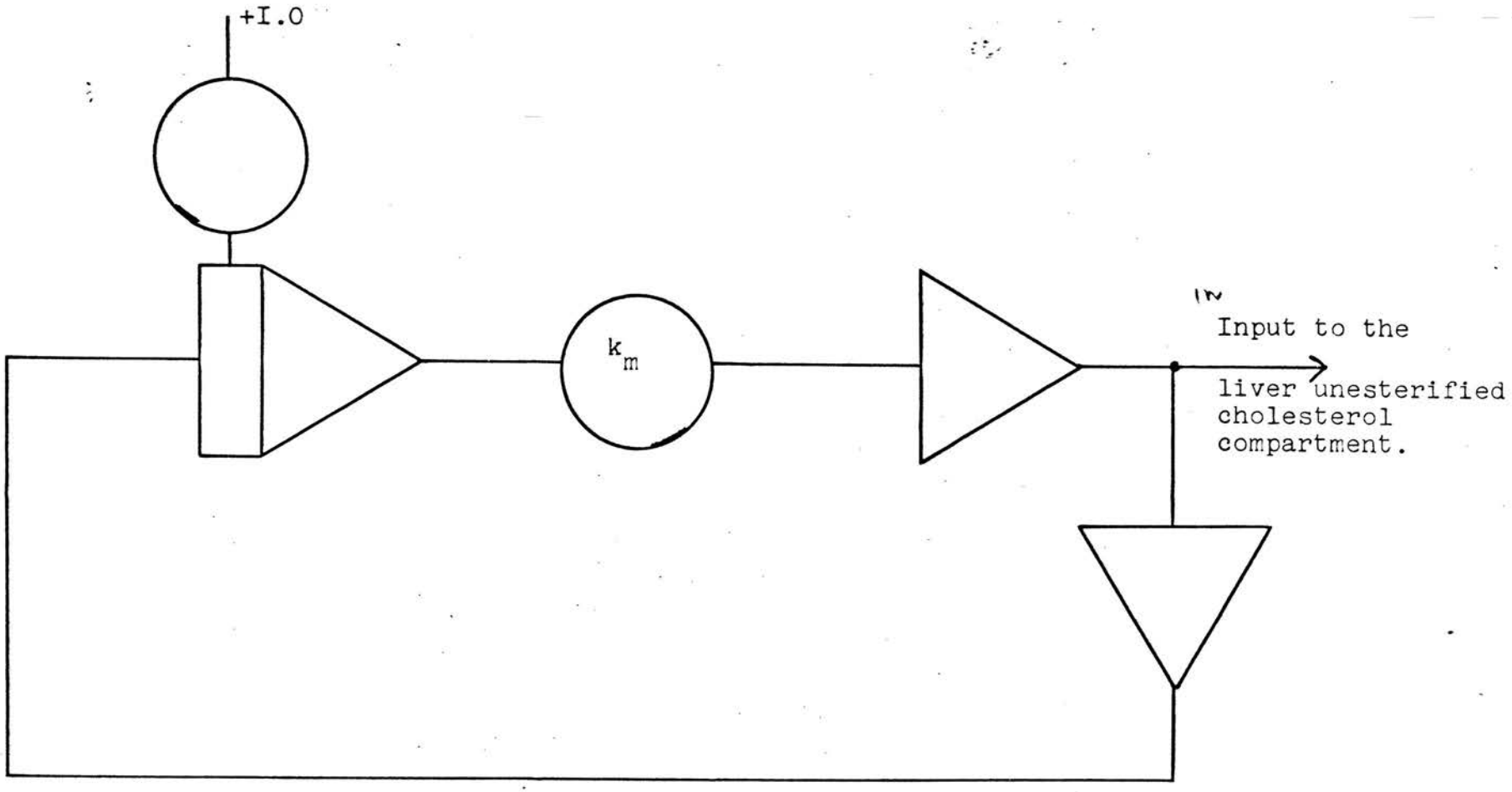


Fig.57. Analogue program to represent an external compartment of mevalonic acid in the main analogue program(see Fig.30).k_m is the rate constant for the transfer of mevalonate into the hepatocyte.

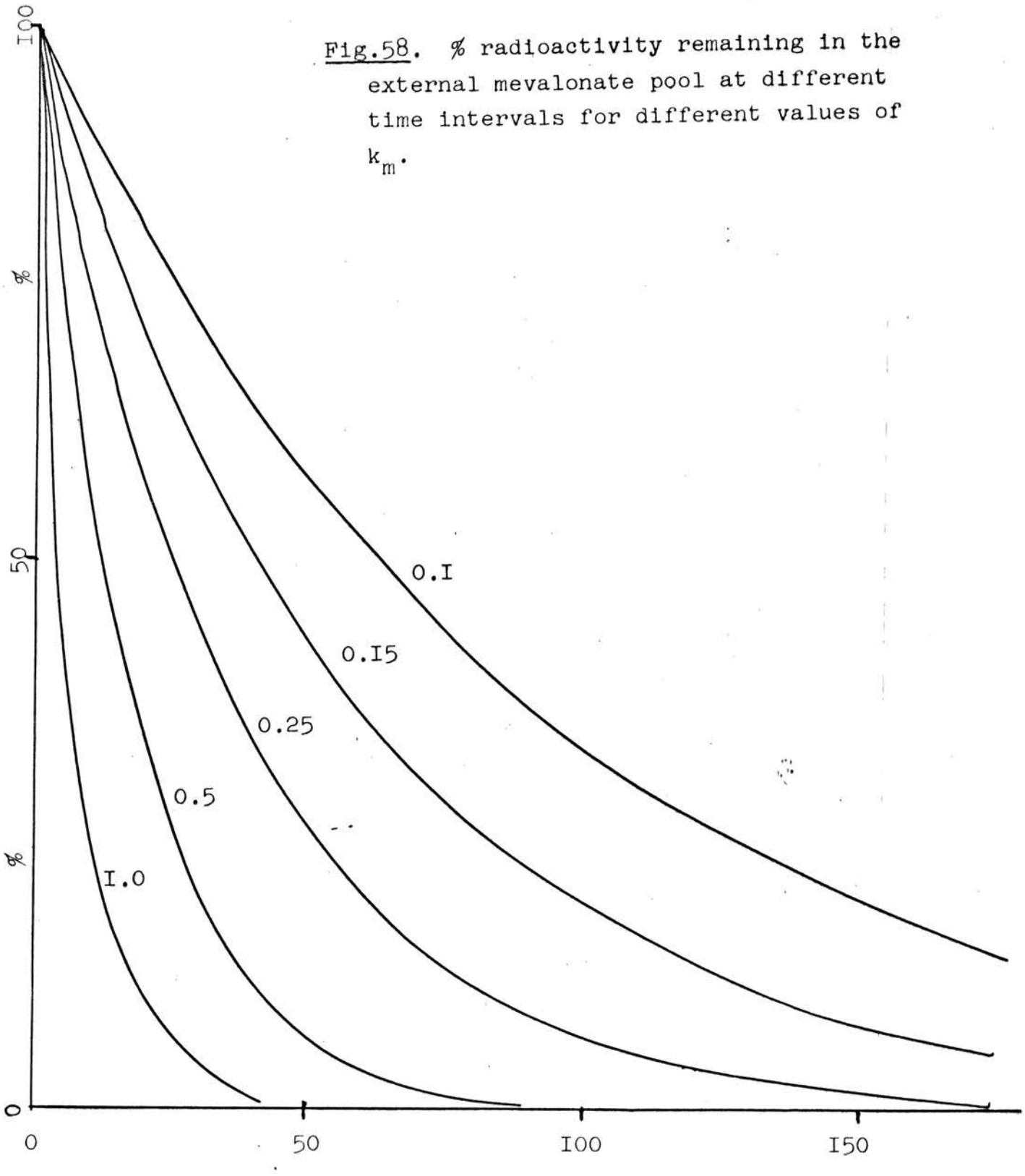


Fig.58. % radioactivity remaining in the external mevalonate pool at different time intervals for different values of k_m.

functional (Fig. 59). These simulated curves were clearly unsuitable to fit the experimental data of Fig. 48 and a modification of the rate constants was needed.

The computing procedure outlined on pages was repeated to obtain a new set of curves which would again approximate to the experimental data (Fig. 48). The new rate constants giving a best visual fit of the experimental and simulated data are shown in Fig. 60.

Using the model with an external mevalonate compartment a better fit of the simulated and experimental curves could be obtained than for the more simple model.

Thus the sum of the residual squares for both models was:-

	Sum of Residual Squares
Model without mevalonate pool	47824
Model with mevalonate pool	18792

Recalculation of the absolute rates of cholesterol transfer between compartments using the modified rate constants (Fig. 60) are shown in Table 13.

Reliability of model parameters.

One of the drawbacks of analogue computing is that it is not possible to obtain estimates of the variance of the rate constants determined for compartmented models of the type used in this study. The best way to obtain some indication of the reliability of the values determined is to alter each one in turn by a fixed percentage and then to generate curves appropriate to the whole model. In cases where major perturbations of the model

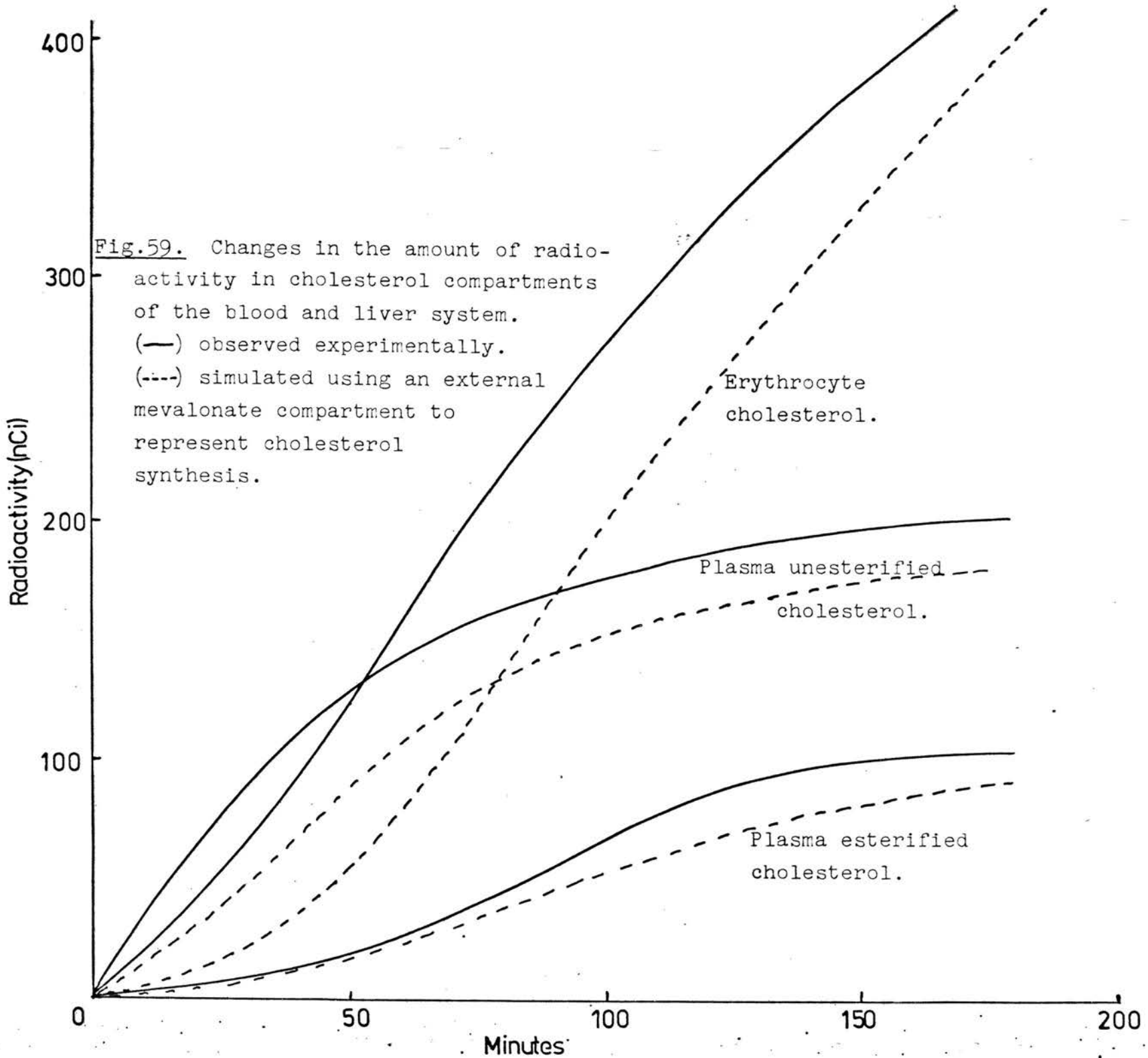


Fig.59. Changes in the amount of radioactivity in cholesterol compartments of the blood and liver system.
(—) observed experimentally.
(----) simulated using an external mevalonate compartment to represent cholesterol synthesis.

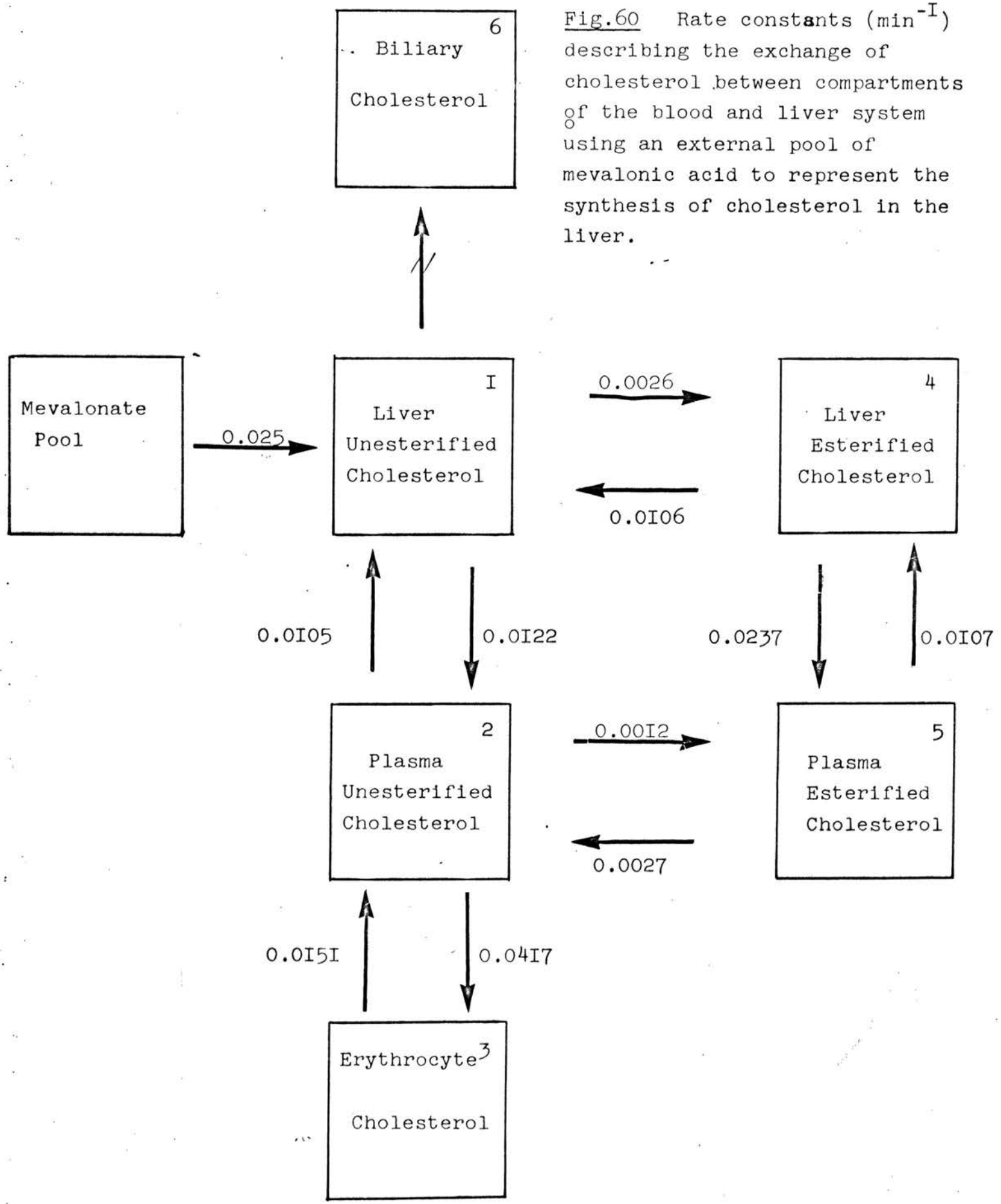


Fig.60 Rate constants (min^{-1}) describing the exchange of cholesterol between compartments of the blood and liver system using an external pool of mevalonic acid to represent the synthesis of cholesterol in the liver.

k	Rate constant (min^{-m})	Turnover time ($1/min^{-1}$)	Pool size (mg)	Transfer rate mg/min
12	0.0105	95	4.2	0.044
21	0.0122	82	11.1	0.135
41	0.0026	384	11.1	0.029
14	0.0106	94	1.1	0.012
54	0.0237	42	1.1	0.026
45	0.0107	93	7.6	0.082
52	0.0012	833	4.2	0.005
25	0.0027	370	7.6	0.021
23	0.0151	66	18.7	0.283
32	0.0417	24	4.2	0.462
61	-	-	-	-

Table 13

The rate constants, turnover times, pool sizes and actual rate of transfer of cholesterol between compartments in the blood and liver system when using an additional compartment to represent mevalonic acid.

occur, then the value of the rate constant can be said to be reliable at ± the percentage alteration used. Conversely, if little change is noted in the generated curves then the percentage limits used are insufficient to define parameter reliability. For those parameters which show little change in the model curves following modification, the variance is increased by fixed percentages until significant changes are noted in the model.

This procedure was applied to the more complex model using the external mevalonate pool. For three rate constants, k_{21}, k_{23}, and k_{32} changes of ± 10% from the nominal value caused marked deviations from the simulated curves for plasma unesterified and erythrocyte cholesterol. An example is shown in Fig. 61 for rate constant k_{21}.

The nominal values for the remaining rate constants (k_{41}, k_{12}, k_{54}, k_{14}, k_{45}, k_{52} and k_{25}) were then changed sequentially by ± 50% and the output of the model program again examined. In this case rate constants k_{21}, k_{23}, k_{32}, k_{41}, k_{12} and k_{54} showed significant changes in the generated curves when compared to those obtained using the nominal values. The changes for rate constant k_{12} at ± 10% and ± 50% are shown in Figs. 62 and 63. Table 14 shows the ranges suggested for the values of the different rate constants, based upon the changes obtained in the simulated curves using these limits.

Rate of equilibration of cholesterol between liver and blood.

The rate at which unesterified and esterified cholesterol in the blood equilibrates with that in the liver is shown in Fig. 64 for the (^{14}C) precursor and in Fig.65 for the (^{3}H) precursor. The graphs were obtained by calculating the relative percentage proportion of the blood cholesterol specific radioactivity to that of the

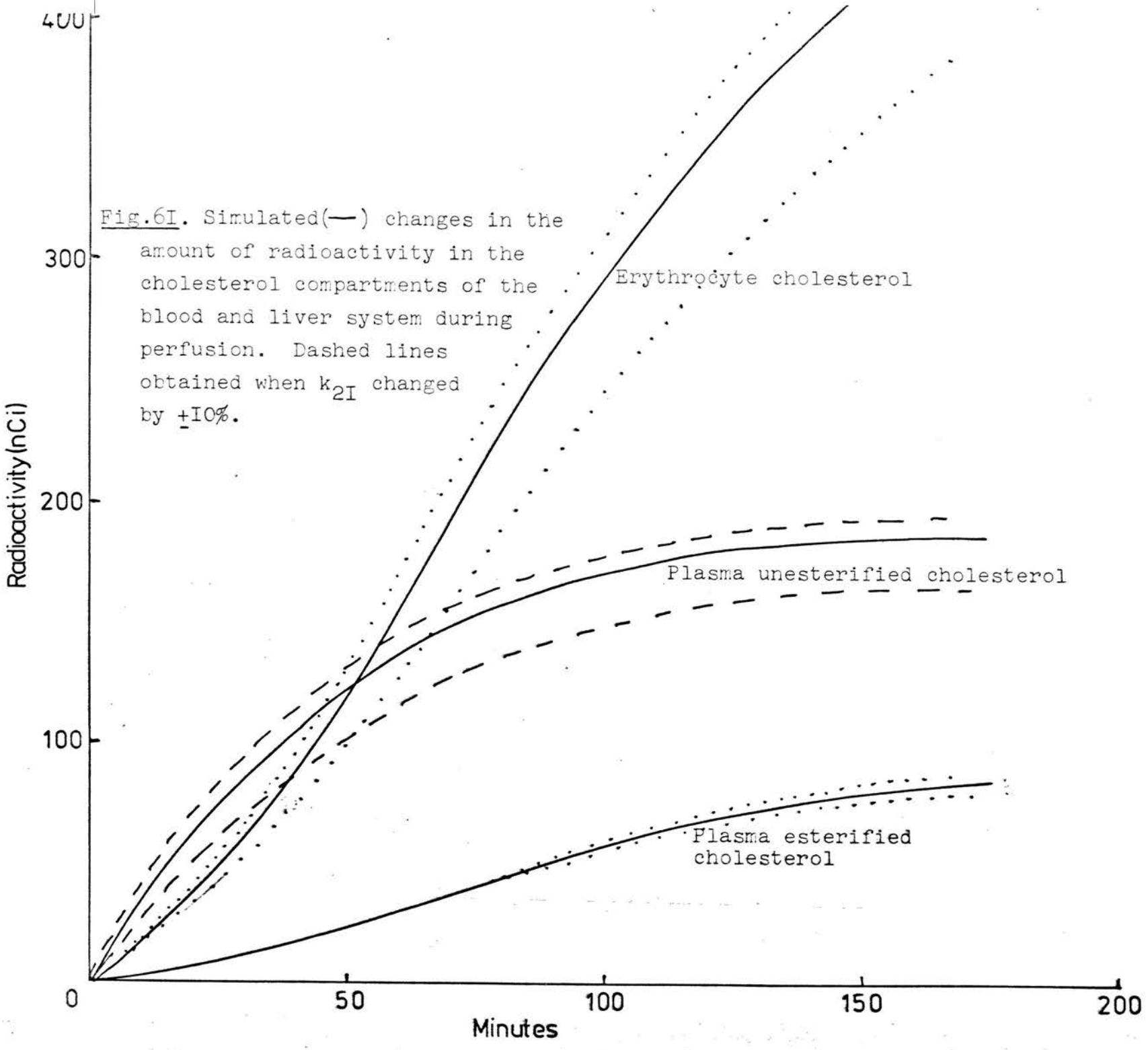


Fig.6I. Simulated(—) changes in the amount of radioactivity in the cholesterol compartments of the blood and liver system during perfusion. Dashed lines obtained when k_{2I} changed by ±10%.

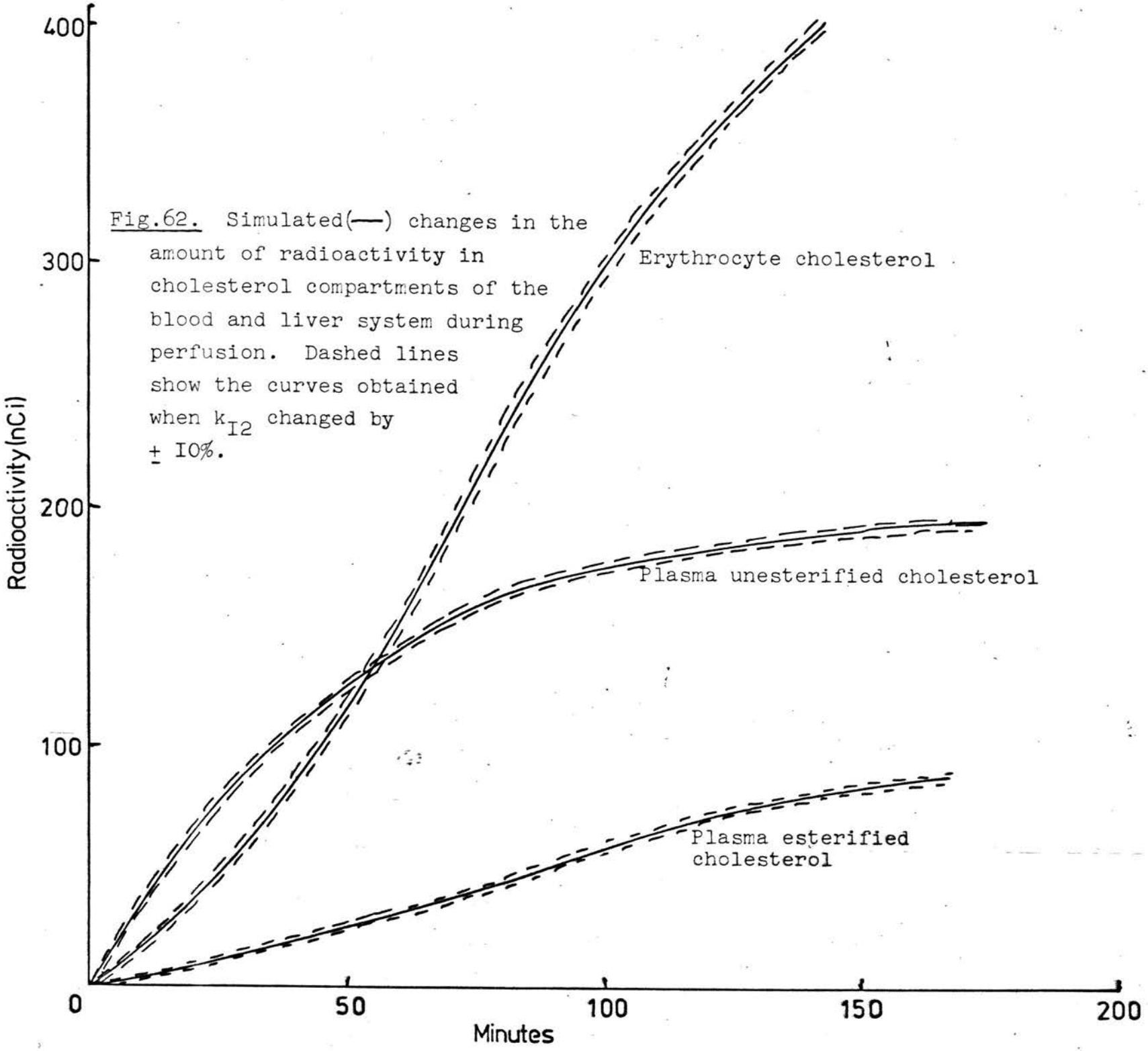


Fig.62. Simulated(—) changes in the amount of radioactivity in cholesterol compartments of the blood and liver system during perfusion. Dashed lines show the curves obtained when k_{I2} changed by ± IO%.

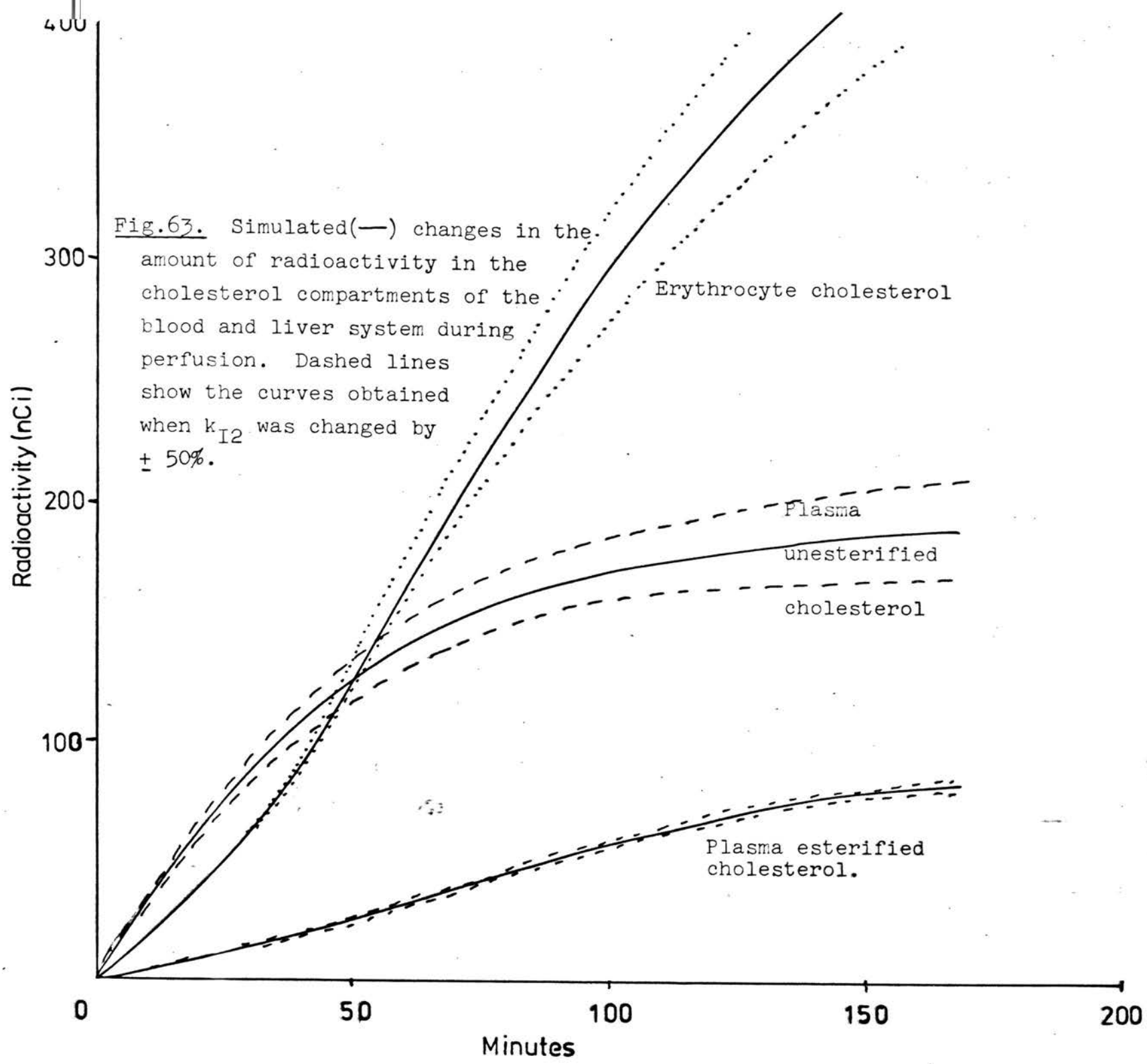


Fig.63. Simulated(—) changes in the amount of radioactivity in the cholesterol compartments of the blood and liver system during perfusion. Dashed lines show the curves obtained when k_{I2} was changed by ± 50%.

Rate constant	Nominal value	Range
k_{21}	0.0122	0.0110 -- 0.0134
k_{23}	0.01514	0.01363 - 0.01665
k_{32}	0.04170	0.03753 - 0.04587
k_{41}	0.00258	0.00129 - 0.00387
k_{12}	0.01054	0.00527 - 0.01581
k_{54}	0.02372	0.01186 - 0.03558
k_{14}	0.01062	Variation greater than $\pm$ 50%
k_{45}	0.01072	
k_{52}	0.00122	
k_{25}	0.00276	

Table 14

Nominal values and suggested ranges for the rate constants describing the exchange of cholesterol between compartments of the blood and liver system.

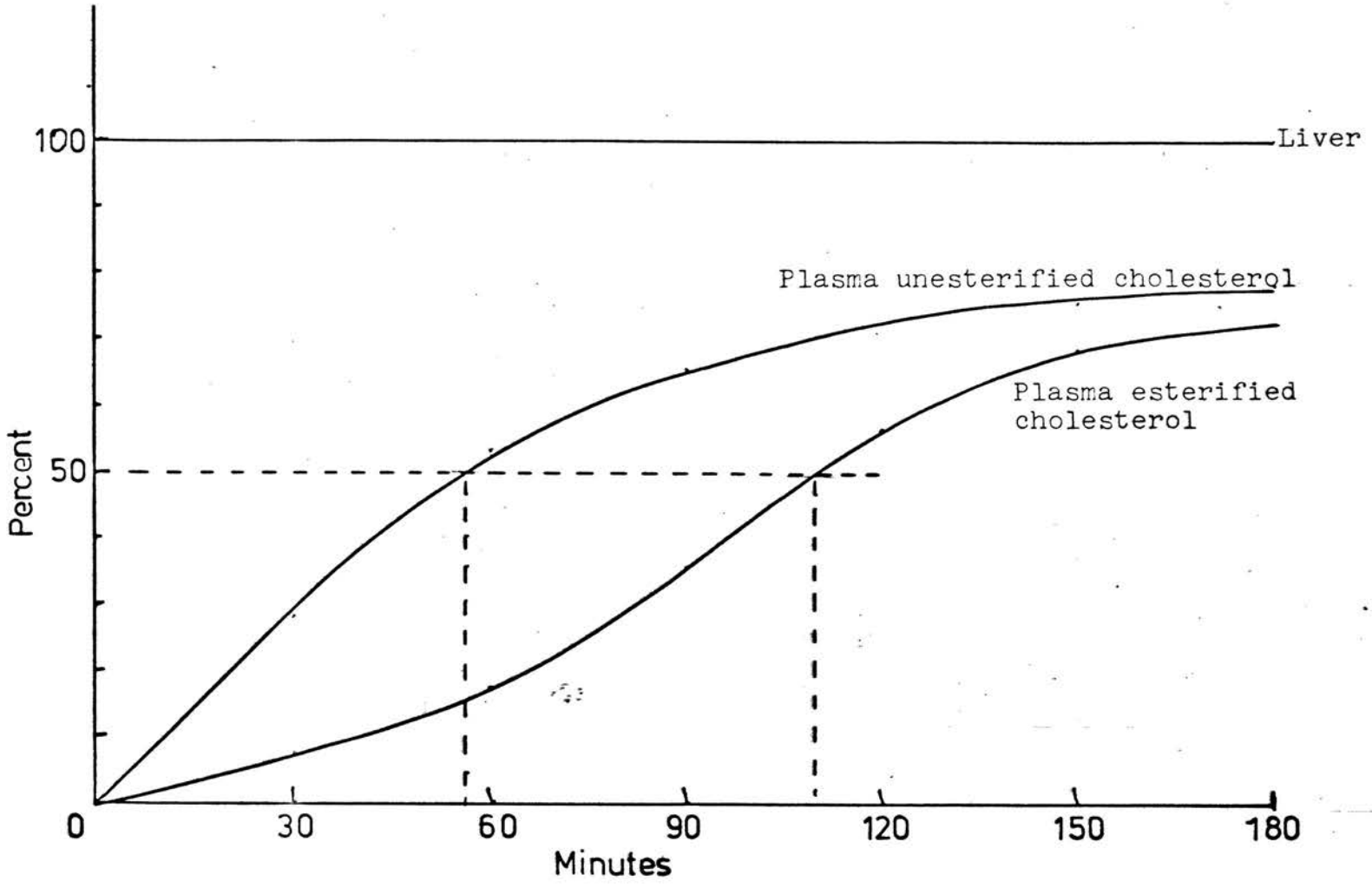


Fig.64. Percentage proportion of the specific radioactivity of plasma (^{14}C)cholesterol to liver (^{14}C)cholesterol during perfusion.

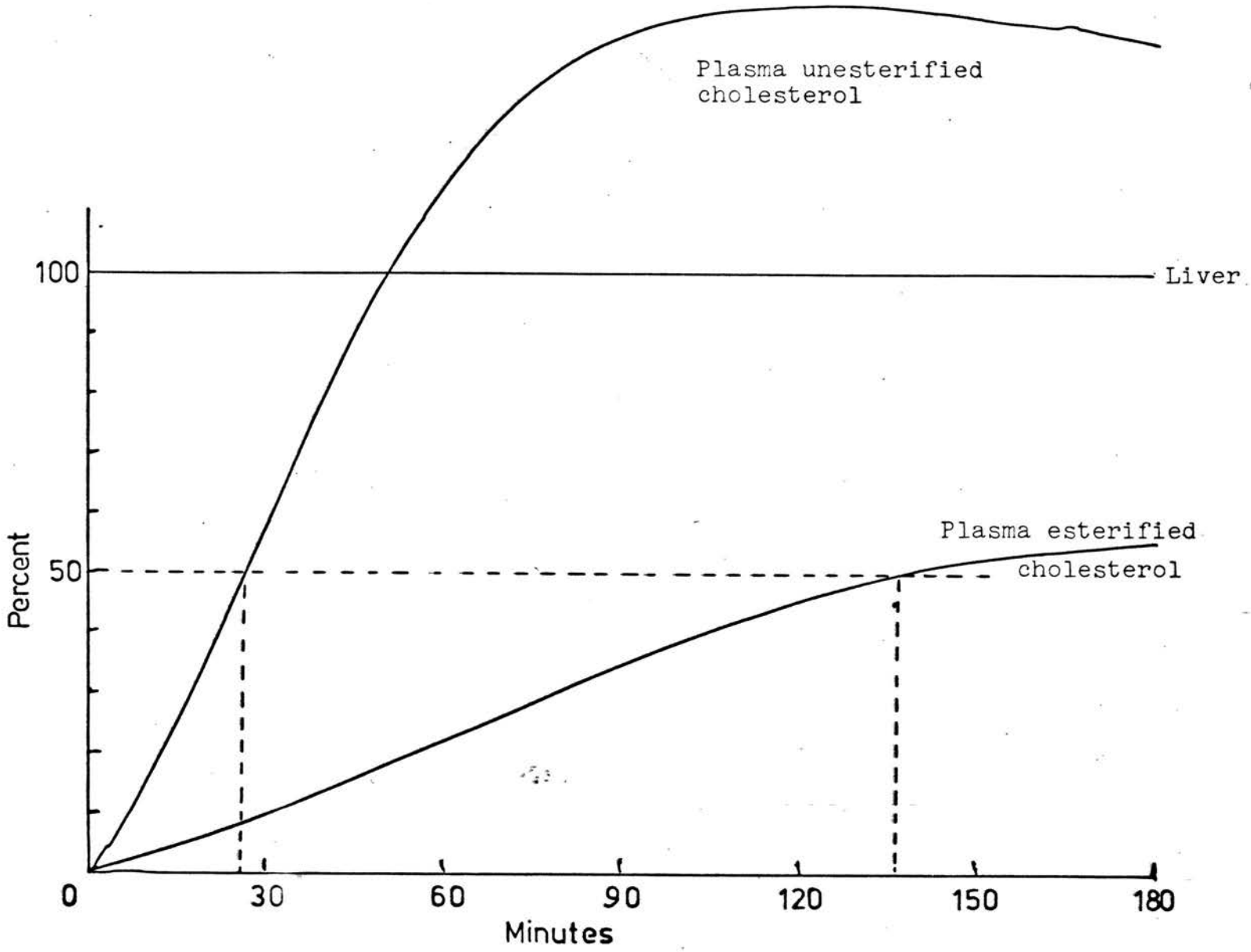


Fig.65. Percentage proportion of the specific radioactivity of plasma (^{3}H) cholesterol to liver (^{3}H)cholesterol during perfusion.

cholesterol in the liver compartment at the end of the perfusion. Table 15 shows the mean specific radioactivty of cholesterol in the liver at the end of the perfusion. The curves obtained are not exactly of these shapes, particularly in their earlier portions, because the relative percentages at times less than 150min would be lower than indicated. This is because the specific radioactivity of the liver compartments was not known for each time point, i.e. the liver was not sampled, and the relative percentages are based upon the terminal specific radioactivity of the liver compartments. The graphs become more correct at the later stages of the perfusion because the specific radioactivity values are converging. Equilibration of (^{14}C) cholesterol in esterified or unesterified form was not achieved with their counterparts in the liver during the perfusion times used in this study. The plot for the unesterified cholesterol in erythrocytes is not shown because equilibration should be mediated via the plasma compartment, no evidence being available that cholesterol in erythrocytes exchanges directly with the cholesterol in the liver compartment.

An interesting feature of these data was the rapid equilibration of tritiated cholesterol between the plasma and liver unesterified cholesterol compartments. Equilibration was found at about 30min and then the specific radioactivity of the plasma compartment rose above that of the liver. This would imply that cholesterol derived from acetate is treated differently from that derived from mevalonate and might suggest a physical compartmentation of cholesterol synthesis in the cell.

	^{14}C	^{3}H
Specific radioactivity liver unesterified cholesterol	74.89nCi/mg	16.6nCi/mg
Specific radioactivity liver esterified cholesterol	22.24nCi/mg	6.42nCi/mg

Table 15

Specific radioactivity of cholesterol in the liver after perfusion of livers from normal rats.

Non-cyclic perfusion

This experiment was carried out to examine the early incorporation of (2^{14}C)DL mevalonic acid into cholesterol. The technique is described in the methods section (p63). After liver perfusion had been satisfactorily established without the presence of any radioactive precursor of cholesterol in the perfusate, the secondary reservoir containing perfusate and radioactive mevalonate was connected to the liver and blood perfusate allowed to pass through the liver without recycling. Samples of the liver effluent were collected at one minute intervals. These samples were analysed for specific radioactivity of the cholesterol. The results are shown in Fig. 66.

The specific radioactivity reached an almost constant value in 4 - 5 min and then slowly increased for the remainder of the sampling period. The initial rise was probably related to the flushing of the vascular bed of the liver with the labelled precursor and was not related to the kinetics of cholesterol synthesis or transport, but to the haemodynamics of hepatic circulation. Thus, although the blood flow rate was between 10 and 12ml per minute, the vascular volume of the liver is probably only about 2ml and this means that if vascular perfusion is complete then the vascular bed must be perfused 5 - 6 times per minute.

The specific radioactivity of the liver unesterified cholesterol at the end of the perfusion was approximately two and a half times that of the plasma unesterified cholesterol.

Recovery of radioactivity in cholesterol was 5.4%. Only a small proportion of the added precursor was therefore

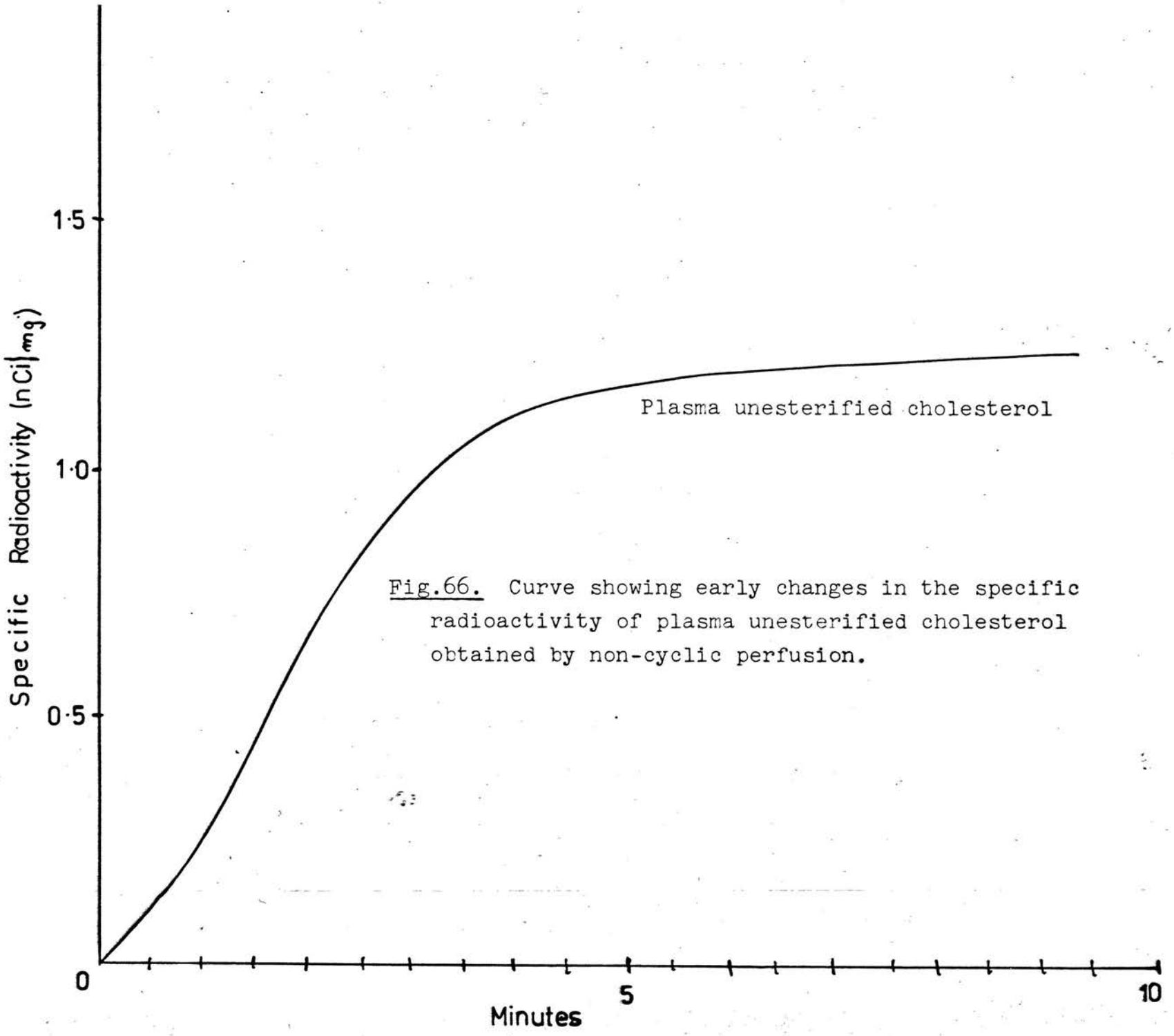


Fig.66. Curve showing early changes in the specific radioactivity of plasma unesterified cholesterol obtained by non-cyclic perfusion.

entering the hepatocyte on a single passage through the liver. If 5.4% is utilised in twelve minutes then approximately 50% of the label would be consumed in two hours. This is in keeping with the findings in the closed perfusion system.

Studies in sub-cellular fractions in normal rat liver.

These experiments were designed to investigate whether the shapes of the specific radioactivity curves found during normal liver perfusion were in any way influenced by the time course of the synthesis of cholesterol and its transfer into the perfusate. If any of the intracellular organelles act as compartments, (i.e. the intracellular cholesterol cannot be considered homogeneous), then the model adopted would have to be modified to take account of this. The exchange of cholesterol between nuclear, mitochondrial, microsomal and the soluble fraction was examined as detailed below.

A group of 14 rats (weight 200 - 240g) were maintained on the soft diet (see Appendix) for three weeks prior to use in this study. Two rats were given an intraperitoneal injection of 5μCi(2^{14}C)DL mevalonic acid lactone in acetone:0.9% saline (1/1), the injections being performed five minutes apart. Two minutes after injection of the first rat it was anaesthetised and bled by cardiac puncture, anti-coagulating with heparin. The liver was excised and flushed via the portal vein with ice-cold 0.44M sucrose. The second rat was then treated in a similar manner. The blood specimens were separately analysed to determine the specific radioactivity in the plasma and erythrocytes. The livers were separately weighed and pulped. Sub-cellular fractions were prepared (p 48), the cholesterol extracted and the specific radioactivity determined.

Consecutive pairs of rats were treated in a similar fashion, sacrificing them at intervals of 2, 5, 15, 30, 60, 90, 120 and 240min after injection of the cholesterol precursor.

The time course of the appearance of radioactive cholesterol in the various fractions is shown in Fig. 67. There was a rapid incorporation of radioactivity into the cholesterol in all subcellular fractions, which was at a maximum around 30min. The specific radioactivity then fell more slowly, equilibrium between the plasma unesterified cholesterol and the subcellular cholesterol fractions occurring at approximately 200min. At this point the plasma esterified and erythrocyte cholesterol had not reached equilibrium with either the plasma unesterified cholesterol or the cholesterol present in the liver.

The slow decline in the specific radioactivity of the plasma unesterified cholesterol was probably associated with exchange into the erythrocytes and plasma unesterified fraction, and transfer to the bile in the form of cholesterol or bile acids.

The relative movement of radioactivity between the subcellular organelles was assessed by determining the ratio of the specific radioactivity of the cholesterol in each liver compartment to that of the plasma unesterified cholesterol at each time point of sampling. As the specific radioactivity of cholesterol in the plasma is the final end product of the movement of cholesterol in the cell, then any divergence of the ratios relating to individual subcellular organelles would be evidence for intracellular compartmentation.

The ratios of the specific radioactivity of plasma cholesterol to the specific radioactivity of liver cholesterol are shown in Fig. 68. The crude liver homogenate, before fractionation, was in equilibrium with

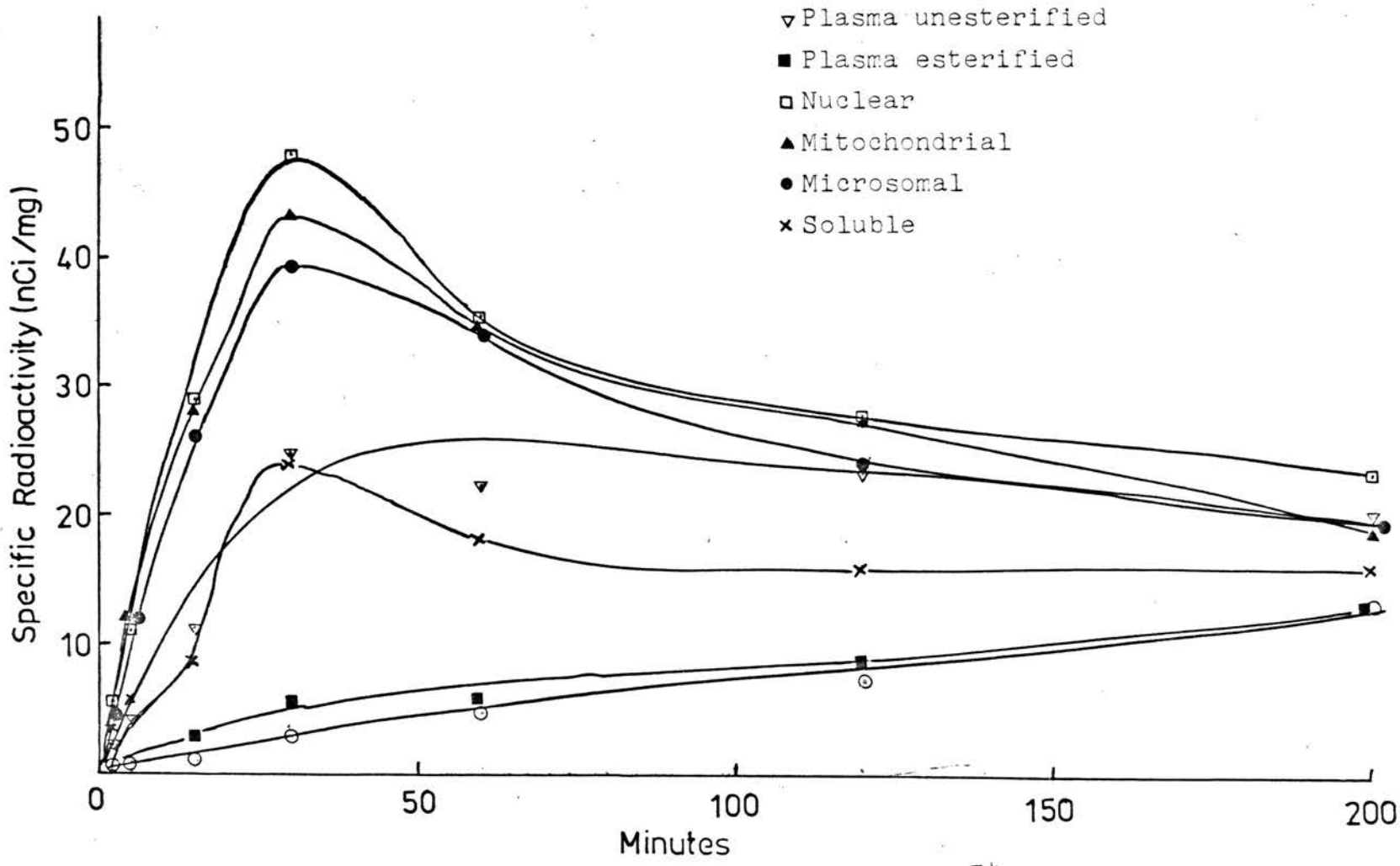


Fig.67. In vivo changes in the specific radioactivity of (^{14}C) cholesterol in various compartments of the blood and liver system after administration of an intraperitoneal dose of radioactive mevalonate.

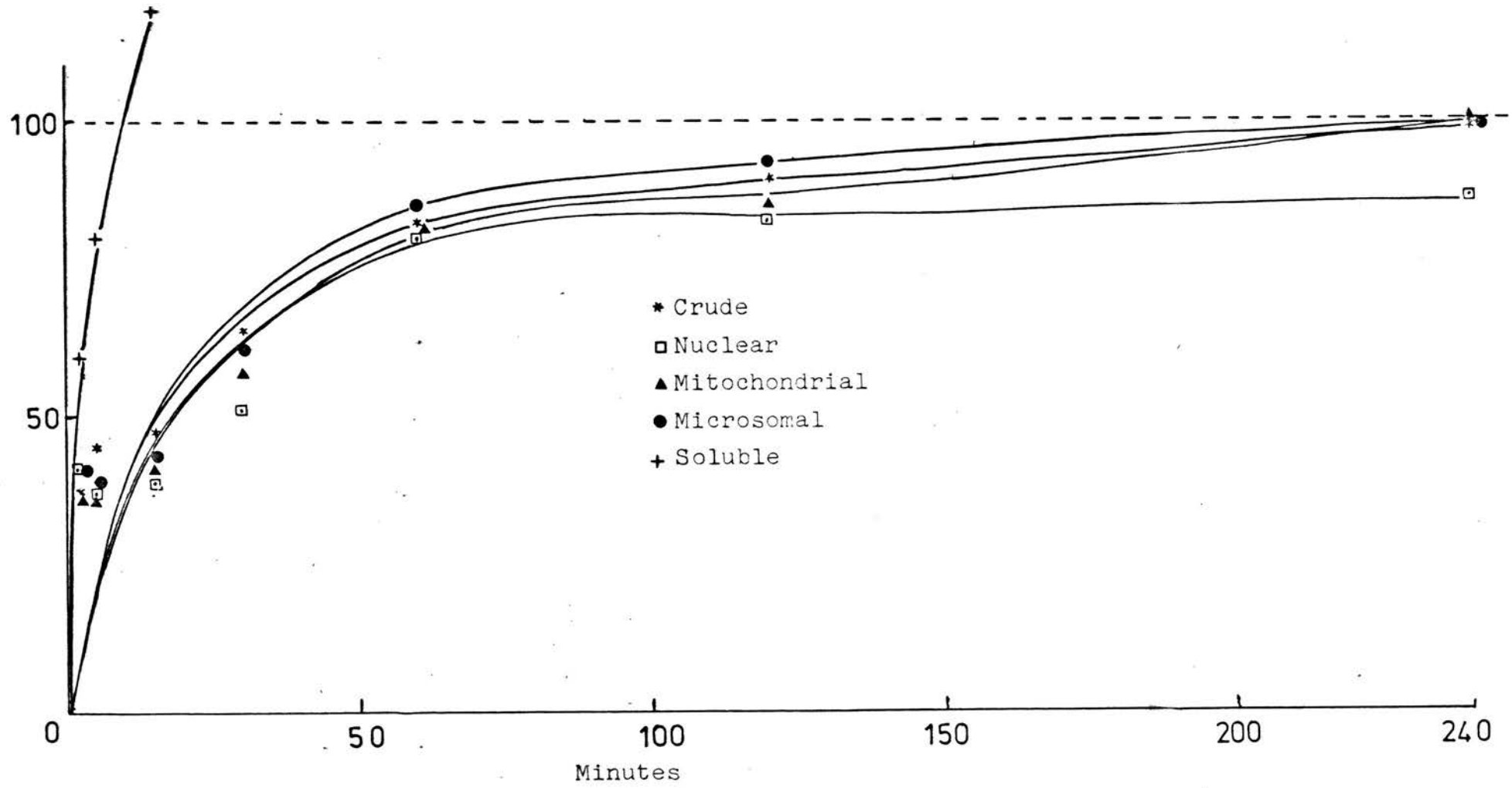


Fig.68. Percentage ratio of the specific radioactivity of plasma unesterified (^{14}C) cholesterol to the specific radioactivity of cholesterol in subcellular fractions of the livers of rats which had had an intraperitoneal dose of radioactive mevalonate.

the plasma unesterified cholesterol at 240min. The mitochondrial and microsomal fractions showed slightly earlier equilibration but were not significantly different from the crude preparation. The nuclear material however had not reached equilibrium at 240min. These results suggest that the cholesterol exchange between nuclear material and the plasma was slower than for the other subcellular organelles.

The specific radioactivity of cholesterol in the nuclear, mitochondrial and microsomal fractions was compared by taking the ratio of their specific radioactivity (Tables 16 and 17). A Student's 't' test on these mean ratio values would suggest that cholesterol transfer between mitochondria and microsomes is more rapid than that between the nuclei and mitochondria (Table 16). It is also more rapid between the microsomal and mitochondrial fractions than between the microsomal and nuclear fractions.

Although this data provides some evidence of intracellular compartmentation of cholesterol the differences between the mean specific radioactivity ratios was small. To expand the biological model to take account of these data was considered unjustifiable because the model parameters could not be defined uniquely (Table 14). It seemed unlikely therefore that addition of extra compartments to the biological model would provide a better accomodation to the experimental data.

Time	Nuclear	Microsomal
2	1.0630	1.1389
	0.8229	1.3046
5	1.0666	1.0668
	1.0398	1.0555
15	0.9524	1.0414
	0.9923	1.1265
30	0.8189	0.9817
	0.9786	1.1950
60	0.9854	0.9932
	0.9250	1.0654
120	1.0510	1.1399
	0.9123	1.0453
240	0.9115	1.0005
	0.7352	0.9067
	$\frac{x}{n}$ = 0.9467	$\frac{x}{n}$ = 1.0750

($p = < 0.001$)

Table 16

Ratio of the specific radioactivity of mitochondrial cholesterol to that in the nuclear and microsomal fractions

Time	Nuclear	Mitochondrial
2	0.9334	0.8780
	0.6308	0.7665
5	0.9997	0.9373
	0.9851	0.9474
15	0.9144	0.9602
	0.8809	0.8877
30	0.8342	1.0187
	0.8190	0.8369
60	0.9921	1.0068
	0.8682	0.9386
120	0.9219	0.8772
	0.8727	0.9566
240	0.9110	0.9995
	0.8108	1.1029
	$\frac{x}{n}$ = 0.8839	$\frac{x}{n}$ = 0.9367

($p = < 0.05$)

Table 17

Ratio of the specific radioactivity of microsomal cholesterol to that in the nuclear and mitochondrial fractions.

Some normal physiological and biochemical values of the rats used in this study.

In order to compare the study reported here with other reports relating to cholesterol metabolism in the rat, it was felt necessary to establish normal values for some of the variables measured in this study. Ten rats of the Wistar strain were used and the following variables measured:-

1. Body weight
2. Liver weight
3. Blood volume (by cardiac puncture)
4. Haematocrit
5. Plasma cholesterol concentration a) unesterified b) esterified
6. Erythrocyte cholesterol concentration a) unesterifed b) esterified
7. Liver cholesterol concentration a) unesterified b) esterified
8. Biliary excretion of cholesterol.

Blood was collected by cardiac puncture and the livers were then excised after flushing the vascular bed with cold saline injected into the portal vein. The liver was then blotted dry and homogenised in saline.

Excretion of cholesterol in bile was measured in a separate experiment in which four rats were subjected to biliary drainage for 44h.

The cholesterol concentration was determined as described previously. The results are shown in Table 18. The ratio of the concentrations of unesterified plasma cholesterol to total plasma cholesterol was 2.61.

Compartment	Total	Unesterified	Esterified
Plasma	46.6mg/100ml ± 7.1	17.9mg/100ml ± 1.94	28.67mg/100ml ± 2.28
Erythrocytes	82.7mg/100ml ± 5.25	82.03mg/100ml ± 2.93	0.63mg/100ml ± 1.2
Liver	1.43mg/g tissue ± 0.093	1.23mg/g tissue ± 0.041	0.20mg/g tissue ± 0.061

Table 18

The concentration of unesterified and ester cholesterol in the liver, erythrocytes and plasma of Wistar rats.

Cholesterol was excreted in the bile at a rate of 23.3 - 45.3µg/h. The weight of the rats used was 201 - 225g (mean 209g), and the liver weights were 7.0 - 8.4g (mean 7.7g). The mean volume of blood obtained by cardiac puncture was 7.8ml and the haematocrit was 39.6% ± 1.8. There was no significant difference between the total amount of cholesterol and the unesterified cholesterol in the erythrocytes. It was therefore assumed that all erythrocyte cholesterol is in the unesterified form.

Incorporation of (2^{14}C)DL mevalonic acid into cholesterol in vivo in normal rats.

A single experiment was performed to obtain information about the rate of incorporation of the radioactive precursor into cholesterol in vivo. This was intended to provide a basis for comparison of the results obtained during isolated liver perfusion.

Six rats were each given an intraperitoneal injection of 5µCi of (2^{14}C)DL mevalonic acid lactone in acetone:0.9% saline (1/1). Two of the rats were bled at intervals of one hour by cardiac puncture and the livers excised and perfused briefly with isotonic saline. The specific radioactivities of the cholesterol present in blood and tissue were measured. The results are shown in Table 19.

The specific radioactivity of the liver unesterified cholesterol was highest after one hour and reached equilibrium with the plasma after about two hours. The specific radioactivity of the esterified cholesterol in the plasma and liver changed in a similar fashion. The accumulation of radioactivity in the plasma cholesterol may be due to the appearance of radioactive cholesterol synthesised at sites other than the liver, particularly from intestinal synthesis (Weiss and Dietschy, 1971).

	1 Hour	2 Hours	3 Hours
Plasma unesterified	18.18	9.55	6.02
Plasma esterified	9.71	3.74	3.64
Liver unesterified	32.29	10.47	3.79
Liver esterified	14.44	3.53	1.0
Erythrocytes	6.35	7.81	4.61

Table 19

Changes in the specific radioactivity (nCi/mg) of unesterified and ester cholesterol in the liver and blood of normal rats after a single intraperitoneal dose of (2^{14}C)DL mevalonic acid.

Van Bruggen and Elwood (1961) showed that mevalonic acid was incorporated into intestinal cholesterol at later time periods than liver incorporation. This could explain the crossover of specific radioactivity of the liver and plasma cholesterol.

Erythrocyte cholesterol reached a maximum specific radioactivity later than the other compartments sampled; this was hardly surprising since the only source of radioactive cholesterol in the erythrocytes was by exchange from the plasma.

Studies in rats subjected to biliary drainage.

This section deals with experiments examining the effects of biliary drainage upon the exchange of cholesterol between liver and blood.

Depletion of the intestinal bile acid pool leads to an increase in the hepatic synthesis of cholesterol. It was thought that this increased synthesis rate may lead to changes in the rate at which newly synthesised cholesterol in the liver transfers into the blood. If this were the case then the model derived for normal liver perfusion could be changed to fit experimental data obtained from perfusions of drained livers. The model might then indicate the point at which the exchange processes between liver and blood are modified by stimulation of intrahepatic cholesterol synthesis.

The most reliable way of depleting the intestinal bile acid pool is by diversion of the bile flow through an external fistula. This was performed in a number of rats before carrying out perfusions of the isolated livers.

Excretion of cholesterol in bile fistula rats.

Five rats (weight 210 - 230g) were subjected to continuous biliary drainage for periods of up to 44h and the bile from each rat collected in one hour discrete samples. The bile was weighed by difference and samples obtained from each rat combined into four lots, each of 10h. One ml of each lot was analysed for esterified and unesterified cholesterol concentration as described in the methods section.

The results showed that there was no significant difference between the concentrations of unesterified and total cholesterol ($p = > 0.7$). Therefore it was assumed that all of the cholesterol was excreted in the

unesterified form. Table 20 summarises the results. Both the amount of bile secreted and the concentration of cholesterol in the bile fell during the first twenty hours, and then increased, reaching the original excretion rate at an estimated 50h after cannulation. These values are similar to, but slightly lower than those reported by Eriksson (1957a).

The average bile wieght over the first three hours of drainage was 0.47g/h and the cholesterol excretion was approximately 34µg/h. This was less than the value obtained during perfusion of the isolated normal liver under which conditions the excretion rate was 48.5ug/h.

Perfusion of livers after previous biliary drainage.

After 44h drainage of bile through an external fistula the livers of the five rats used in the previous experiment were removed and perfused with heparinised rat blood as described in the methods section (p52). Addition of the radioactive precursor of cholesterol and analysis of samples obtained during perfusion was performed as described previously (p38).

The mean specific radioactivity curves for (^{14}C) cholesterol observed in some of the compartments of the proposed model are shown in Fig. 69. These are similar to those obtained for perfusion of the normal rat livers, but in each case the mean specific radioactivity curve was lower for the bile drained rat liver. A statistical comparison, by means of a Student's 't' test, (see Appendix), for each compartment over a three hour perfusion period showed no significant difference between the curves obtained for normal and bile drained livers, for the plasma unesterified or esterified cholesterol (($p = > 0.5$ and $p = > 0.9$ respectively). There was

Time after cannulation(h)	Weight of bile (g)	Total cholesterol excreted (µg)	ug/cholesterol/g bile	Cholesterol (µg/h)
0 - 10	4.82	380	78.8	38
10 - 20	3.56	235	66.0	23.5
20 - 30	4.02	283	70.4	28.3
30 - 40	4.19	306	73.03	30.6
30 - 44	5.58	437	78.31	31.2

Table 20

Weight of bile and cholesterol excreted bile fistula rats.

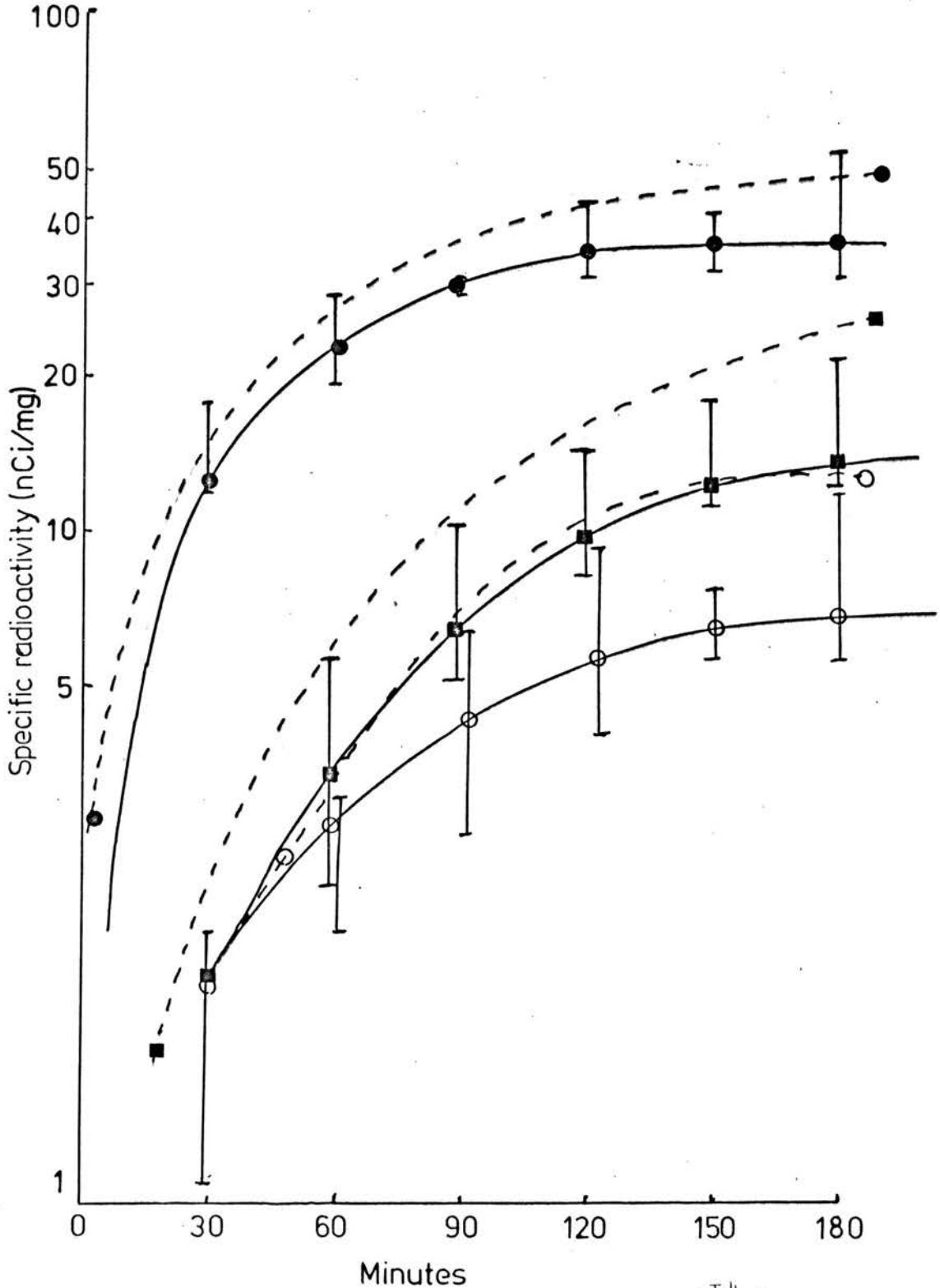


Fig.69. Changes in the specific radioactivity of (^{14}C) cholesterol in the plasma unesterified(●), esterified(O) and erythrocyte (■) compartments during perfusion of livers taken from bile drained rats. Dashed lines show changes obtained for normal liver perfusion.

however, a significant difference between the erythrocyte curves obtained during normal and bile drained liver perfusion (= < 0.05). In addition the mean specific radioactivity of the liver cholesterol at the termination of the perfusions was significantly lower ($p = < .05$) in perfusions of livers from bile fistula rats than in perfusions of normal rat livers (Table 21).

The mean biliary excretion of cholesterol during perfusion was 46ug/h from livers which had bee previously subjected to biliary drainage and 48ug/h from normal livers. This is somewhat greater than the excretion rate which would have been predicted in vivo (Table 20).

The specific radioactivity of biliary cholesterol was not significantly different ($p = > 0.7$) in comparing normal and bile drained livers, thus the mean specific radioactivity of the biliary cholesterol was 23.28nCi/mg in perfusions of normal liver and 21.76nCi/mg in perfusions of bile drained livers.

Perfusate cholesterol concentration.

The concentration of cholesterol in the perfusate increased during perfusion in a manner similar to that found in normal liver perfusion. It was of interest to establish whether this increase was significantly different from the increases found during normal liver perfusion. This was tested using a Student's 't' test on the means of the slopes of the regression equations, describing the relationship of perfusate cholesterol with time, both for normal and bile drained liver perfusions.

No significant difference was found between the two types of perfusion for plasma unesterified cholesterol ($p = > 0.5$) or total cholesterol ($p = > 0.5$).

	Normal liver perfusion	Bile drained liver perfusion
Unesterified cholesterol	74.89	40.16
Esterified cholesterol	22.24	25.96

Table 21

A comparison of the specific radioactivities (nCi/mg) of cholesterol in the perfused livers of normal and bile drained rats.

Equilibration times between liver and blood.

The rate at which radioactive unesterified cholesterol in the blood equilibrated with radioactive cholesterol in the liver is shown in Fig. 70. The construction of this graph and a critique of the method have been previously described (p.92).

The most notable feature of the graph is the rapid rate of equilibration between liver and plasma unesterified cholesterol, equivalent specific radioactivity values being obtained at about 110min. This was in marked contrast to the results obtained in normal liver perfusion when equilibration between plasma and liver unesterified cholesterol was not found at the termination of the perfusion (c.f. Fig. 64). After 110min the specific radioactivity curve of the unesterified plasma cholesterol rose above the equilibrium point and continued above the 100% datum line for the remainder of the perfusion. Care must be taken in interpreting the equilibration curves obtained for the bile drained system because the rate of equilibration is probably much less rapid. This can be ascribed to the increased loss of radioactivity into the bile as bile acids and therefore a lower specific radioactivity of the liver unesterified cholesterol at the end of, and during, the perfusion when compared to the normal liver perfusion. This was supported by finding a mean specific radioactivity of the unesterified cholesterol at the end of normal liver perfusion of 74.89nCi/mg and a value of 40.16nCi/mg in the bile drained preparation.

The esterified cholesterol equilibration curve was sigmoid in shape and was similar to that found in normal liver perfusion (Fig. 64). In this instance the degree of equilibration attained was considerably less than in

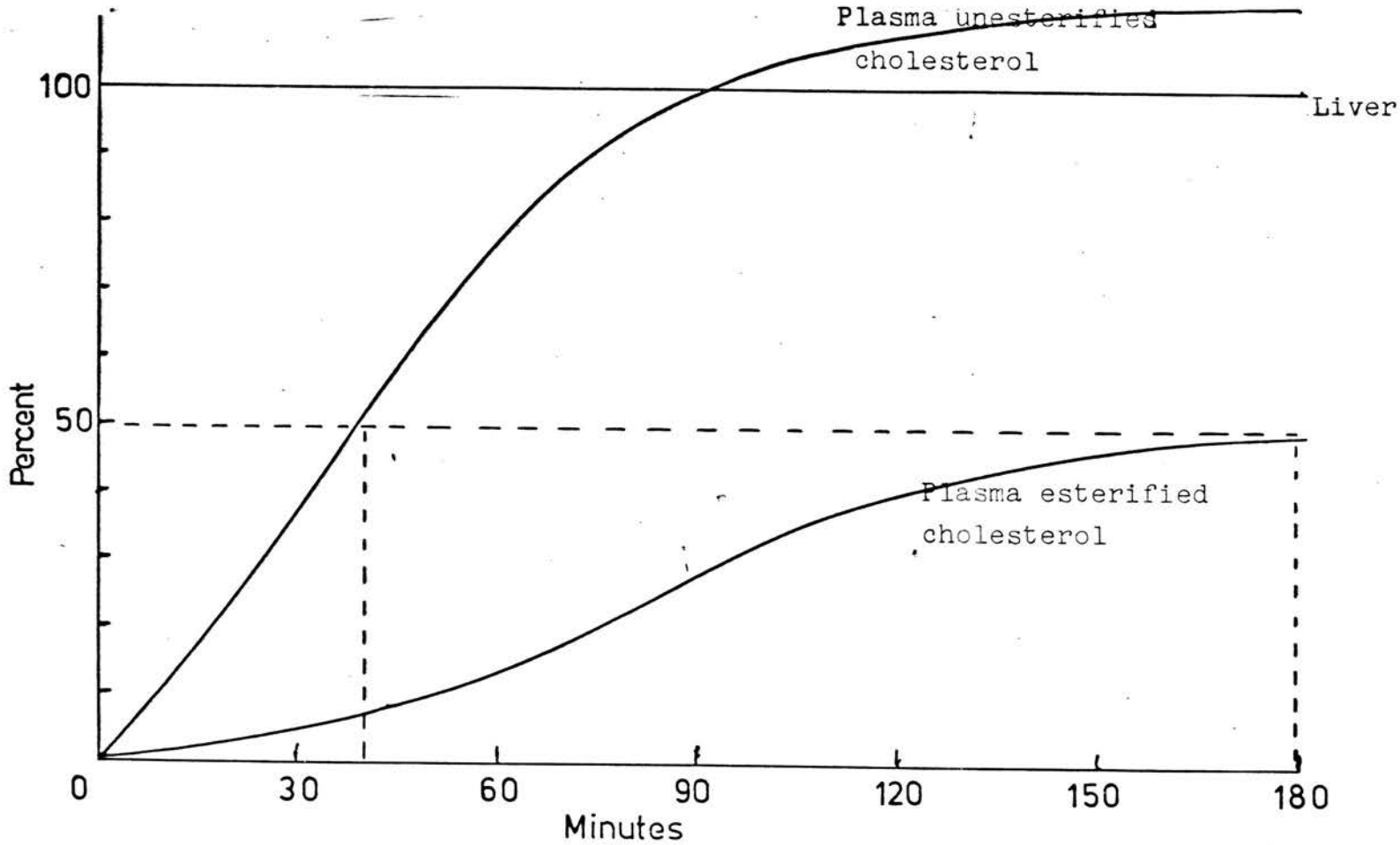


Fig.70. Percentage proportion of the specific radioactivity of plasma (^{14}C) cholesterol to liver (^{14}C) cholesterol during perfusion of livers taken from bile drained rats.

the normal liver perfusion studies even though the specific radioactivity of the liver esterified cholesterol was greater than that in the normal rat liver perfusions (Table 21).

Radioactive recovery.

The recovery of radioactivity at the end of the perfusions was determined by multiplying the compartment sizes by the terminal specific radioactivity, and then summating the values obtained. This showed a mean recovery of 33% of the total radioactivity injected or 66% of metabolisable label, due allowance being made for the counting efficiency. This is in contrast to the values found in the normal perfusion when 47.5% of the total radioactivity or 95% of the metabolisable label was recovered.

This reduction in the amount of label recovered can possibly be attributed to three factors:-

1) an increase in the rate of incorporation of the precursor, mevalonic acid into non-cholesterol products.

2) an increase in the rate of incorporation of cholesterol into other products which were not measured in this study, e.g. bile acids or steroids.

3) an increase in the physical loss of label from the perfusion system into the bile as either cholesterol or its metabolic derivatives.

The most likely explanation is the third one. It is well established that bile acid synthesis and excretion is stimulated by bile drainage(Myant and Eder, 1961) and therefore in the experimental model of an isolated

perfused liver there will be an apparent loss of label from the system. An estimate of this loss can be obtained from the work of Eriksson (1957a) and Percy-Robb and Boyd (1970). Their studies would suggest that about 1/5 - 1/7 of the cholesterol in the perfusion system would be transformed to bile acid in the bile drained liver. This means that up to 20% of the label would not be recovered as cholesterol.

On the basis of the recovery obtained in this study, the incorporation of cholesterol into bile acids might be even greater than that found by the aforementioned authors.

Simulation with experimental data.

The data from the perfusions on the bile drained livers were transformed to show the absolute amount of radioactivity associated with any of the sampled compartments at each point in time. This was performed in a similar manner to that described previously (p.86).

A comparison of the radioactivity changes observed experimentally in each compartment for normal and bile drained liver perfusions is shown in Fig. 71. The general shape of the plasma curves was the same, with the plasma unesterified cholesterol curve for the bile drained livers slightly below that for the normal liver perfusion. The curves describing radioactivity changes in the erythrocyte compartments of normal and bile drained livers were markedly different.

The loss of radioactive label from the system as bile acid can be simulated on the model by increasing the rate constant k_{61} (see Fig. 8). Based upon the work of Percy-Robb and Boyd (1970) it can be shown that a

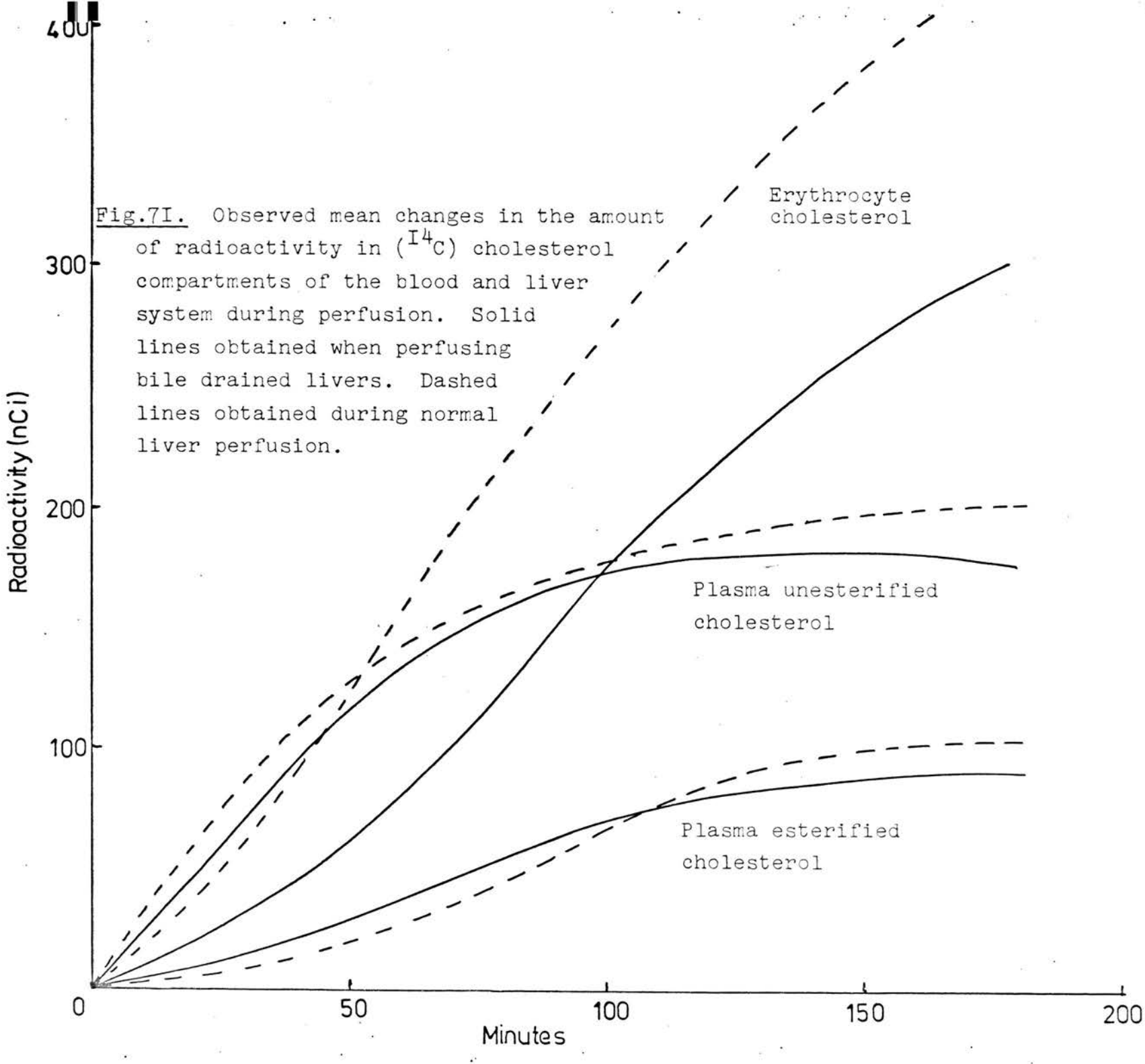


Fig.7I. Observed mean changes in the amount of radioactivity in (^{14}C) cholesterol compartments of the blood and liver system during perfusion. Solid lines obtained when perfusing bile drained livers. Dashed lines obtained during normal liver perfusion.

fifteen fold increase in this rate constant would accommodate the increased loss of labelled cholesterol as bile acids. Rate constant k_{61} was increased by factors of 10 in the model programmed on the analogue computer and curves simulating radioactivity changes in the three blood cholesterol compartments were generated. Fig. 72 shows the results of this simulated experiment. Although increases in rate constant k_{61} allowed the model to accommodate to the lower curves of the erythrocyte compartment obtained in the bile drained preparation, this also reduced the unesterified cholesterol curves. An increase in k_{61} of the order of x 10 would allow for bile acid excretion, give better correspondence to the curves for plasma unesterified cholesterol in the normal perfusion and also partly satisfy the requirements for lower erythrocyte curves.

New model parameters could be found to fit the reduced erythrocyte curves by changing rate constants k_{21}, k_{12}, k_{23}, or k_{32} (Fig. 8). Reduction of k_{21} for example, would indirectly reduce the accumulation of radioactivity in the erythrocyte compartment, but would also lead to a reduction in the plasma unesterified compartment, and so deviate from the experimental data. The best way to accommodate the normal model to the bile drained experimental data was to reduce the rate constant k_{32}. It is however, difficult to conceive of a mechanism whereby the stimulation of the metabolic pathway from cholesterol to bile acid could directly affect the exchange of cholesterol between plasma and erythrocytes.

The best way to explain the reduced erythrocyte curve is probably on the basis of increased excretion of radioactive label in the bile, and by recognising that the erythrocyte compartment size was smaller in the

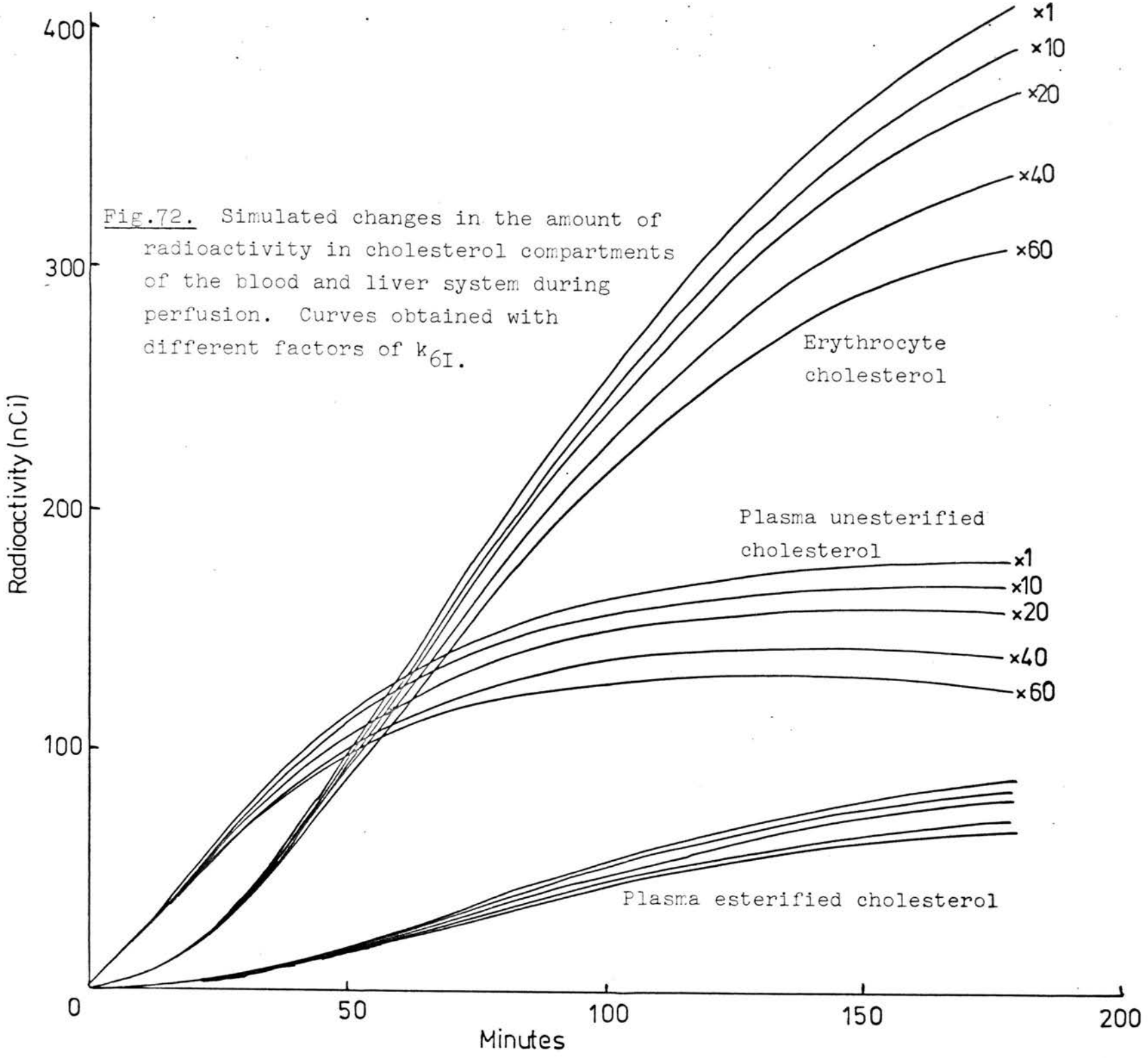


Fig.72. Simulated changes in the amount of radioactivity in cholesterol compartments of the blood and liver system during perfusion. Curves obtained with different factors of k_{6I}.

bile drained preparation than in the normal

Mean erythrocyte compartment size	=	18.68mg normal liver perfusion
Mean erythrocyte compartment size	=	17 2 mg bile drained liver perfusion.

Studies in rats subjected to thyroidectomy.

Thyroidectomy has been shown to reduce hepatic cholesterol synthesis in rats (de Matteis, 1969: Marx et al, 1953). At the same time the hypothyroid rats have an increased concentration of cholesterol in the blood (Byers et al., 1970). A series of perfusion experiments were performed on livers obtained from thyroidectomised animals to see if a lack of thyroid hormone had any effect on the exchange of cholesterol occurring between liver and blood. Any changes brought about by the absence of the hormone may be accommodated by changing the normal model to fit the new experimental data. This may allow prediction of the site of action of thyroid hormone.

Sixty rats were subjected to surgical thyroidectomy as described in the methods section (p54) Relevant checks on the success of the operation were performed (p55).

The blood used as perfusate was obtained from thyroidectomised animals by cardiac puncture. It was felt that this perfusion medium should more closely simulate physiological conditions in that any reduction in hormonal substances (e.g. thyroxine) in the blood due to thyroidectomy, should be present during perfusion of livers obtained from treated animals. If blood obtained from normal animals had been used to perfuse livers obtained from thyroidectomised rats, then any changes in cholesterol metabolism in the perfused liver system would have been more difficult to interpret.

Perfusions and analyses of specimens obtained were carried out as described previously (methods section p 38 ,) 4μCi ($2^{14}C$)DL mevalonic acid in lactone were added to the perfusate after establishing a viable preparation. Five perfusions were performed on the livers of

thyroidectomised animals whose thyroidectomy was apparently successful as judged by measurement of heart rates and body weights.

Excretion of cholesterol in bile.

During isolated liver perfusion, cholesterol was excreted at an average rate of 17µg/h. This was significantly less than that found during normal liver perfusion which averaged 48.5µg/h ($p = < 0.01$). The actual weight of bile formed during perfusion was also less than normal ($p = < 0.001$) but this does not fully explain the reduced excretory rate of cholesterol because the concentration of cholesterol per gram of bile was also decreased in thyroidectomised animals ($p = < 0.05$).

The specific radioactivity of biliary cholesterol was not significantly different from normal ($p = > 0.1$). A summary of the results is shown in Table 22 with the corresponding normal values shown in brackets.

Both bile weight and the amount of cholesterol excreted fell during perfusion and were similar to the changes noted during normal perfusion but at a correspondingly lower level. The specific radioactivity of the biliary cholesterol did not change significantly during perfusion in contrast to the findings during normal liver perfusion when the specific radioactivity of the biliary cholesterol showed an increase. No obvious changes in the concentration of biliary cholesterol were noted during perfusion.

Time after commencing perfusion	Weight of bile (g)	Cholesterol excreted per hour (µg)	Specific radio-activity cholesterol (nCi/mg)	µg Cholesterol/g bile
1.	0.255 (0.315)	24.3 (57.4)	14.92 (16.49)	95.4 (182.2)
2.	0.168 (0.258)	14.2 (46.8)	17.36 (26.07)	84.5 (181.4)
3.	0.163 (0.258)	15.6 (32.6)	13.29 (27.41)	95.47 (126.4)
Mean	0.195 (0.277)	16.9 (48.5)	15.19 (23.28)	91.8 (163)

Table 22

The weight of bile and cholesterol excreted during perfusion of livers from thyroidectomised animals. (Normal values are shown in the brackets).

Perfusate cholesterol concentration.

The cholesterol concentration in the perfusate rose steadily throughout the perfusion period in a manner similar to that found during normal liver perfusion. No significant statistical difference could be found between the rate at which the perfusate cholesterol concentration rose in normal or thyroidectomised rat liver perfusions.

However, when the mean slope of the regression equations for unesterified cholesterol/time was compared to the mean slope obtained for total cholesterol during perfusion of livers from thyroidectomised animals, then a 't' test showed a significant difference ($p = < 0.05$) with the total cholesterol concentration/time having a greater slope.

This was in contrast to the results obtained during normal liver perfusion when no significant difference could be found between the rate of increase of unesterified and total cholesterol in the perfusing blood.

Changes in the specific radioactivity of cholesterol during perfusion.

The curves describing the changes in the cholesterol specific radioactivity in each of the ^{14}C labelled compartments are shown in Fig. 73 with the corresponding curves for normal liver perfusion indicated by dotted lines. It is apparent that the general shape of the paired curves is very similar.

In every case the curves obtained from perfusions of the livers from thyroidectomised rats were significantly lower than for perfusions of normal livers. For plasma unesterified cholesterol $p = < 0.001$, plasma esterified cholesterol $p = < 0.02$ and for erythrocyte cholesterol $p = < 0.005$. The lower specific radioactivity curves

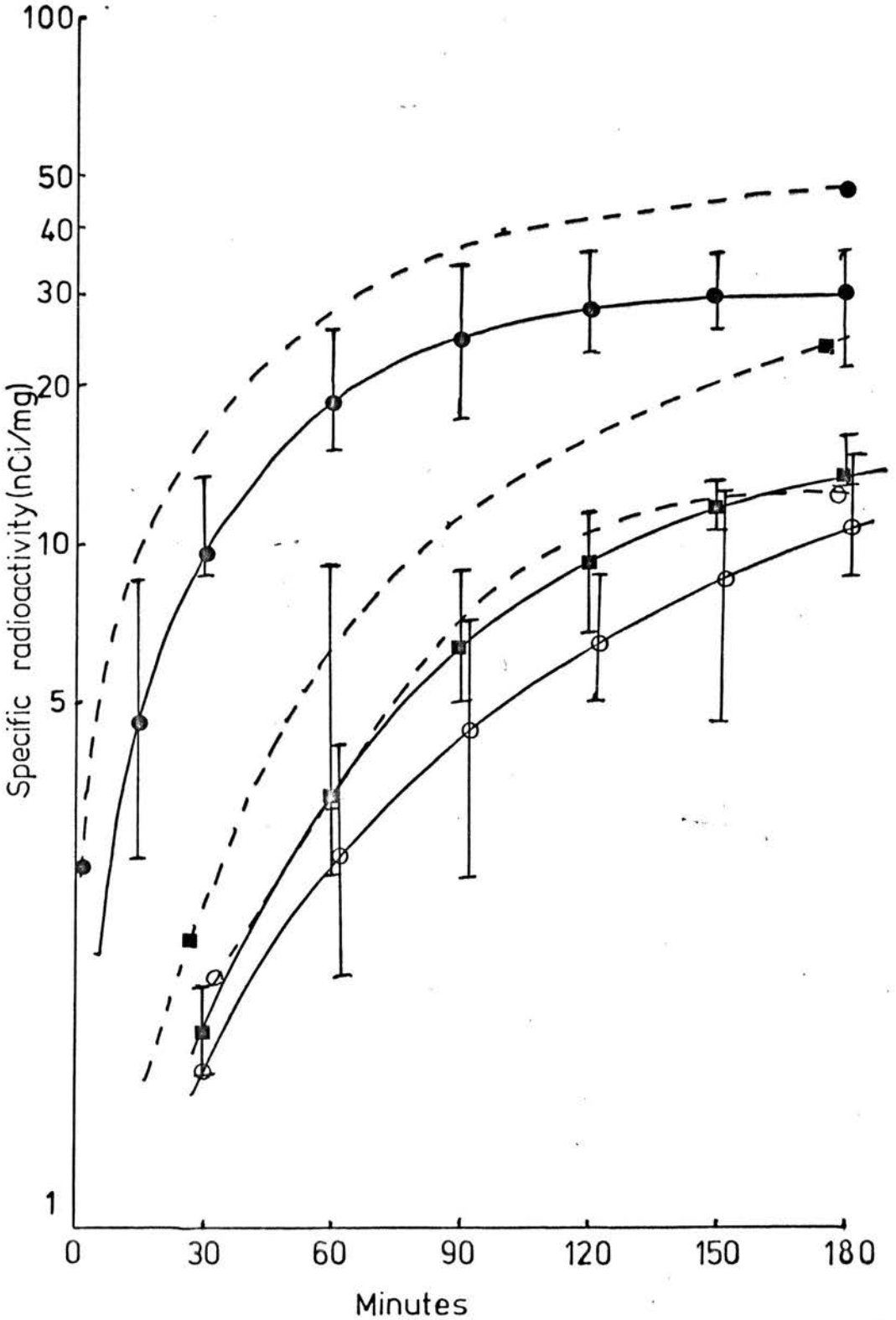


Fig.73. Changes in the specific radioactivity of (^{14}C) cholesterol in the plasma unesterified(●), esterified(O) and erythrocyte (■) compartments during perfusion of livers from thyroidectomised rats. Dashed lines show changes for normal liver perfusion.

found in perfusions of livers obtained from thyroidectomised rats can be explained partly by the expanded compartment sizes observed. Table 23 contrasts the compartment sizes in the normal and thyroidectomised rat liver perfusions.

Recovery of radioactivity.

The recovery of radioactivity in the form of cholesterol and bile acids at the end of the perfusions was only on average 65% of the metabolisable label and this included an estimate of the amount of radioactivity which had been incorporated into bile acids. This estimate of the bile acid secretion rate was derived from the work of Percy-Robb (1968) where the experimental conditions approximated to those in this series of experiments.

As the major rate limiting step in cholesterol biosynthesis lies before its precursor mevalonic acid, then it is difficult to explain this finding without postulating an effect of thyroid hormone on steps in the biosynthetic pathway after mevalonic acid. Alternatively, the hepatocellular uptake of mevalonate may be modified by the thyroid hormone. The major component of the reduction in recovered label was due to the lower final specific radioactivity of the liver cholesterol when compared to the normal liver perfusion. The mean specific radioactivity of the liver cholesterol in the normal and thyroidectomised rats at the end of the perfusion is compared in Table 24. It is evident that whilst the incorporation of label into the unesterified cholesterol is reduced, that into esterified cholesterol was increased suggesting an enhancement of the esterification processes in the thyroidectomised rat liver. The change in specific radioactivity in the thyroidectomised rat livers was a

	Normal	Thyroidectomised
Plasma unesterified	4.82	5.10
Plasma esterified	8.61	9.40
Erythrocyte	18.7	19.1
Liver unesterified	11.11	10.71
Liver esterified	1.06	1.09

Table 23

A comparison of the sizes of the cholesterol compartments of the blood and liver system in normal and thyroidectomised rats. (mg)

real one because the pool sizes were similar to those found for the normal rat livers.

Equilibration times between liver and blood.

The rate at which the plasma cholesterol compartments equilibrated with their counterparts in the liver is shown in Fig. 74. This was derived as described previously (p 92). The rate at which unesterified cholesterol in the plasma equilibrated with that in the liver was very similar to the results found during normal liver perfusion (Fig. 64). For esterified cholesterol the two plots were quite dissimilar, the final equilibrium point being 46.5% in the perfusions using livers from thyroidectomised rats as compared to 76% for normal rat liver perfusion. This may be due to two factors, firstly the mean specific radioactivity of the esterified cholesterol in the liver at the termination of the perfusions was greater than that for normal liver perfusion (Table 24), and secondly, the actual specific radioactivity of the plasma esterified cholesterol was lower than that for normal liver perfusion. These factors in conjunction would tend to lower the percentage equilibration at all time points.

Simulation with experimental data.

The data from the liver perfusions on the thyroidectomised rats was reduced to show the absolute amount of radioactivity associated with each cholesterol compartment sampled (see p 86). A plot of the mean of five perfusions is shown in Fig. 75. A comparison with the simulated results obtained by modelling normal liver perfusion (Fig.76), showed that the curves obtained from perfusions of livers

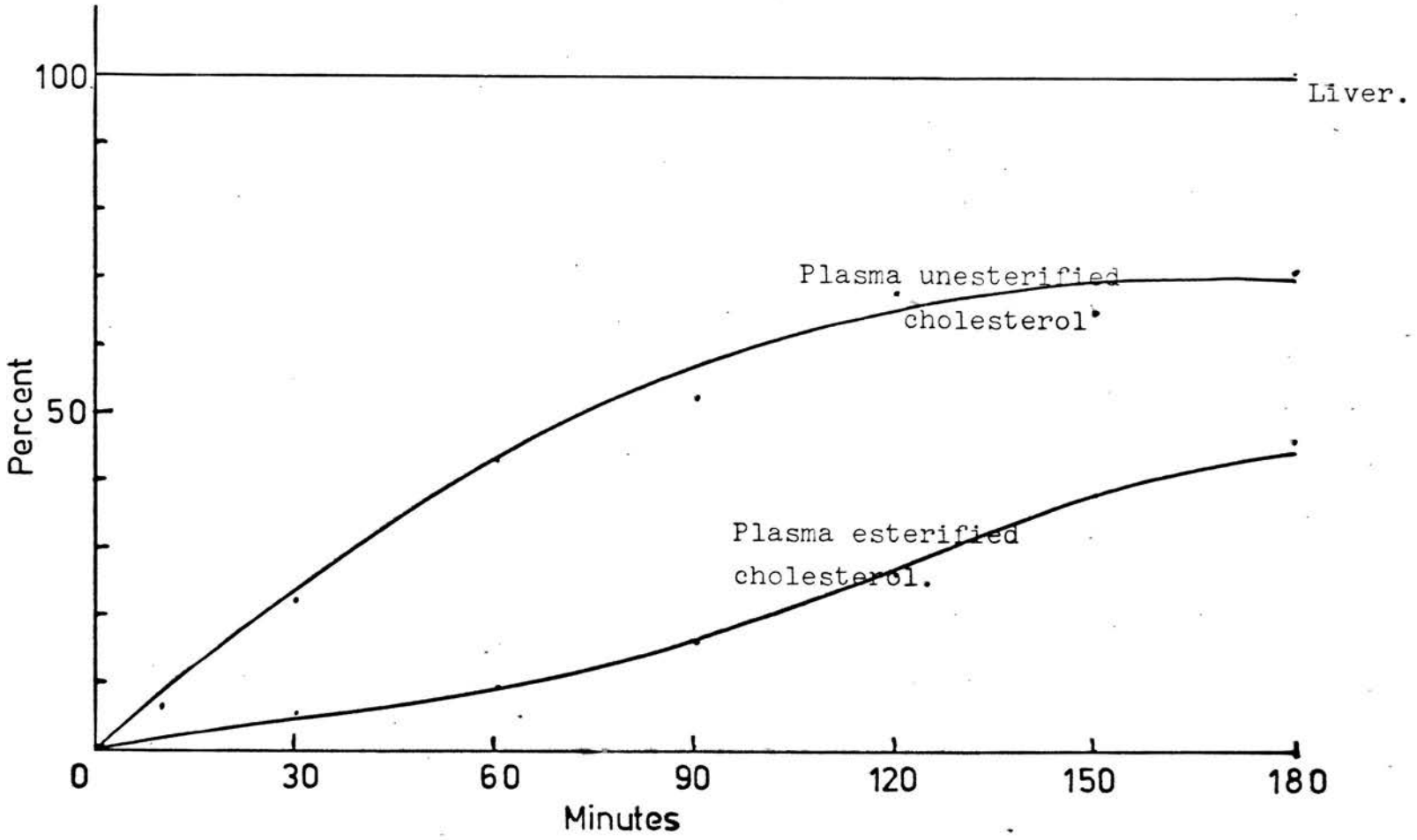


Fig.74. Percebtage proportion of the specific radioactivity of plasma (^{14}C) cholesterol to liver (^{14}C) cholesterol during perfusion of livers taken from thyroidectomised rats.

	Normal liver perfusion	Liver perfusion after thyroid-ectomy
Total cholesterol	71.09	51.01
Unesterified cholesterol	74.89	53.72
Esterified cholesterol	22.24	25.50

Table 24

A comparison of the specific radioactivities (nCi/mg) of cholesterol in the perfused livers of normal and thyroidectomised rats.

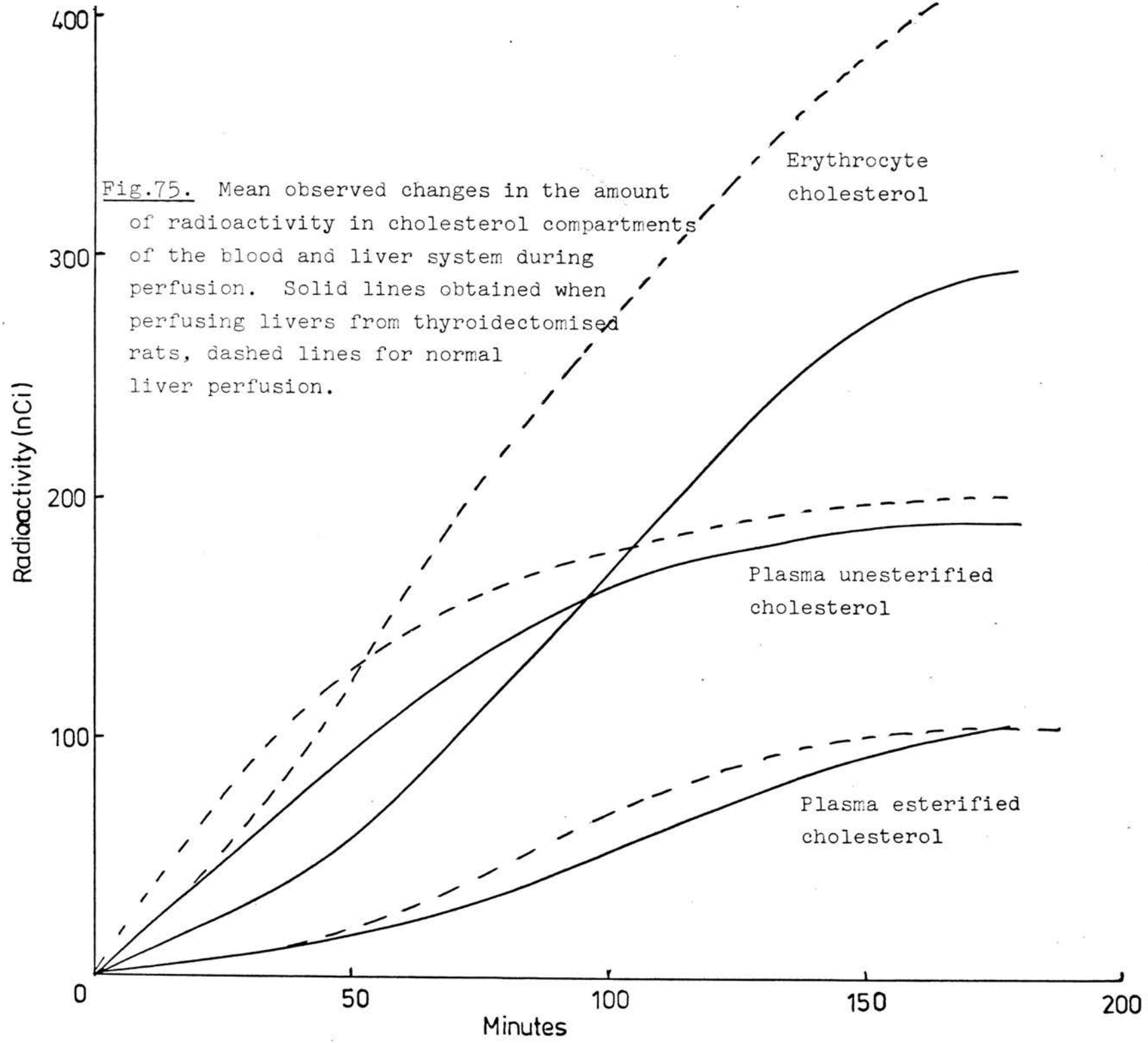


Fig.75. Mean observed changes in the amount of radioactivity in cholesterol compartments of the blood and liver system during perfusion. Solid lines obtained when perfusing livers from thyroidectomised rats, dashed lines for normal liver perfusion.

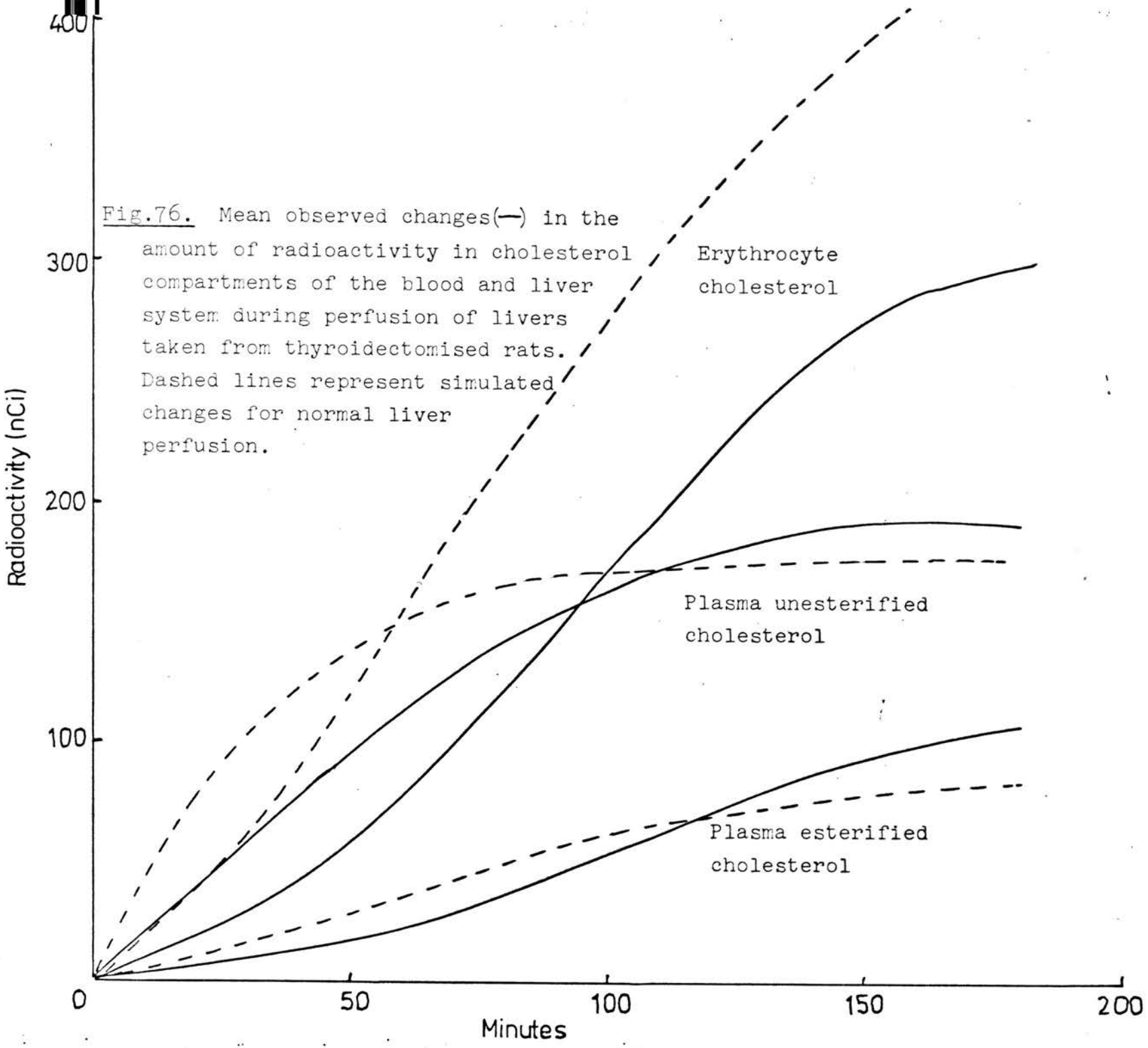


Fig.76. Mean observed changes(—) in the amount of radioactivity in cholesterol compartments of the blood and liver system during perfusion of livers taken from thyroidectomised rats. Dashed lines represent simulated changes for normal liver perfusion.

from thyroidectomised animals were consistently lower than those obtained during normal simulated liver perfusion. Statistical examination showed significant differences between the absolute number of counts in the blood compartments sampled when compared with the normal perfusion results.

New values of the rate constants for the transfer of cholesterol between liver, blood and bile in the thyroidectomised rat were not determined. The reason for this was that as the radioactive recoveries were not 100% of the metabolisable label, then the reduced curves found might simply have been a reflection of the reduced incorporation of mevalonic acid into cholesterol with a consequent reduction in the amount of radioactivity appearing in the blood compartments. If this were the case then one could not assume that the rate constants had changed from the appearance of reduced specific radioactivity curves.

A better fit to the reduced curves could be obtained by changes in the rate constant k_m i.e. the rate constant controlling the incorporation of labelled mevalonic acid into liver unesterified cholesterol. Estimation of the radioactive recovery showed that only 65% of the metabolisable label was recovered; a loss of 35%. The approximate value of k_m needed to simulate this experimental result was determined by generating curves of the type shown in Fig. 58 which illustrate the residual non-metabolised label over a 180min period for different values of k_m.

Using the relationship $x = e^{-k_m t}$ a value of $k_m = 0.05$ would simulate a recovery of 65% of the metabolisable label in the compartments sampled after 180min.

This value of k_m was introduced into the six compartment model which had been modified by the inclusion of a

compartment to represent the mevalonate pool and the new curves for the amount of radioactivity associated with each compartment generated. As can be seen from Fig. 77 this change leads to a better fit to the experimental data (compare with Fig. 76). A small revision of k_m to 0.08 gave a better composite fit and showed that 18% of the label was still not accounted for. This is not entirely in agreement with the recovery value found in the perfusion experiments where 35% of the label was not recovered but does support the hypothesis that the reduced recovery could be explained in part by a failure to incorporate the mevalonic acid into cholesterol, and not by losses of cholesterol into other metabolic pathways.

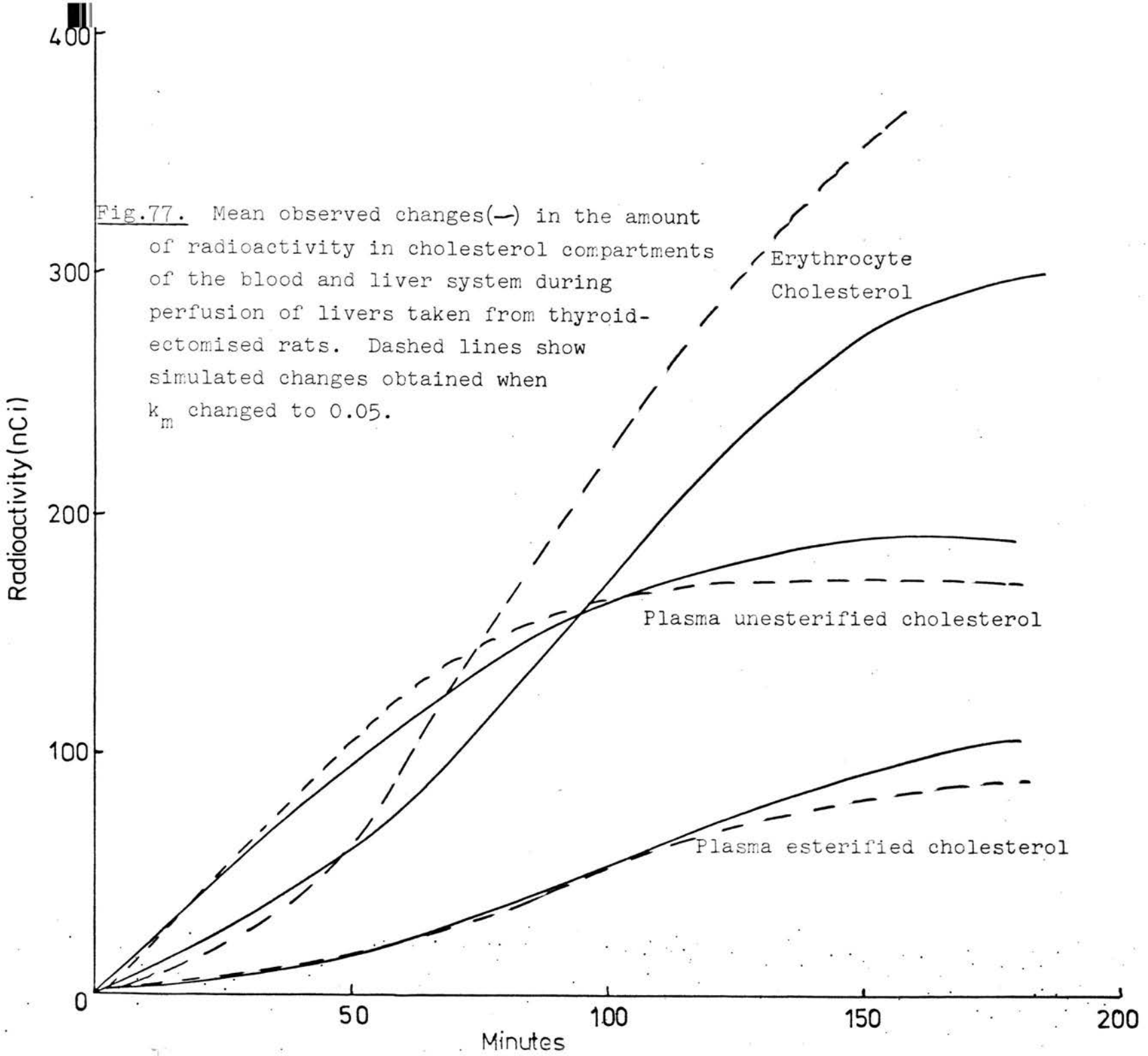


Fig.77. Mean observed changes(—) in the amount of radioactivity in cholesterol compartments of the blood and liver system during perfusion of livers taken from thyroidectomised rats. Dashed lines show simulated changes obtained when k_m changed to 0.05.

Studies related to the action of the drug ethyl chlorophenoxyisobutyrate (Atromid-S 500; C.P.I.B.)

This drug is known to reduce the concentration of cholesterol in the blood of rats (Thorp and Waring, 1962). The mode of action of the drug is still conjectural but two theories have been advanced. Firstly that hepatic cholesterol synthesis is reduced (Avoy et al., 1965) and secondly, that the rate of release of newly synthesised lipoproteins (which contain cholesterol) is also diminished (Duncan et al., 1965). An examination of the effect of the drug on the kinetics of the distribution of newly synthesised cholesterol in the perfused liver preparation may allow further interpretation of the mode of action of the drug. This would be achieved by manipulation of the normal model to accommodate the experimental data derived from perfusions in which the drug was used.

Two types of experiments were carried out to investigate the mode of action of C.P.I.B. The first type was an acute experiment where the effect of the drug on cholesterol metabolism in a normal rat liver perfusion was examined by adding C.P.I.B. to the perfusate during perfusion. In the second type, termed chronic experimentation, the liver donor animals were pretreated with the drug for periods of up to three weeks.

In both these groups of experiment two different precursors of cholesterol were administered. This was considered important because Avoy et al. (1965) have shown that inhibition of cholesterol biosynthesis by C.P.I.B. takes place at a point prior to the reduction of HMG-CoA to mevalonic acid. The use of sodium (^{3}H) acetate and (2^{14}C)DL mevalonic acid in the perfusion system allowed examination of the kinetics of cholesterol biosynthesis and transport, under the influence of C.P.I.B., when the

cholesterol had been labelled by using precursors which were incorporated before and after the rate limiting step in the biosynthesis.

Acute experiments.

Perfusion was established with a liver taken from a normal rat. After 10min perfusion, a solution of chlorophenoxyisobutyrate, sodium salt, in 0.1M phosphate buffer (pH 7.4) was added to the perfusate to produce a final concentration of 200µg/ml of perfusate. This is the serum level found in rats by Thorp and Waring (1962) after administering the drug as a 0.25% supplement in the diet. At the same time 4µCi (2^{14}C)DL mevalonic acid and 10µCi sodium (^{3}H) acetate were also added. Sampling of the perfusate and the determination of the specific radioactivity of the various cholesterol containing fractions was performed as described in the methods section. Two experiments were performed and Fig. 78 shows the average specific radioactivity curves for ^{14}C labelled cholesterol. The general shape of the curves were similar to those found in perfusions using normal rat livers. Statistical tests showed no significant differences between curves obtained from normal and drug treated livers. The curves found in C.P.I.B. experiments did however, fall below the mean curves obtained in the 'normal' set of perfusions. These data are similar to the findings of Azarnoff et al., (1965) who demonstrated a decrease in the rate of release of cholesterol into the perfusate of isolated rat livers. They concluded that the decrease was not statistically significant. Since the small differences in specific radioactivity were not significant no attempt was made to try to fit the curves to the model by manipulating the rate constants.

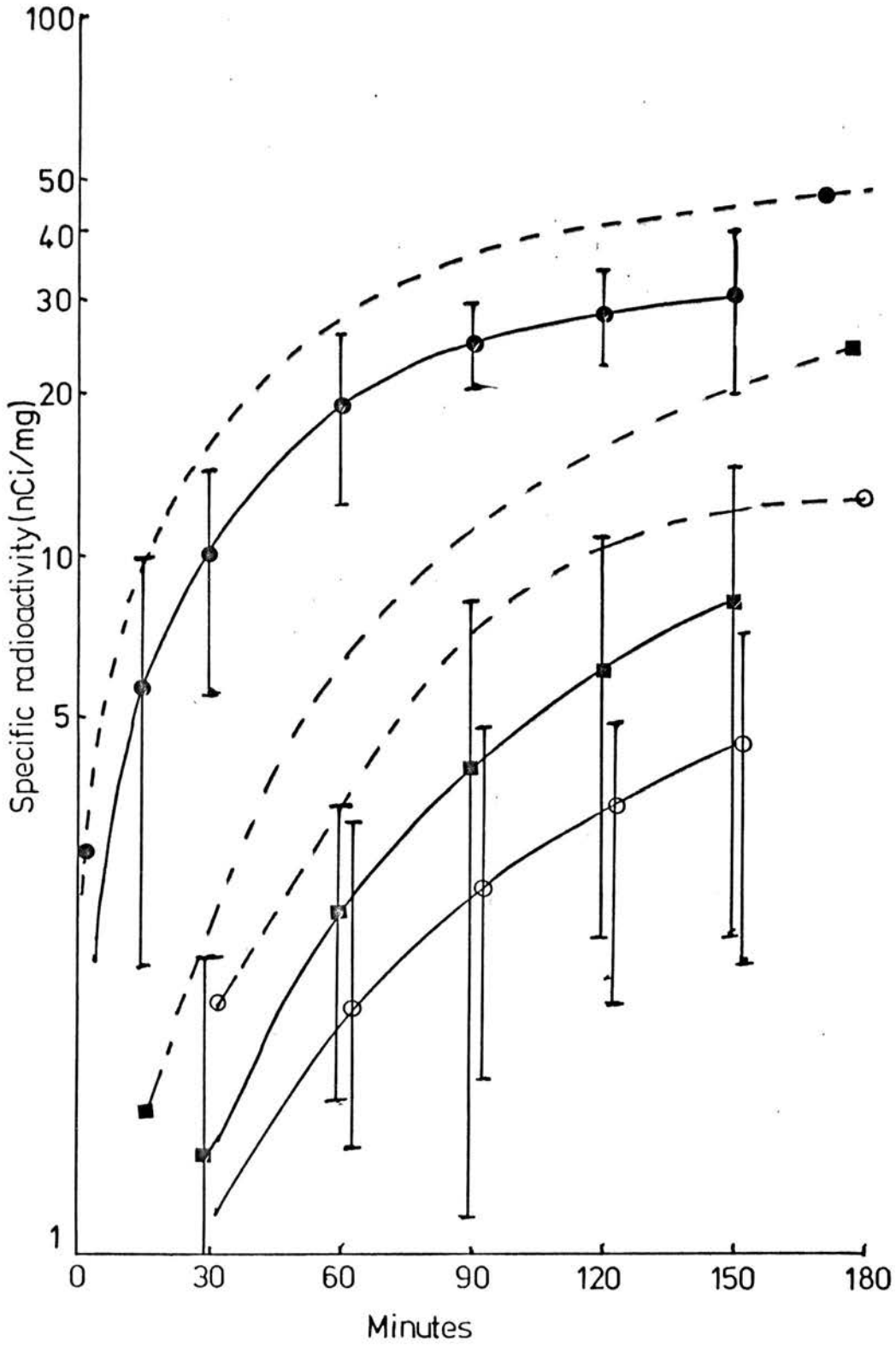


Fig.78. Changes in the specific radioactivity of (^{14}C) cholesterol in the plasma unesterified(●), esterified(○) and erythrocyte(■) compartments during normal liver perfusion when CPIB had been added to the perfusate. Dashed lines show curves obtained without any drug present.

The specific radioactivity curves of ^{3}H labelled cholesterol following the addition of C.P.I.B. were quite different from those found during normal perfusions (Fig.79), and confirm the findings of Avoy et al. (1965) and of Dexter and Stanley (1970) who showed marked inhibition of incorporation of sodium (^{3}H) acetate into cholesterol, both in vivo and in vitro. The interesting feature of the experiments reported here was that the effect of C.P.I.B. on sodium (^{3}H) acetate incorporation seemed to be immediate and did not require extended exposure times of the liver to the drug. Thus the specific radioactivity of the plasma unesterified cholesterol stopped increasing after about 25 - 30min.

The rate at which plasma cholesterol reached equilibrium with that of the liver was similar to that found in normal rats (Fig. 80).

Cholesterol biosynthesis in the liver, estimated from the incorporation of sodium (^{3}H) acetate was decreased by about 85% as judged by the relative radioactivity values obtained for liver cholesterol in normal and C.P.I.B. treated livers, Table 25. Avoy et al. (1965) found smaller reductions using rat liver slices. The specific radioactivity of liver unesterified cholesterol labelled with carbon-14 was not significantly different from the values found for the livers of normal rats after perfusion ('t' test $p = > 0.20$)

The average ($n = 2$) amount of cholesterol excreted in the bile was 11.25µg/h. This was significantly less than the excretion found in normal rat livers during perfusion, this rate being 48.5µg/h. The specific radioactivity of the biliary cholesterol was also lower than for normal rats, having a mean value of 11.50nCi/mg as compared to that found in the normal rat of 23.28nCi/mg.

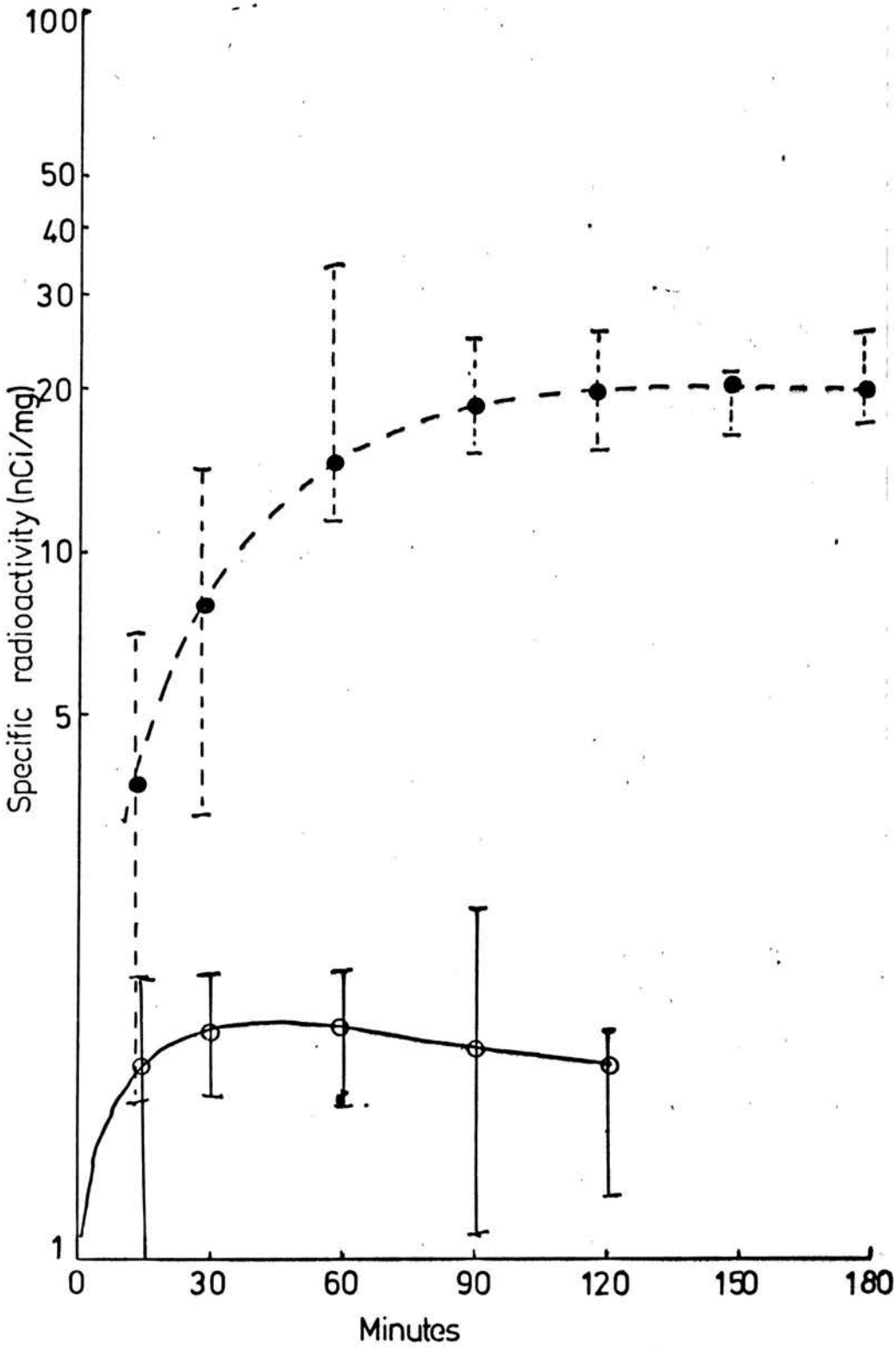


Fig.79. Changes in the specific radioactivity of unesterified (^{3}H) cholesterol in the plasma during normal liver perfusion (●) and after the addition of CPB to the perfusate(O).

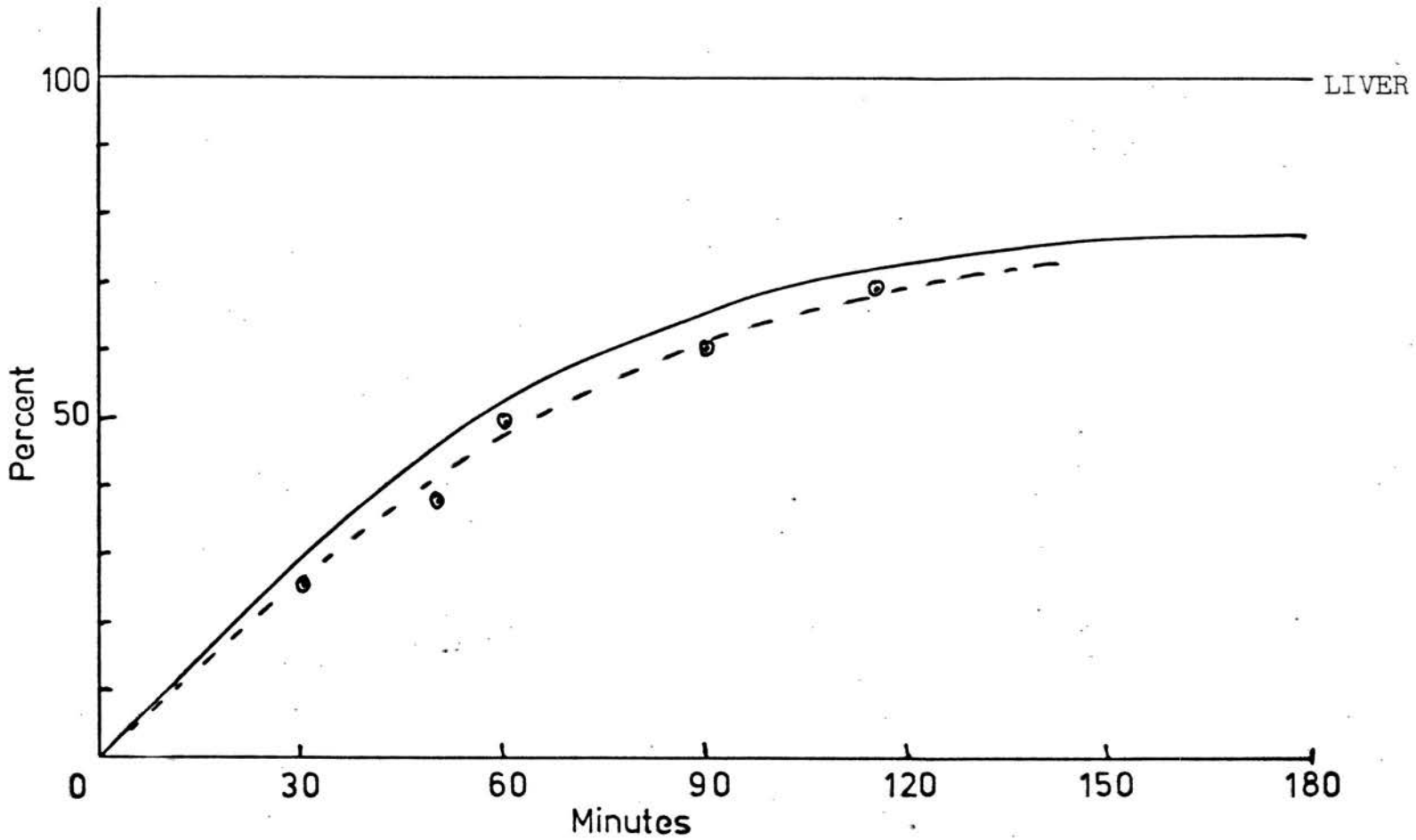


Fig.80. Percentage proportion of the specific radioactivity of unesterified (^{14}C) cholesterol in the plasma to that in the liver during perfusion. Solid line obtained for normal liver perfusion, dotted line obtained when CPIB added to the perfusate.

	Normal (3)	C.P.I.B. (Acute)(2)	C.P.I.B. (Chronic)(2)
Mevalonate ^{14}C	74.89	80	79
Acetate ^{3}H	31	3.5	8.6

Table 25

A comparison of the specific radioactivities (nCi/mg) of unesterified cholesterol in the perfused livers from normal and C.P.I.B. treated rats.

The small number of acute experiments (2) carried out does not permit reliable interpretation of these data.

Chronic experiments.

Female rats (200 - 240 grams) were fed on the soft diet supplemented with 0.25% C.P.I.B. for a period of three weeks. This dose level has been shown to have a maximal effect on serum cholesterol levels without producing any toxic effects (Thorp and Waring, 1962). The drug was dissolved in ether and sprayed onto the surface of the diet with continuous mixing. The ether was then allowed to evaporate (60°C) and a final thorough mixing performed before using as a stock diet. Perfusion was performed as described previously, using livers from the treated animals. The blood used as perfusate was also obtained from pre-treated animals by cardiac puncture.

Figs. 81 and 82 show the specific radioactivity curves obtained for ^{14}C and ^{3}H labelled cholesterol. The incorporation of (2^{14}C)DL mevalonic acid into cholesterol was not statistically significantly different from that obtained in the acute experiments or in the experiments in which normal livers were perfused. The incorporation of (^{3}H) acetate was markedly reduced and was similar to the results found in the acute experiments(Table 25). The rate of equilibration of ^{I4}C labelled cholesterol between plasma and liver appears to be more rapid than in the acute experiments, Fig.83 (compare with Fig.80).

The mean specific radioactivity of the plasma unesterified cholesterol was significantly higher ($p < 0.005$) in the chronic experiments than in the acute experiments. A similar difference was found for plasma esterified cholesterol ($p < 0.0I$). Average biliary excretion of cholesterol was similar to that found in the acute experiments being 8.2μg/h.

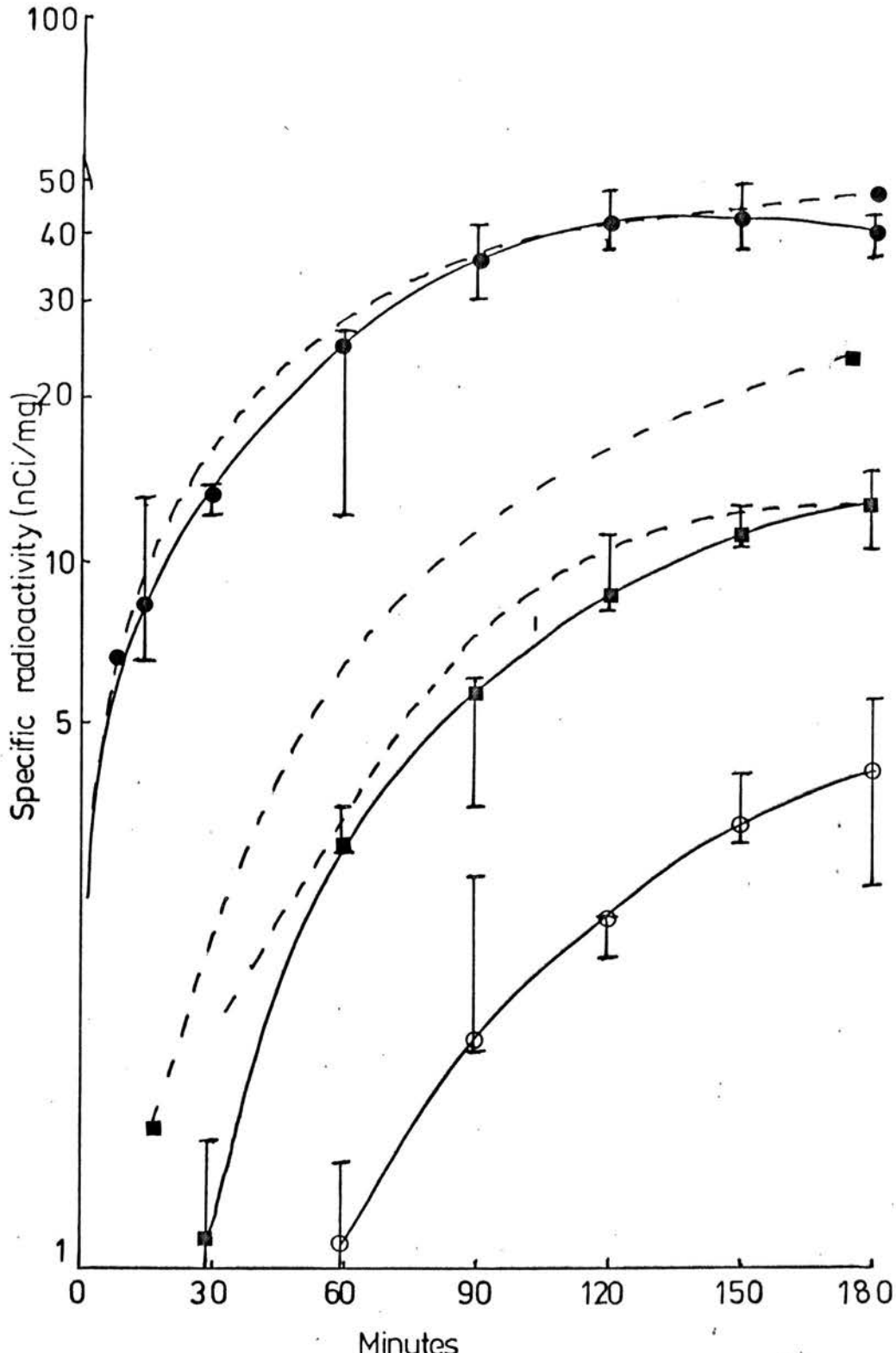


Fig. 8I. Changes in the specific radioactivity of (^{14}C) cholesterol in plasma unesterified(●), esterified(■) and erythrocyte compartments during perfusion of livers from rats which had been pretreated with CPIB. Dashed lines show curves obtained for normal liver perfusion.

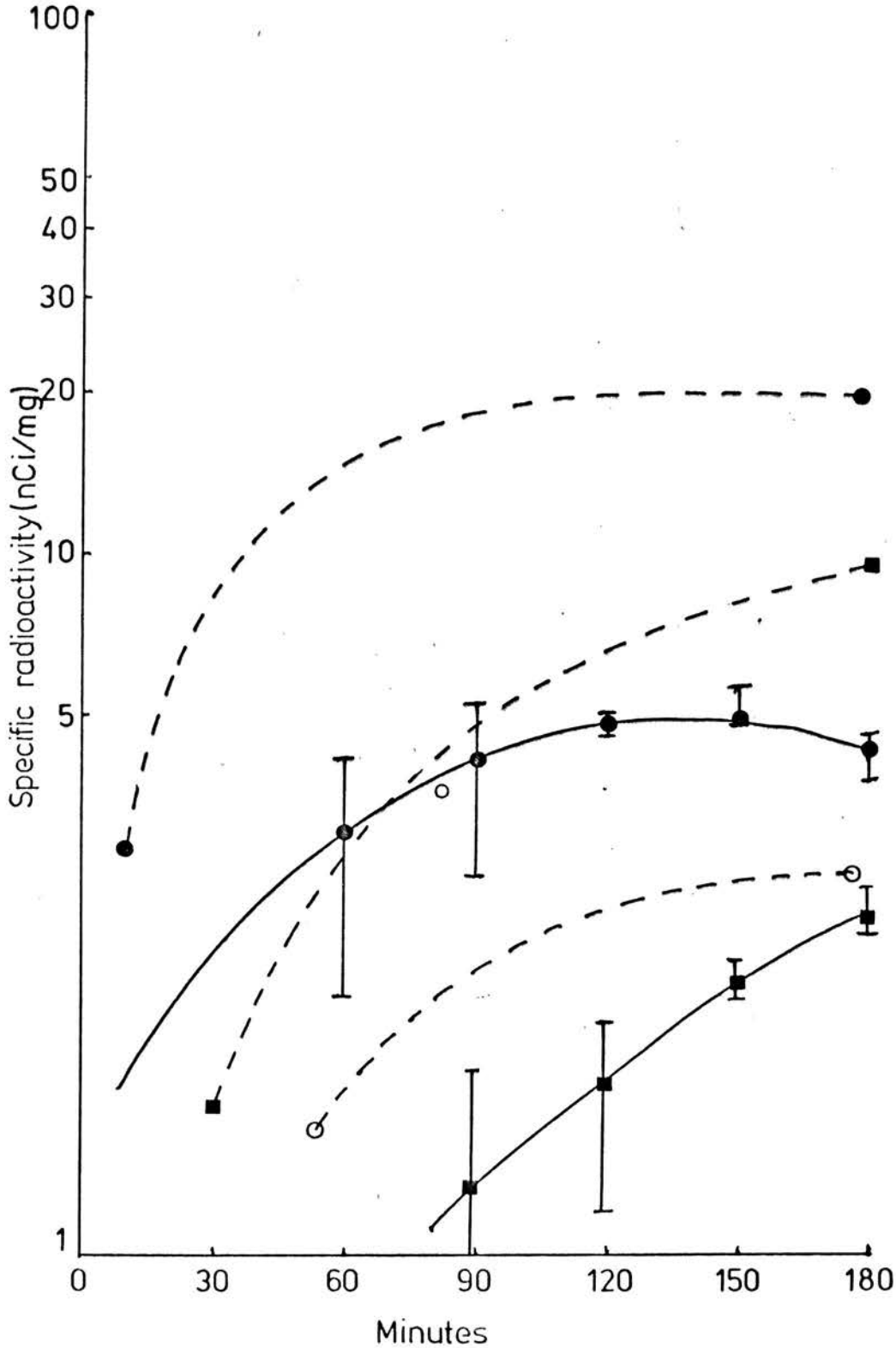


Fig.82. Changes in the specific radioactivity of (^{3}H) cholesterol in plasma unesterified(●) and erythrocyte(■) compartments during perfusion of livers taken from rats which had been pre-treated with CPIB. Dashed lines obtained during normal liver perfusion.

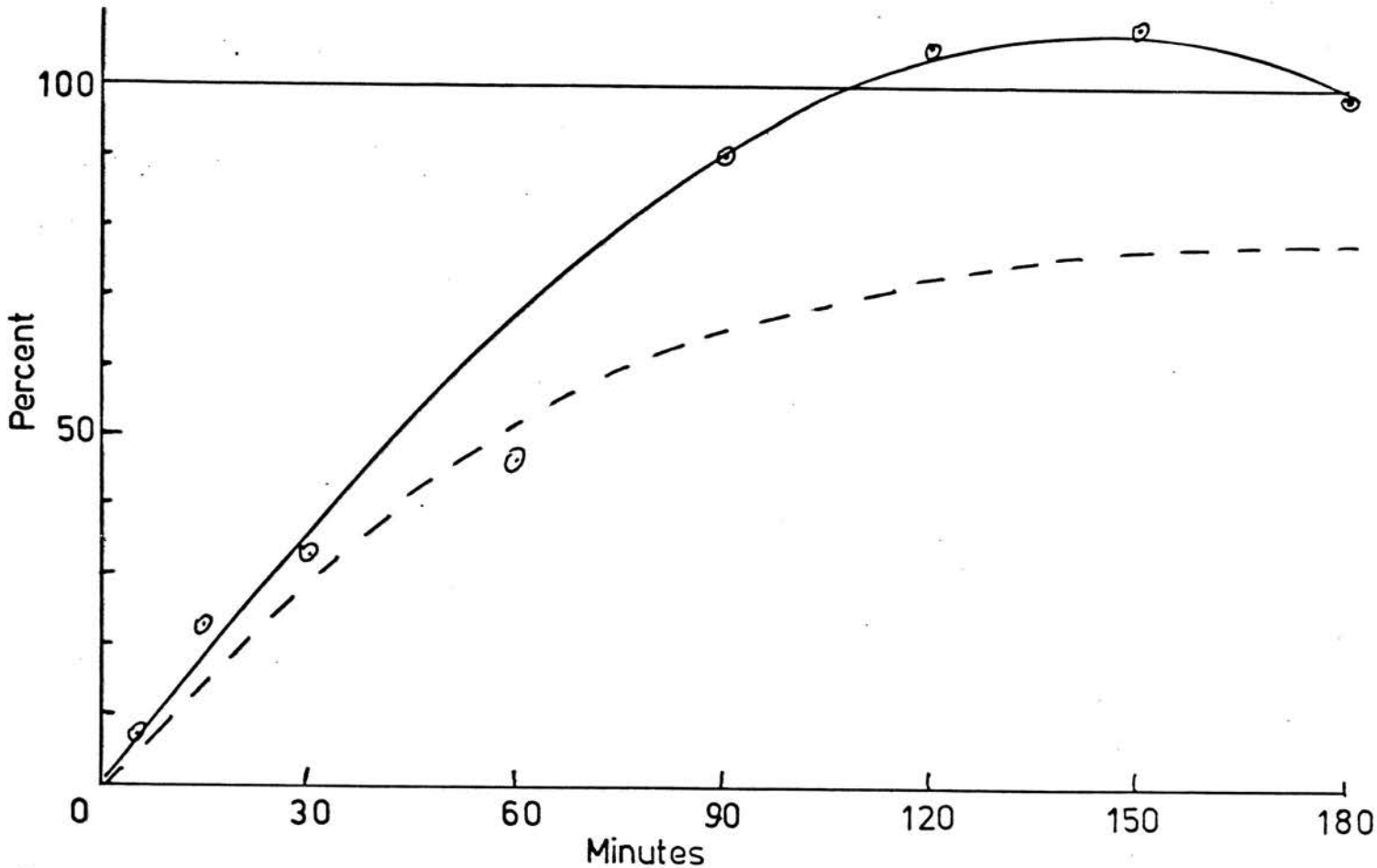


Fig.83. Percentage proportion of the specific radioactivity of unesterified plasma (^{I4}C) cholesterol to liver (^{I4}C)cholesterol during perfusion of livers taken from rats which had been pre-treated with CPIB. Dashed line obtained for normal liver perfusion.

DISCUSSION

Liver perfusion

In attempting to obtain information about conditions in vivo using isolated organ techniques, it is of prime importance that the in vitro preparation should simulate the conditions existing in vivo. To obtain qualitative information, e.g. to establish metabolic pathways, then the maintenance of in vivo physiological and biochemical conditions is not so important as when quantitative information is sought. When dealing with the liver, the functional reserve is usually so great as to make extended perfusion possible, if only qualitative information is required, even if parts of the organ are not viable. For an examination of whole liver function from a quantitative standpoint it is desirable that biochemical and anatomical relationships should be preserved.

There are reports of rat liver perfusion where the perfusion periods ranged from three hours (Morris and French, 1958), to twenty five hours (Brauer et al., 1951). Many of these studies did not fulfill the criteria for an adequately functioning liver. In this study the minimal requirements for the acceptance of results of experiments using isolated liver perfusion were:-

1) a continuous production of bile

2) a constant blood flow which, although allowed to oscillate, should not require continual increases in perfusion pressure to maintain the constant value.

3) well marked deoxygenation of the arterialised blood perfusate.

4) normal histology at the end of the perfusion period.

Several studies have been reported where the liver has been perfused but in which the bile secretion rate has not been given. For example, Lueck and Miller (1970) perfused for five hours without measuring bile flow rates and Ryoo and Tarver (1968) carried out perfusions for twelve hours even though the secretion of bile had failed after six hours. This type of study is considered unacceptable for examining kinetic problems. Brauer et al. (1951), using bile secretion as an index of survival, reported that secretion is positively correlated with the weight of the rat and found adequate secretory rates in livers from heavier rats (270 - 300g) for periods of up to ten hours. These authors used an artificial medium as perfusate. Using whole blood as perfusate there is good evidence that, on average, survival time judged on the basis of bile flow rate, histological appearance and several metabolic functions (Percy-Robb and Boyd, 1970), is between three and four hours. It is of interest in this context that Morris and French (1958) carried out perfusions for only three hours in their investigations on the metabolism of chylomicron fat, but had been able to perform perfusions for up to eight hours in their preliminary studies. This suggests that they felt that in vitro perfusions for more than three hours would not produce reliable information about in vivo conditions.

Gibbon (1959) performed perfusion experiments on dogs with the liver retained in situ. He then reconstituted the anatomical relationships and showed in 'reconstituted' animals after three hours perfusion that the survival rate was only 50% indicating features of irreversible damage. In a similar study in dogs, Daly et al. (1954) reported distension and oedema of the liver with a watery vacuolation of the parenchymal cells and with many of the Kupfer cells detached and necrotic. Perfusion was again for periods of up to three hours.

Several types of perfusion media have been used. These range in complexity from saline (Trowell, 1942) to whole heparinised rat blood (Percy-Robb and Boyd, 1970). One disadvantage in the use of whole blood is that perfusions of livers from small animals are limited to the use of pooled homologous blood. In order to obviate this difficulty many authors have diluted blood with saline or Krebs-Ringer solution and dilutions of 1/3 - 1/2 have been commonly used (Nestel and Steinberg, 1963; Lueck and Miller, 1970). These dilutions have also permitted improved perfusion rates since the viscosity of undiluted blood has appeared to be a barrier to adequate and well maintained perfusion. Because this study set out to examine factors affecting the equilibria in the complex inter-relationships between the fractions containing cholesterol in the blood and liver system, it was felt necessary to use whole blood as the perfusing medium. It was considered that, if a partially synthetic medium had been used, then the rates of transfer of cholesterol from the liver might have been very much dependent upon the composition of the perfusate.

The necessity to use whole blood in the perfusion apparatus raised a number of experimental problems. These were related to the comments of Gibbon (1959) who suggested that the most important factor in the maintenance of a viable perfusion was not one of metabolic homeostasis but one of damage to the elements of the blood which adversely affected the circulation and indirectly the metabolic status of the perfused organ. The main problem in the study reported here was of erythrocyte destruction by mechanical abrasion.

This effect was partially overcome by the selection of optimal pumping and oxygenating conditions so that haemolysis was minimised at the same time as maintaining a good flow of oxygenated blood to the liver. Under the

conditions of this study 1.6% of the blood cells were disrupted every four hours. This was similar to the values reported by Ostashever et al. (1960), Percy-Robb (1967) and Seglen and Jerrel (1969). With more sophisticated equipment it should be possible to reduce this rate as was shown in the study of Cahill and Kolf (1959) during extracorporeal perfusion in man, where only 0.1% haemolysis occurred.

Several methods of oxygenation were tried in combination with different pumping modules and it was concluded that a stationary thin-film oxygenator caused least haemolysis. Whenever possible, glass tubing or glass surfaces were used in the blood circuitry because of adverse reports about the effects of substances leached from plastic upon the organ under perfusion (Duke and Vane, 1968). Plastic (Tygon) tubing has also been shown to be thirty times more harmful than glass in producing haemolysis in canine blood, after exposure times of two hours (Hirose et al., 1963).

Continuous filtration of the perfusate always showed evidence of protein denaturation and thrombus formation by the accumulation of debris on the surface of the filter at the end of the perfusion. The filter assembly was of the low volume, high surface area type allowing comparatively slow flow rates across the mesh which had a pore size of 40u.

On physiological grounds it is desirable that pulsatile flow should be applied to any organ undergoing perfusion. Ogata et al. (1960) examined the haemodynamic and biochemical alterations induced in whole animals by pulsatile or steady blood flow and concluded that those animals with pulsatile flow remained more nearly normal than those in which a steady flow was used.

The perfusion pressure used was selected on the basis

of the report by Brauer et al. (1954) who found a relationship between bile flow and perfusion pressure or perfusate flow. When the perfusion pressure had reached 12cm of perfusate, the bile flow was approaching a stable value and at perfusate flow rates above 20ml/min the bile flow rate was again reaching a stable value. If, however, the perfusate flow rate dropped to below 8ml/min then there was a marked reduction in bile flow rate. In another report (Brauer et al., 1951), blood flow rates in vivo were estimated to be between 8 and 12 ml/min, although this was at variance with a study from the same group (Brauer et al., 1953) when in vitro flow rates of 50 - 70ml/min were found. However, in the latter study a synthetic medium was used for perfusion.

Direct comparison with other studies in assessing liver function is difficult because of the different conditions of perfusion. Nevertheless, conditions in this study appear adequate as judged by the information presented in Table 26. Perfusion flow rates and pressure, bile flow, perfusion time and the ability of the liver to synthesise glucose and urea in this study were similar to many of the conditions used by previous investigators. This perfusion system was based upon the earlier work of Percy-Robb and Boyd (1970) who carried out a detailed examination of the biochemical functions and anatomical relationships in the liver and concluded that for periods of up to four hours the in vitro preparation was suitable for use as a model of behaviour in vivo.

In order to simulate the normal dynamic equilibria which exist in vivo it was desirable that the blood volume should approximate to that of a single rat. For example, consider a first order process of synthesis of some substance in the liver. Its relationship with the blood is shown diagramatically in Fig. 84.

Table 26. Showing conditions of perfusion used by a number of investigators.

Period of Perfusion(h)	Perfusate Volume (ml)	Perfusate Pressure(cmH_2O)	Perfusate Flow (ml/m)	Bile Volume ml/h	Glucose	Urea	Author
1	50	2 - 3	6 - 8	-	-	-	Heimberg et al. (1958)
3	25	15	10 - 15	0.44			Burton et al. (1960)
4 - 5	-	11	160	-	-	-	Kay and Entenman (1961)
3 - 4	120	-	12 - 15	0.5		-	Schiff and O'Donnell (1962)
4	200	-	16 - 19	0.5	-		Ostashever et al. (1960)
up to 10	140	-	9 - 11	0.2 - 2.0	-	-	Ryoo and Tarver (1968)
4	135	13.5	12.5 - 21	1.0	-	-	Swell et al. (1968)
3	100	18	8 - 12	0.2 - 0.5	-	-	Morris and French (1958)
12	1000(rabbit)	30	150	0.3 - 1.4			Young et al. (1955)
4	200	21.5	150 - 200	0.3	-	-	Tuttle et al. (1962)
2	25	16	8.4 - 11.3	22.0		-	Seglen and Jervell (1969)
4	20	14	9 - 19	0.25			Percy-Robb (1967)
3	50	16	8 - 14	0.3			This study
In vivo							
-	-	-	-	0.46 - 0.75	-	-	Byers and Friedman (1952)
-	-	-	-	0.5	-	-	This study

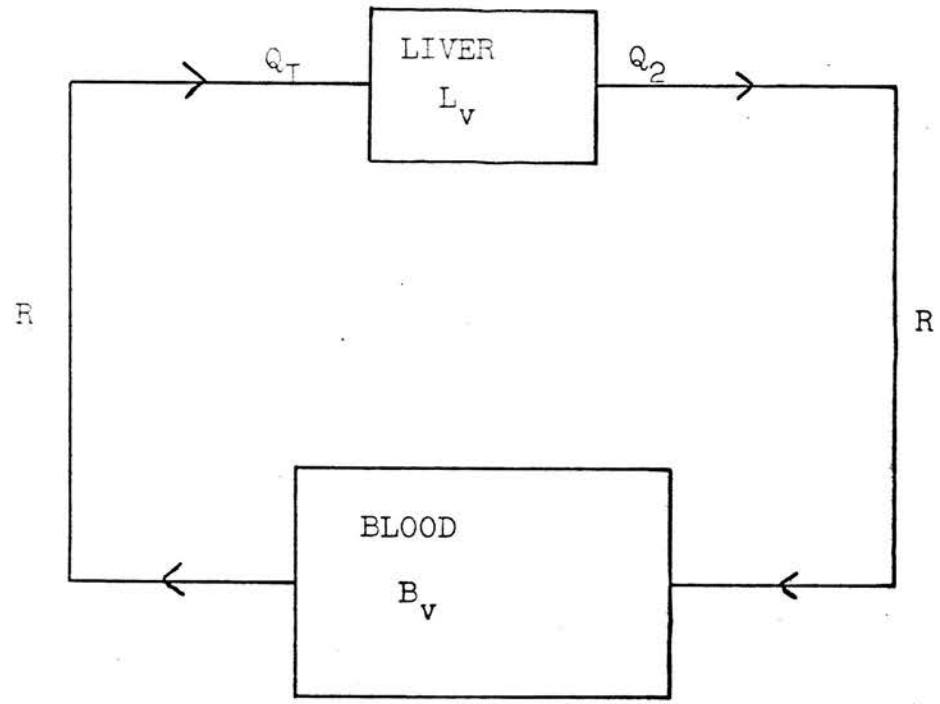


Fig.84. Schematic diagram of the synthesis of substance Q in the liver and its distribution and circulation in the blood. L_v= vascular volume of liver, B_v= volume of extrahepatic blood, R= rate of flow of perfusate, Q_I= concentration of Q entering the liver, Q_2= concentration of Q leaving the liver.

Then:-

1) $\frac{B_v \, dQ_1}{dt} = R(Q_2 - Q_1)$ Rate of change of concentration of Q_1 in the blood is equal to the blood flow rate multiplied by the concentration difference across the liver.

2) $Q_2 = Q_1 e^{kt}$ where k is the first order rate constant for the synthesis of the substance.

3) $t = \frac{L_v}{R}$ t is the transit time of the substance through the liver.

4) $\frac{dQ_1}{dt} = \frac{R}{B_v} (Q_1 e^{kt} - Q_1)$ rearranging 1) and 2)

Substitute for t (given by 3)

5) $\frac{dQ_1}{dt} = \frac{R}{B_v} (e^{kL_v/R} - 1)Q_1$

6) $\frac{Q_1}{Q_1} = k \text{ observed} = \frac{R}{B_v} (1 + \frac{kL_v}{R}) - 1$

(if $\frac{kL_v}{R}$ is small

Maclaurin's Theorem for exponential series)

$= \frac{R \, kL_v}{B_v \, R}$

7) $= K \frac{L_v}{B_v}$

i.e. The observed rate constant is a function of the reciprocal of the blood volume. Therefore in attempting

to establish rate constants for cholesterol transfer within the blood and liver system the blood perfusate volume should be 15 - 20ml, which is approximately the blood volume of a rat in the weight range 180 - 250g.

It was clearly impossible to use such a small volume in the study reported here because a reasonable number of samples of perfusate had to be taken from the in vitro system. A total of about twelve samples were taken, each with a volume of up to 2ml and thus amounted to a total removal of about 24ml perfusate. The unavoidable dead space in the perfusion apparatus was about 10ml, and some additional volume was necessary to allow for manipulation of the equipment (this amounted to another 10ml of blood). In addition a further 5 - 8ml of blood was used to prime the apparatus. This was done to allow for losses into the thorax of the donor rat during cannulation of the liver. The total priming volume of the equipment was therefore about 55ml and once perfusion was established, about 50ml.

A difficulty thus arises in the interpretation of the calculated rate constants because the values would be smaller than if the calculations had been derived on the basis of a 20ml volume, i.e. the blood volume of the normal adult rat. A second problem derives from the relationship shown in Equation 7 (p I25) because multiple sampling from the perfusate will cause changes in the observed rate constant for each time point unless corrections are made to accommodate this blood volume change. Such a correction was made in the perfusion experiments by taking account of the progressively decreasing pool sizes which occurred throughout the perfusion.

In this study it was assumed that the rate of incorporation of mevalonic acid into cholesterol was rapid; in the perfused rat liver undergoing non-cyclic perfusion data were presented (Fig. 66) which would suggest that the uptake of mevalonic acid into the liver, and the synthesis of cholesterol from this precursor, can be considered as a single stage process in a kinetic sense. Thus the specific radioactivity of the labelled cholesterol did not increase appreciably after the initial rise. This suggested that all of the active isomer of mevalonic acid which was taken up from the perfusate was incorporated into cholesterol. If this were not so, then the amount of mevalonic acid available in the hepatocyte would increase with time and, assuming first order kinetics for the synthesis of cholesterol, the specific radioactivity of the intracellular cholesterol would increase. This in turn would be reflected in an increasing specific radioactivity of the plasma cholesterol. However no significant change was observed after the initial rise.

During non-cyclic perfusion the time taken for a stable specific radioactivity value for cholesterol to be established is a rough indication of the time for the whole vascular bed of the liver to be perfused. This is true only if the uptake of mevalonate, synthesis of cholesterol and transfer of the newly synthesised cholesterol into the blood perfusate can be considered to be very rapid and do not constitute rate limiting steps. With this proviso Fig. 66 indicates that between 3 and 4 minutes are needed to flush the whole liver vascular system. Assuming a perfusate flow rate of 12ml/min with a total liver volume less than 10ml, then the whole vascular bed should be perfused in less than one minute. The data presented in Fig.66 would therefore suggest that some of the perfusate was recycling faster than the whole perfusate and there was an element of haemostasis present in the vascular bed.

Studies in Normal Rat Liver.

I In vivo

Acetate is only incorporated into cholesterol to a minor degree *in vivo*. For example, Gould *et al*. (1955) showed in man that only about 1% of an oral or intravenous dose of ^{14}C acetate was utilised for cholesterol synthesis *in vivo*. In marked contrast virtually all mevalonic acid can be considered to be incorporated into cholesterol (Goodman, 1963). This is because it lies beyond the rate limiting step of the conversion of β -hydroxy- β methyl glutaric acid to mevalonate in the synthetic pathway leading to cholesterol.

The results of the *in vivo* experiments (Table 19) showed maximum incorporation of the label after one hour into liver and plasma cholesterol and after two hours into erythrocyte cholesterol. The delayed incorporation found in erythrocytes was expected because it is simply a function of the rate of appearance of radioactive cholesterol in plasma. These results are consistent with the report of Elwood and Van Bruggen (1961) who examined the time course of mevalonate metabolism *in vivo*. They found that within 30min of an intraperitoneal injection of (2^{14}C)DL mevalonic acid 47.6% of the biologically active isomer was incorporated into the non-saponifiable lipid fraction. The liver fraction reached a maximum within 30min whereas the maximum incorporation into other tissue lipids was found after at least two hours. This late maximum was probably related to two factors, firstly an active redistribution of lipid labelled in the liver into other tissues, and secondly extrahepatic synthesis proceeding at a slower rate than that in the liver. The rapidity of the conversion of mevalonate to cholesterol is supported by the study *in vivo* of Berkowitz *et al*. (1963) who found that 50% of the injected mevalonate had been

decarboxylated within thirty minutes and had proceeded along the metabolic pathway as far as squalene. Chesterton (1968) however found only 3.2% of a dose of intravenous mevalonate had been incorporated into liver lipid within thirty minutes. This lower recovery may be related to the mode of administration of the labelled precursor. Intravenous injection would allow a more rapid assimilation into cholesterol in the organs having the most active synthesis rates. Consequent upon this rapid synthesis would be an equally rapid redistribution of labelled cholesterol throughout the body and thus an early peak in the amount of label in the liver cholesterol may be missed by sampling at thirty minutes. However the model of the perfusion experiments performed in the study reported here suggested that the mevalonic acid precursor may be utilised over quite a long time period. This could extend up to 180min after addition of the mevalonic acid into the blood, whether in vivo or in vitro.

The results of the in vivo experiment showed that after the maximal specific radioactivity of each compartment had been achieved it then declined steadily as a function of time, presumably as isotopic equilibrium was established with the extrahepatic tissues. In the early part of the experiment the liver unesterified cholesterol had the highest specific radioactivity followed by plasma unesterified cholesterol, liver esterified cholesterol, plasma esterified cholesterol and erythrocyte cholesterol in descending order. Similar curve relationships were found by Gould et al. (1955) and by Eckles et al. (1955) in their studies on dogs. They found radioactive equilibrium between plasma and liver unesterified cholesterol occurred between two and three hours after administration of (^{14}C) acetate, which compares well with the time period found here of about 2.25h.

The specific radioactivity curves of the plasma and liver unesterified cholesterol in the *in vivo* experiment then crossed and the specific radioactivity of the unesterified plasma cholesterol was still greater than that of unesterified cholesterol in the liver at the termination of the experiment. This suggests that either there is a pool of plasma unesterified cholesterol which turns over more slowly than other plasma pools (possibly an α lipoprotein) or that the plasma activity is being supplemented by labelled cholesterol derived from synthesis in extrahepatic tissues. The latter explanation is probably the most likely one.

The unesterified cholesterol in the plasma and in the erythrocytes had not reached isotopic equilibrium by the end of the *in vivo* experiment (3h) which is in agreement with the report of Eckles *et al*. (1955) but at variance with the *in vitro* results reported here. The *in vitro* experiments which examined the exchange of cholesterol between plasma and erythrocytes in isolation from other tissues showed that equilibration occurred within 4h. The discrepancy is probably related to the closed system used for the *in vitro* experiments where no loss of labelled cholesterol occurred by exchange with other tissues. The investigation performed *in vivo* by Gould *et al*. (1955) in man extended over a longer time period (up to 48h after administration of oral acetate) and they were able to show equilibration of unesterified cholesterol in plasma and erythrocytes in 10h.

When considering esterified cholesterol in the plasma or liver a peak specific radioactivity was recorded at one hour. (Table 19). In the earlier studies of Eckles *et al*. (1955) in dogs and Gould *et al*. (1955) in man, the specific radioactivity of the plasma ester cholesterol either rose steadily or, after rising, remained at a constant level.

The absence of a peak specific radioactivity may be related to the presence of a cholesterol ester hydrolase. (Such a hydrolase is known to be present in dog plasma, Swell and Treadwell 1950). A similar peak radioactivity for plasma cholesterol ester was reported by Porte and Havel (1961) in their studies in dogs but this occurred at about 18h after the start of their in vivo experiments.

This early maximum found for the specific radioactivity of the plasma ester cholesterol is almost certainly related to the species used as the experimental model. There is still some doubt as to the primary source of the plasma cholesterol esters in man, but in rats the liver has been shown to release cholesterol esters into the perfusing blood (Roheim et al., 1963).

It is apparent that the synthesis and decay curves of the liver cholesterol fractions rise and fall together (Table 19) and this is also evident for the plasma compartments. Unesterified cholesterol is released from the liver and since the decay curves for both ester and unesterified cholesterol are similar then this might be taken as support for the release of cholesterol ester also. Equilibrium between unesterified and esterifed plasma cholesterol has not been reported within seven hours and this study confirms these findings.

There is a distinct possibility that the rate at which liver and plasma cholesterol reach equilibrium may be governed by events taking place within the hepatocyte. For example, several intracellular components are involved in the synthesis of cholesterol but the actual site of completion of synthesis is unknown. With such intimate anatomical location it might be expected that the whole cell acts as a homogeneous pool of cholesterol. The results of the in vivo experiments suggest some element of compartmentation of intracellular cholesterol. Thus

after an intraperitoneal injection of radioactive mevalonic acid the cholesterol in the microsomal and mitochondrial fractions of the liver cell reached equilibrium with the cholesterol in the plasma after about 180min (Fig. 68). However, the specific radioactivity of the cholesterol in the nuclear fraction was above that of the plasma throughout the experiment. It is likely therefore, that there is a pool of cholesterol in the nuclear material which turns over more slowly than cholesterol in other intracellular organelles. By contrast the cholesterol in the soluble fraction achieved very rapid equilibrium with plasma cholesterol in about 15min (Fig. 68). The specific radioactivity of plasma cholesterol then remained above that of the soluble fraction for the remainder of the experiment. There was also a marked difference between the specific radioactivity of cholesterol in the soluble fraction and that in the other intracellular organelles. Thus, in the early part of the experiment the specific radioactivity of the soluble fraction was about half that of the other intracellular organelles: by the end of the experimental period (4h) the ratio had increased considerably and approximated to 0.75.

Overall these results would suggest three pools of cholesterol in the liver cell, a pool associated with the nuclear fraction, one with the combined microsomal and mitochondrial fractions and finally a pool of cholesterol in the soluble fraction. The pool of cholesterol in the soluble fraction is probably the direct precursor of plasma cholesterol.

Other investigators have also found evidence of intracellular compartmentation using ^{14}C labelled mevalonic acid as precursor; Pronczuk and Fillios (1968) were able to demonstrate differential rates of

incorporation of label into the cholesterol in rough or smooth endoplasmic reticulum. They also reported that the peak specific radioactivity in the microsomal fraction occurred between 20 and 40min after administering the label. This is similar to the results reported here (Fig.67) The in vivo experiments were of short duration (4h). At the end of the experiment the microsomal and soluble fractions had not reached equilibrium and a study by Hollander and Kramsch (1967) using dogs would suggest a very slow rate of exchange between these two fractions. They showed that 28 days after administering radioactive cholesterol there was a significant difference between the specific radioactivity of the microsomal fraction and the cell supernatant or soluble fraction.

The results presented by Chesterton (1968) showed that not only is there a difference in specific radioactivity of cholesterol in the microsomal and soluble fractions, but that there is also a marked difference between the microsomal and the 'nuclear fraction' soon after injection of (^{14}C) mevalonate. This latter difference tended to disappear after 30min. In contrast the difference in specific radioactivity of cholesterol in the mitochondrial and microsomal fractions actually increased over the time period used (30min). This is at variance with the results reported here and by Swell et al. (1968) where the microsomal and mitochondrial cholesterol had closely similar specific radioactivity values over a four hour time period (Fig. 67).

The study of Swell et al. (1968) was performed in vitro using an isolated perfused rat liver preparation. They found that the plasma unesterifed cholesterol did not reach equilibrium with the liver microsomes or mitochondria during the four hour liver perfusion. The

results of the *in vivo* study reported here showed the equilibration to be almost complete after 150min (Fig.68), but as this investigation was *in vivo*, the results are not strictly comparable because of synthesis of cholesterol by extrahepatic tissues which also equilibrate with the blood cholesterol pool. However the *in vitro* perfusion studies performed here confirm the results obtained *in vitro* by Swell *et al*. (1968). It was found that the specific radioactivity of unesterified cholesterol in the whole liver cell was greater than that of the plasma after three hours of perfusion.

In conclusion it would seem that the intracellular compartmentation of cholesterol is a real phenomenon, although some disagreement exists as to the actual sequence of steps in the exchange processes.

II In vitro

During the perfusion of the isolated liver taken from a normal rat the actual mass of cholesterol synthesised over a period of 3 - 4h was negligible in comparison with the mass of cholesterol present in the system. The perfusate cholesterol concentration rose in a linear fashion by an average of 17% over a three hour period. This was due to the haemoconcentration taking place as a consequence of the experimental technique because circulation of blood in the apparatus without the presence of a liver produced similar changes . Other authors have found minimal or erratic changes in perfusate cholesterol concentration with time. These inconsistencies are probably a reflection of the different perfusion techniques used (Kay and Entenman, 1961; Percy-Robb 1968).

Surprisingly, the erythrocyte cholesterol concentration did not demonstrate the significant trend which would have been expected if a redistribution of cellular water had taken place. The change in cholesterol concentration in the whole perfusate is therefore probably less than the 17% found for plasma only.

Parallelism was observed for the regression lines defining the changes in total and unesterified cholesterol in the plasma during perfusion (Fig. 42). Subtraction of unesterifed cholesterol from total cholesterol should give an index of the change in ester cholesterol with time. As the regression lines for total and unesterified cholesterol were parallel then the ester regression line must have a zero slope. Therefore because haemo-concentration should equally affect plasma ester and unesterified cholesterol this would imply an actual uptake of plasma cholesterol ester into the liver to maintain a zero slope regression line. This does not preclude an

exchange of ester cholesterol between liver and blood. Such an exchange must occur since the specific radioactivity of plasma ester cholesterol rose during perfusion. It is unlikely that this radioactive esterified cholesterol is derived from unesterified cholesterol within the blood compartment since during incubation *in vitro* of plasma containing labelled cholesterol, the labelled plasma ester did not show changes in specific radioactivity. These changes might have been expected because the specific radioactivity of the unesterified plasma cholesterol was initially much higher than that of the plasma ester cholesterol. The apparent stability of the specific radioactivity of the esterified cholesterol in the plasma confirms the earlier finding of Hagerman and Gould (1951) who demonstrated in their experiments *in vitro* that no significant changes in the specific radioactivity of esterified cholesterol took place in 4h.

According to Sperry (1935) and Glomset *et al*. (1962) there is an active enzyme present in human serum capable of esterifying cholesterol. However their studies extended over a minimum time period of 24h and if the enzyme lecithin cholesterol acyl transferase (LCAT) is active during the first three hours of incubation, then in this study and that of Hagerman and Gould (1951) the shape of the specific radioactivity curve of esterified cholesterol should have been of a quite different shape. Thus in the experiments reported here in which radioactive cholesterol in the plasma exchanged with cholesterol in the erythrocytes, there was no significant change in the specific radioactivity of ester cholesterol in the plasma and it must be concluded that either there is a lag phase in the enzyme action extending beyond the time period of the investigation, or that the experimental conditions were such that the enzyme was rendered inactive.

Similar rate constants for the intravascular formation and hydrolysis of cholesterol esters were obtained in the smaller model (Fig. 31) describing exchange of cholesterol *in vitro* between plasma and erythrocytes. If the reactions (i.e. synthesis and hydrolysis) were simple first order, then on the basis of the differences in the sizes of the esterified and unesterified cholesterol compartments it would be predicted that the rate constants should not be equal. Since they were equal, this would suggest that the amount of cholesterol esters being formed was less than the amount being hydrolysed. However, the rate constants could be equal when other exchanges in the model are taken into consideration. Although the model predicted this exchange it is possible that the data could have been fitted in another way so as to delete this exchange process from the model.

The predicted hydrolysis of cholesterol esters is not born out in the studies of other investigators. Swell and Treadwell (1950) examined the serum of humans, dogs, rats, guinea pigs and rabbits and found that only dog serum contained an enzyme capable of catalysing the synthesis and hydrolysis of cholesterol esters. Although Glomset *et al*. (1962) showed a continuous fall in unesterified cholesterol concentration, this would not preclude the possibility of a hydrolytic enzyme provided its activity relative to that of the synthesising enzyme was comparatively low. On balance the prediction by this model of an intravascular hydrolytic process is suspect.

During incubation *in vitro* of erythrocytes and plasma there were no discernible changes in the mass of esterified or unesterified cholesterol associated with the plasma or the erythrocytes.

This was not surprising because mature mammalian erythrocytes do not synthesise cholesterol de novo (Nelson, 1967).

Murphy (1962) on the other hand, demonstrated a shift of cholesterol into the esterified form within twenty four hours. Cooper and Jandl (1969) maintain that it is this shift of unesterified cholesterol into the esterified form which is responsible for the fall in erythrocyte cholesterol content found in their studies in vitro with normal human blood. An equimolar transfer of the cholesterol into the erythrocytes does not occur even if the total plasma cholesterol is elevated eightfold (Brun, 1939).

The hypothesis is that the lipoproteins in the plasma become depleted of unesterified cholesterol because of the action of L.C.A.T. and thus show an avidity for the unesterified cholesterol in the erythrocytes. Although the study reported here and those of Quarfordt and Hilderman (1970) and Hagerman and Gould (1951) provide no evidence of an interaction of this type, it does seem a reasonable proposal that in vivo in the longer term, the exchange of cholesterol between plasma and erythrocytes should be intimately correlated with the intravascular esterification process. Although there was little change in the specific radioactivity of plasma ester cholesterol during the in vitro experiments where plasma containing labelled cholesterol was incubated with erythrocytes containing unlabelled cholesterol, this was not the case in the perfusion studies. The specific radioactivity of the plasma ester cholesterol rose steadily during perfusion. Whilst it is recognised that newly synthesised unesterified cholesterol is released from the liver, such a mechanism for ester cholesterol in man is

controversial, although Swell and Law (1971) have demonstrated the appearance of cholesterol esters derived from an isolated rat liver, in a synthetic perfusate which had very low levels of L.C.A.T. activity.

The specific radioactivity decay curves, for the compartments examined during perfusion (Fig. 43) showed no maxima and no points of intersection, i.e. isotopic equilibrium was not found between the cholesterol in the plasma ester or unesterified fractions, or the cholesterol in the erythrocyte compartment.

In all perfusions (8) the unesterified plasma cholesterol showed the highest specific radioactivity value at the end of the perfusion followed by erythrocyte cholesterol and by esterified plasma cholesterol. These results are similar to those found in the *in vivo* studies and agree with the *in vivo* investigations of Eckles *et al.* (1955). Because of the restricted time period used in determining the results reported here it was not possible to determine equilibration times between compartments but estimates would be made by extrapolation of the specific radioactivity time curves.

A period of at least 6h would be necessary to achieve equilibrium between cholesterol in the plasma unesterified compartment and that in the erythrocytes. This is similar to the report of Eckles *et al.* (1955) who found that during studies in dogs the cholesterol in the erythrocytes and in the plasma unesterified pool reached equilibrium in 6h. In contrast to this, Hagerman and Gould (1951) and the in vitro study reported here of cholesterol exchange between erythrocytes and plasma showed equilibrium in 3h. This discrepancy can probably be explained by considering the other exchanges available to the plasma unesterified cholesterol compartment *in vivo*.

Quarfordt and Hilderman (1970) quote times for isotopic equilibrium *in vitro* between erythrocytes and high density lipoproteins (HDL) of about four hours and between erythrocytes and low density lipoproteins (LDL) of twelve hours. This is consistent with the results reported here becuase the rat α lipoproteins (i.e. the high density lipoproteins) carry most of the plasma cholesterol (Jevons and Glover, 1968).

Estimates of the time for equilibration to occur between plasma ester and unesterified cholesterol are unreliable but would be in excess of ten hours. Most *in vivo* studies suggest that periods ranging from 20 - 50h are necessary, dependent upon the species considered. The initial rise in the specific radioactivity of plasma ester cholesterol *in vivo* probably derives from the liver and is subsequently supplemented by the action of L.C.A.T.

Isotopic equilibrium was not found between the plasma and liver (^{14}C) cholesterol (ester or unesterified) within 3h. Examination of Fig. 64 shows that plasma ester and unesterified cholesterol equilibrated at different rates with their counterparts in the liver. This would suggest that there were two different mechanisms operating in the exchange of these two forms of cholesterol between liver and plasma. Swell *et al.* (1968) also found that equilibrium between unesterified plasma cholesterol and cholesterol in the liver microsomes or mitochondria was still incomplete after four hours perfusion of an isolated rat liver. Hepatic and plasma unesterified cholesterol have a $t_{\frac{1}{2}}$ of about 55min, which is similar to that reported by Gould *et al.* (1955) in man, but considerably longer than the time found in dogs of 18-19min Eckles *et al.* (1955). The rate of exchange of ester cholesterol between the liver and plasma is estimated to

have a $t_{\frac{1}{2}}$ of 115min. However, treating the ester cholesterol as a single pool may be an oversimplification because Goodman and Shiratori (1964) found that within the plasma compartment, monosaturated esters had the most rapid turnover. In addition they reported that the mono- and di- unsaturated esters in the liver turned over more rapidly than the saturated esters. Thus the average figure of $t_{\frac{1}{2}}$ reported here could have a wide range of values about this observed mean to accommodate the different ester components.

When using (^{3}H) acetate as precursor the $t_{\frac{1}{2}}$ time for ester cholesterol in liver and plasma was about 135min (Fig. 65) and is similar to that using mevalonic acid as precursor. The most interesting feature of these experiments is the very rapid equilibrium between plasma and liver for unesterified cholesterol, a $t_{\frac{1}{2}}$ of 30min being observed. This is approximately 50% of the value found when using mevalonic acid as precursor (55min) and might suggest that acetate is being taken up and used by the hepatocyte more rapidly than mevalonate. The specific radioactvity of the plasma unesterified cholesterol labelled with (^{3}H) remained higher than that of the liver after 30min. It might therefore be suggested that there is a plasma cholesterol pool with a slow turnover and operating exclusively on cholesterol derived from acetate. No direct support for this hypothesis is available.

The shape of the specific radioactivity time curves found in this study are different from those studies reported by Swell et al. (1968) and Percy-Robb and Boyd (1970) in which both groups used isolated liver perfusion. These authors found that the specific radioactivity of unesterified and total cholesterol rose in almost a linear mode throughout the perfusion period and the initial incorporation of the labelled mevalonic acid into

plasma cholesterol was appreciably slower than in this study. This can be accounted for because the sampling intervals used in those studies were large, (approximating to one hour) and more frequent sampling might have produced a different shape of curve. The curve shapes demonstrated in this *in vitro* study compare well with those found *in vivo* by Eckles *et al.* (1955) in dogs, who showed very rapid increases in the specific radioactivity of plasma unesterified cholesterol during the first hour after dosing the animals with labelled acetate. Assuming that all of the radioactive mevalonate is rapidly incorporated into cholesterol and that a first order reaction defines the transfer of unesterified cholesterol into the plasma from the liver, then curves of the type demonstrated in the study reported here would be expected. Therefore it seems likely that this isolated perfused rat liver preparation acts as a credible model for assessing *in vivo* conditions.

However, on theoretical grounds the rate of increase of the specific radioactivity curves for the perfused rat liver should be greater than for the *in vivo* situation because, if the model of feedback control of cholesterol synthesis by chylomicrons proposed by Weiss and Dietschy (1969) is accepted, then inhibition of cholesterol synthesis in the isolated perfused rat liver is removed. Cholesterol synthesis would increase and the rate of appearance of labelled plasma unesterified cholesterol would not represent the true rate which would be found *in vivo*.

When the cholesterol was labelled with tritium derived from radioactive acetate, the curves found were somewhat different in form to those obtained from (2-^{14}C)DL mevalonic acid. The initial rise in specific radioactivity was

similar to that obtained with mevalonate but rapidly reached a constant value for the unesterified plasma cholesterol (Fig.44) after about 90min. The specific radioactivity of the plasma esterified cholesterol showed similar characteristics but took longer to reach a stable value(about 160min.). The specific radioactivity of the (^{3}H) cholesterol in the erythrocytes continued to rise and mirrored the curve for (^{14}C) cholesterol in erythrocytes (Fig.47). The similar shapes of the decay curves of (^{3}H) and (^{14}C) labelled cholesterol in erythrocytes confirms that the plasma is the primary source of cholesterol exchange with the erythrocytes.

All of the (^{3}H) labelled curves had lower specific radioactivities than their counterparts labelled with (^{14}C), even though more than twice as much (^{3}H) label was added. These results were not unexpected when consideration was given to the number of metabolic pathways available to 'acetate' in the liver. It is this effect that accounts for the rapid flattening of the (^{3}H) labelled specific radioactivity curves, although if all the other metabolic exits for acetate are first order then the (^{3}H) labelled curves should still show evidence of small increases at the later time points. The apparent plateau effect is probably a technical artefact because the analytical techniques used were not sufficiently sensitive to demonstrate very small changes in the specific radioactivity.

The specific radioactivity of the biliary (^{14}C) cholesterol rose and approached a constant value about 150min after the start of perfusion, the mean value of 23.28nCi/mg being less than half that of the unesterified cholesterol in the liver at the end of the perfusion. The specific radioactivities of unesterified cholesterol in both plasma

and liver at the end of the perfusion were greater than that of biliary cholesterol. The results therefore did not allow one to speculate as to whether biliary cholesterolis derived from newly synthesised hepatic cholesterol or from the plasma cholesterol. It is most likely that biliary cholesterol is derived from both sources, the relative contributions of each being dependent upon the activity of the synthetic pathway operating in the liver. Byers and Friedman (1952) demonstrated that the rate of biliary excretion of cholesterol was a function of the rate of hepatic synthesis of cholesterol, suggesting that biliary cholesterol represents a portion of the cholesterol synthesised in the parenchymal cell. Additionally, Percy-Robb (1967) using an isolated perfused rat liver, found that (^{14}C) cholesterol added as lipoprotein to the perfusate, appeared in the bile as bile acids thereby indicating equilibrium with intracellular cholesterol which acted as a precursor for bile acid synthesis. If the pool of cholesterol in the endoplasmic reticulum is homogeneous then some of the intrahepatic cholesterol derived from the plasma would be excreted into the bile.

In contrast to the results obtained using (^{14}C)DL mevalonic acid as precursor, the results using (^{3}H) acetate showed that the specific radioactivity of biliary cholesterol approximated closely to the values found for the unesterified plasma cholesterol. This confirms the report of Rosenfeld and Hellman (1959) who found the same specific radioactivity for biliary and unesterified plasma cholesterol <u>in vivo</u> after oral dosage of human subjects with (^{3}H) or (^{14}C) acetate. This suggests that acetate is utilised more rapidly than mevalonate by the hepatocyte and provides support for this suggestion earlier when considering the rate of equilibration between liver and

plasma cholesterol labelled with tritium.

The actual mass of cholesterol excreted in the bile during a 3h perfusion was on average 48µg/h. This was greater than that found in the bile obtained from a biliary fistula which had been established for 10h (38µg/h). It is likely that this difference has a real biological significance since in the isolated state, feedback controls of cholesterol synthesis existing in the whole animal would be absent. This should lead to an increased synthesis of cholesterol and consequently an increased concentration of cholesterol in the bile. (Byers and Friedman, 1952). Both of these secretion rates are similar to the rates reported by other investigators; Eriksson (1957a) found nearly 40µg/h for the first 24h in biliary fistual rats, Byers and Friedman (1952) again using rats demonstrated nearly 50µg/h in vivo and Kay and Entenman (1961) reported 80µg/h during the first 4h of an isolated rat liver perfusion. Surprisingly, the latter authors also demonstrated the presence of an appreciable amount of cholesterol esters in the bile; the results reported here indicate that no cholesterol esters were present in the bile. The mass of cholesterol excreted by bile fistula rats is this study changed with time reaching a minimal value after about 15h drainage and paralleled the volume of bile secreted. Similar findings have been reported by Eriksson (1957a)

Interpretation and justification of the predicted model.

It was possible to fit the data obtained from the perfusion studies to the biological model represented by Fig. 8. This did not validate the model as unique but at least established that it was a feasible representation of the conditions existing *in vivo*. The selection of this model can be justified in two ways, firstly by reviewing the qualitative evidence that the transfers shown in Fig. 8 actually do occur, and secondly by establishing points of similarity for the kinetic parameters obtained in this study and those in the reports of other investigators.

The transfer of unesterified cholesterol from the liver into the plasma has been well established for many species, both *in vivo* Gould et al. (1955), Eckles *et al.* (1955) and *in vitro* Swell et al. (1968) and this study. The transfer in the opposite direction i.e. from plasma to liver is also well known, thus Gould *et al.* (1951) found accumulation of radioactivity as cholesterol in the livers of dogs given intravenous injections of radioactive cholesterol in a natural lipoprotein. In a similar type of study, but using *in vitro* techniques Percy-Robb and Boyd (1970) showed an excretion of labelled bile acids after addition of radioactive lipoprotein to the perfusate of an isolated rat liver. This must imply an uptake of cholesterol into the liver to act as part of the precursor pool for bile acid synthesis. Unfortunately, since lipoproteins contain both ester and unesterified cholesterol, these experiments do not entirely establish the uptake of unesterified cholesterol into the liver. It is possible that the unesterified cholesterol appearing in the liver is related to the uptake of plasma ester cholesterol with an intrahepatic hydrolysis subsequently producing

unesterified liver cholesterol. One way in which this question could be directly resolved would be to use artificial substrates such as that described by Porte and Havel (1961) in which all of the cholesterol was in the unesterified form. They showed that the synthetic 'lipoprotein' disappeared from the circulation, in dogs, in a manner similar to that found for natural lipoproteins. They did not present results to confirm that some of this material was entering the liver, but this point could be taken by inference from their specific radioactivity curves for plasma cholesterol.

A criticism of this approach is that the transfer between plasma and liver for naturally occurring lipoproteins containing unesterified cholesterol cannot be convincingly demonstrated using artificial or synthetic lipoproteins. However it seems reasonable to assume that esterified and unesterified cholesterol in the lipoproteins absorbed by the liver would be treated in a similar manner and that the predicted transfer of unesterified cholesterol between the plasma and the liver is a real one.

All cholesterol in the erythrocytes is unesterified (Nelson, 1967) and exchanges with the compartments other than the plasma unesterified cholesterol need not be considered. It is of course possible, but improbable, that exchange occurs directly with the hepatocyte without exchange through the plasma cholesterol pool.

The early studies of Hagerman and Gould (1951) demonstrated that <u>in vitro</u> there is an exchange of unesterified cholesterol between erythrocytes and plasma. This has been confirmed in many subsequent investigations. The transfer between plasma and erythrocytes also occurs <u>in vivo</u> (Porte and Havel, 1961) and although no reports could be found of an exchange occurring <u>in vivo</u> it would be difficult to rule this out, especially on the basis of the <u>in vitro</u> studies.

Cholesterol synthesised in the liver is esterified *in situ* (Swell *et al.*, 1955) with the indvidual esters having different turnover rates (Klein and Martin, 1959). A slow hydrolysis occurs in the liver to produce unesterified cholesterol (Quarfordt and Goodman, 1967). The intrahepatic cholesterol may then be re-esterified to produce a different ester to that originally formed or absorbed by the liver. There is also some evidence that a direct exchange of fatty acids takes place, analogous to that occurring in the esterification of cholesterol in the blood (Swell and Law, 1966).

Esterification of cholesterol is known to take place in human plasma incubated *in vitro* (Sperry, 1935; Glomset *et al.*, 1962b). However the hydrolysis of cholesterol esters in plasma has only been shown to occur in the dog (Swell and Treadwell, 1950) and these authors concluded that such an enzyme did not exist in rat plasma. The model predicted here would suggest that intravascular hydrolysis could take place but as the model is not unique then the results should be interpreted with care.

The origin of the plasma cholesterol esters in man is still not clear. Although it has been demonstrated that the liver is responsible for the removal of the ester cholesterol of the chylomicrons (Quarfordt and Goodman, 1967), the contribution of the liver to the plasma cholesterol ester pool is debatable. Several studies have been reported which would suggest that either the liver, or esterification in the blood is responsible for the presence of cholesterol ester in plasma. Swell *et al.* (1971) were able to show that an isolated perfused rat liver was capable of releasing cholesterol esters into the perfusate. This could not have been derived from L.C.A.T. activity in the perfusate because the perfusate medium was artificial and contained no L.C.A.T.

activity. The study by Roheim et al. (1963), again using an isolated perfused rat liver, found evidence for direct transfer of cholesterol into the perfusate and selective incorporation of the esters into different plasma lipoproteins. On the other hand Glomset (1962a) observed in vitro in human serum that the rate of intravascular esterification of cholesterol was sufficient to account for the turnover of all the cholesterol esters in plasma in vivo. It may therefore be postulated that plasma cholesterol esters derive from both processes, although there is little information about the relative contribution of either one.

Cholesterol is excreted in the bile of most mammalian species and appears predominantly in the unesterified form, although there is a report that esterified cholesterol is present in rat bile (Kay and Entenman, L961). This could not be confirmed in this study and since all of the biliary cholesterol was considered to be unesterified it can only originate by direct transfer from the hepatic unesterified cholesterol pool.

From the foregoing evidence it would seem reasonable to suggest that the cholesterol transfers shown in Fig. 8 may be taken to represent conditions existing in vivo.

Turning to the quantitative evidence reference is made to Table 14 which shows the predicted rate constants and to Table 13 which shows the actual amount predicted for the transfer of cholesterol between compartments.

The plasma unesterified cholesterol compartment is the most active in terms of its exchange rate with the erythrocyte cholesterol and has a $t_{\frac{1}{2}}$ value of about 12min. It also represents the most active mass transfer with a value of 0.46mg/min. The transfer in the opposite direction, i.e. from erythrocyte to plasma is less active with a $t_{\frac{1}{2}}$ of about 30min. The rate constants

describing the exchange of cholesterol between plasma and erythrocytes are greater by a factor of about ten than those found in the investigation reported by Quarfordt and Hilderman (1970).

This is not entirely unexpected because the mean ratio of the size of the plasma unesterified cholesterol compartment was 1:10 in the study reported here and should be compared to the ratio of 1:1.5 used in the study of Quarfordt and Hilderman (1970).

The exchange rates between plasma and erythrocytes were greater than those found for cholesterol exchange between liver and plasma. The study *in vivo* of Eckles *et al.* (1955) using dogs suggested that the most active exchange occurs between plasma and liver. This discrepancy probably arises because of the *in vitro* technique used in the experiments reported here; thus although the relative pool sizes for the plasma and erythrocyte unesterified cholesterol are comparable to those existing *in vivo*, the relative pool size of the liver and plasma unesterified cholesterol is disturbed, with a greatly enlarged pool of plasma unesterified cholesterol.

However, ignoring the question of relative pool sizes, on *a priori* grounds it is possible that the exchange rate between plasma and erythrocytes could occur at a greater rate than between plasma and liver. Thus not only is there a more intimate juxtaposition of erythrocytes and plasma but the exchange is continuous, in contrast to the exchange between plasma and liver which can only occur while the plasma is in contact with the liver cell.

The smallest rate constants were found for equilibrium between unesterified and esterified cholesterol in the plasma. This is consistent with the *in vitro* results obtained in this study when examining the exchange

of radioactive cholesterol between plasma and erythrocytes as an isolated system. In those experiments no significant changes could be seen in the specific radioactivity of the plasma ester cholesterol over a three hour period. These results are also in agreement with the reports of Sperry (1935) and Glomset (1962b) when long incubation periods were necessary to produce any appreciable esterification of cholesterol. Gould et al. (1955) and Eckles et al. (1955) also showed in vivo that the increase in specific radioactivity of the plasma cholesterol esters was slow. The activity of L.C.A.T. both in vivo and in vitro must therefore be very low.

When comparing the values of the rate constants for the exchange of cholesterol ester between the plasma and liver with those defining the exchange of cholesterol between the plasma unesterified and ester compartments it is immediately apparent that the former exchange is the most active. The mass transfer of ester cholesterol from the liver to the plasma is about five times as great as the mass of cholesterol esters derived by esterification of unesterified cholesterol in the plasma and supports the hypothesis that the liver is the primary source of plasma cholesterol esters (Swell and Law, 1971; Swell et al.,1955). The increase in specific radioactivity of the cholesterol esters in the plasma found during perfusion can probably be ascribed to the direct release from the liver, especially as over this experimental time period no increase in specific radioactivity of cholesterol in the plasma esters could be demonstrated in in vitro experiments where cholesterol exchange between plasma and erythrocytes was studied in isolation.

When the specific radioactivity data obtained from normal liver perfusion was transformed to show the amount

of radioactivity in each compartment, the plasma ester data always showed a sigmoid shape when plotted on a linear/linear graph. This suggests that the liver esterase enzyme undergoes a lag phase, with a consequent delay in the appearance of radioactivity in the plasma cholesterol esters. Alternatively the transport mechanism extruding cholesterol ester from the liver may be phasic. There is some suggestion that the latter alternative may be more correct. Thus the rate of equilibrium between liver and plasma ester cholesterol is initially slower than that for the unesterified form of cholesterol (Fig.64).

The major points arising out of the studies in normal rats are:-

1) The liver perfusion preparation produced results comparable with those obtained in vivo by other investigators. It can therefore be used with some confidence to allow investigation of hepatic cholesterol metabolism without having to consider other blood and tissue interactions. These normally make the results obtained from whole animal experiments difficult to interpret.

2) The most rapid exchange of cholesterol occurs between plasma and erythrocytes.

3) Plasma cholesterol esters are derived primarily from the liver.

4) The activity of L.C.A.T. was very low under the conditions used in this study.

5) The amount of cholesterol excreted in the bile is insufficient to perturb the exchanges occurring within the liver and blood system.

6) The uptake of mevalonic acid and its incorporation into hepatic cholesterol may be considered as a single stage process when compared to the time needed to exchange cholesterol between liver and blood.

7) The uptake of mevalonic acid into the cell can be described by a first order reaction.

8) 'Acetate' may be absorbed across the hepatocyte more rapidly than 'mevalonate', suggesting that one is not dealing with a simple diffusive process.

9) Different mechanisms exist for the transfer of ester and unesterified cholesterol into the plasma from the liver.

10) There are three pools of cholesterol in the liver with respect to their turnover with plasma cholesterol. They are i) the nuclear fraction ii) the soluble fraction iii) the mitochondrial and microsomal fractions.

Perfusion studies on livers of rats subjected to previous biliary drainage.

Having established a reasonable model, in qualitative and quantitative terms, to represent exchanges of cholesterol occurring in the blood and liver system, a study was made of the effect of stimulating hepatic cholesterol synthesis on the exchanges taking place.

Stimulation of hepatic cholesterol synthesis from acetate, but not from mevalonate has been demonstrated by Myant and Eder (1961) in rats which had been subjected to previous biliary drainage. Coincident with the increase in cholesterol synthesis the synthesis of bile acids also increased. The predominant bile acids in the rat, cholic and chenodeoxycholic acid, are derived from a common pool of unesterified cholesterol in the liver (Ogura et al., 1971). This pool of cholesterol can be of endogenous or exogenous origin (Percy-Robb and Boyd, 1970) although Ogura et al. (1971) considered that the endogenous cholesterol would show a more rapid incorporation into bile acids. This was supported by the report of Percy-Robb and Boyd, (1970) who showed that there was no increase in the rate of transfer of lipoprotein cholesterol into the liver of rats with biliary fistulae even though bile acid synthesis was markedly increased, thus indicating that extrahepatic cholesterol is a secondary source of bile acids and that the endogenous cholesterol in the liver is the primary source. The latter authors, using an isolated perfused rat liver, demonstrated that the incorporation of (2^{14}C)DL mevalonic acid into the perfusate cholesterol occurred more rapidly in perfusions where the livers had been subjected to previous biliary

drainage (44h) than in livers taken from control rats. This is somewhat at variance with one of their other conclusions, namely that the rate of transfer of cholesterol into the liver was not increased for the bile drained liver. If this were the case, then as the rate of release of cholesterol from the liver is increased it might be expected that the perfusate cholesterol concentration would rise and the authors reported that this did not occur.

Increased incorporation of mevalonate into cholesterol was not confirmed in the study reported here as judged by the appearance of radioactivity in the plasma, moreover the specific radioactivity curves obtained during perfusion of the bile drained livers, were consistently below those obtained during perfusion of normal livers (Fig. 69) although statistical tests showed that no significant difference for the decay curves of plasma ester and unesterified cholesterol generated in both types of perfusion.

If one accepts the model of cholesterol homeostasis proposed by Weiss and Dietschy (1969) then the regulator of hepatic cholesterol synthesis is not blood or hepatic bile acids but rather the amount of cholesterol presented to the liver in the plasma lipoproteins. If this concept is correct, then in the perfusions performed here on livers obtained from normal and bile drained rats where the perfusate cholesterol concentrations were similar, it is not surprising that little difference in the specific radioactivity curves could be demonstrated. This does not rule out an increase in bile acid synthesis under the stimulus of reduced hepatic or blood bile acid concentrations and indeed this is known by inference to take place (Eriksson, 1957a), but it does not necessarily follow that cholesterol synthesis is

stimulated even though cholesterol is the bile acid precursor.

The recovery of radioactivity in cholesterol in the perfusion of drained livers was only 66% compared to 95% for the control perfusions. This means that either the mevalonic acid was not being utilised, which is unlikely, or that the stimulus to bile acid formation is causing an apparent loss of radioactivity, because of the catabolism of newly formed labelled cholesterol to bile acids. This may well be the case since the final specific radioactivity of unesterified cholesterol in the livers of bile drained rats (mean = 40.15nCi) was only about one half that for control rats (mean = 74.89nCi). As the specific radioactivity values for the plasma esterified and unesterified cholesterol were similar in both experimental situations, then the reduced specific radioactivity found for hepatic cholesterol in the bile drained liver is probably a result of increased degradation of cholesterol, leading in turn to an increased excretion of radioactive bile acids in the bile. The studies performed by Eriksson (1957a) and Percy-Robb and Boyd (1970) suggested that about 1/7 - 1/5 of the cholesterol present in the perfused rat liver preparation would be incorporated into bile acids. On the basis of the radioactive recoveries found in the study reported here it would appear that this may be an underestimate. The mass and specific radioactivity of the cholesterol excreted in the bile were similar in both series of the experiments and therefore the differences in the specific radioactivity of hepatic unesterified cholesterol cannot be directly related to biliary excretion of cholesterol.

The time needed to achieve equilibrium between unesterified cholesterol in the plasma and liver was considerably reduced in the bile drained preparation (compare Figs. 64 and 70). In the normal perfusion studies

equilibrium was not found at the end of the perfusion period. In contrast isotopic equilibrium was complete at about 110min in the bile drained preparation and the specific radioactivity of the plasma unesterified cholesterol remained above that of the liver cholesterol throughout the perfusion period. It is unlikely that this represents a change in the transport kinetics between liver and blood. It is probably specifically related to the increased synthesis of bile acids which draws upon the hepatic unesterified cholesterol pool. If this is the explanation then one must conclude that cholesterol synthesised de novo is the preferential precursor of bile acids and not the pre-existing hepatic pool of unesterified cholesterol since the specific radioactivity of liver unesterified cholesterol was reduced at the end of the perfusion.

The reduced accumulation of radioactivity in the erythrocytes found during perfusion of bile drained livers (Fig. 71) could be due to the reduced amounts of radioactivity associated with the unesterified cholesterol in the plasma. However the magnitude of the reduction in plasma radioactivity is insufficient to accommodate the marked reduction of radioactivity in the erythrocytes and one must therefore postulate some direct effect on the exchange of cholesterol between plasma and erythrocytes. Bruckdorfer and Green (1967) showed that this exchange is dependent upon a number of factors, e.g. pH, ionic strength of suspension media, temperature, but these were similar in the bile drained and control experiments. The major difference lies in the stimulation of bile acid synthesis in the bile drained experiments where there is an enhanced excretion of bile acids. Cooper et al. (1972) have shown that the transfer of unesterified cholesterol between plasma and erythrocytes is dependent upon the concentration of bile

acids in the blood. It is possible that a reduction in perfusate bile acid concentrations might explain the reduced accumulation of radioactivity in the erythrocytes. Cooper et al. (1972) also found that the concentration of cholesterol in the erythrocytes increased when the concentration of bile acid was raised, probably at the expense of the cholesterol associated with the α lipoprotein. An earlier study by Cooper and Jandl (1969) showed that 38% of the plasma cholesterol was 'loosely bound' to lipoprotein and could exchange with erythrocytes. This confirms the study of Ashworth and Green (1964) who showed that a larger proportion (60%) of the cholesterol in α lipoprotein was freely available for exchange with erythrocytes.

The actual mechanism of stimulated transfer of cholesterol into the erythrocyte under the influence of bile acids is unknown. Bile acids, as with other detergent substances have a potent effect on the hydrophobic bond with a consequent exposure of the non-polar regions of the erythrocyte membranes to the aqueous medium. This should facilitate cholesterol exchange between the erythrocyte and the plasma lipoprotein or decrease the exchange in media with low bile acid concentrations. If this is the explanation for the reduced incorporation of radioactivity into erythrocytes during perfusion, the the results reported here are presumptive evidence for a reduced bile acid concentration in the blood during external diversion of the bile flow, even though hepatic bile acid synthesis is increased. Swell et al. (1968) found that both the specific radioactivity and mass of cholesterol in bile increased markedly when sodium taurochlorate was added to the perfusate of an isolated rat liver. In the studies reported here the specific radioactivity of the biliary

cholesterol obtained from bile drained livers did not change appreciably when compared to the control values and it seems reasonable to suppose that the perfusate bile acid concentrations remained unchanged or possibly even fell because of the increased excretion of bile acids in the bile.

The results reported here showed no significant change in the rate of appearance of radioactive cholesterol esters in the plasma when compared to the control experiments, although this might have been expected on the basis of a report by Swell and Treadwell(1950). They found in vitro that increasing the bile acid concentrations reduced the rate of esterification of cholesterol. Therefore reduced blood bile acid concentrations, as postulated in the perfusate of bile draind livers, should show increased rates of intravascular esterification. This was not found and suggests that either the experimental time period (3h) was too short to show an effect or that only minimal changes in perfusate bile acid concentrations occurred.

By simulating an increase in the loss of cholesterol as bile acids from the isolated blood and liver system it was possible to obtain a better correspondence between the observed results in the bile drained preparation and the simulated results derived from the normal model. This increases the credibility of the normal model.

Some of the conclusions that may drawn from the studies on livers of rats which had been subjected to previous biliary drainage are:-

I) Stimulation of hepatic cholesterol synthesis has little effect upon the exchange of cholesterol between plasma and liver.

2) The rate of synthesis of cholesterol from mevalonate is not affected .

3) A large proportion of the newly synthesised cholesterol is excreted, not as cholesterol, but some metabolite, probably bile acids.

4) The unesterified cholesterol synthesised de novo is the prferential precursor of bile acids since the final specific radioactivity of unesterified cholesterol in the liver was lower in the bile drained preparation.

5) Unesterified cholesterol synthesised de novo exchanges firstly with the blood cholesterol and only secondarily is used as a precursor of bile acids. This must be the case because the specific radioactivity curves in both experimental situations were similar, even though radioactivity recoveries were diminished in the bile drained liver preparations.

6) The concentration of bile acids in the blood may affect the transfer of cholesterol between plasma and erythrocytes.

Perfusion studies on the livers of rats subjected to thyroidectomy.

Although stimulation of cholesterol synthesis in the liver does not affect the exchange of cholesterol between plasma and liver it was of interest to examine the converse situation, i.e. where hepatic cholesterol synthesis is reduced.

Thyroid hormones have a marked effect on the concentration of cholesterol in the blood (Kritchevsky, I960; Boyd,I963) and this is said to be due to an altered rate of cholesterol turnover(Rosenman et al.,I952). In the hypothyroid state the synthesis of cholesterol is decreased (Fletcher and Myant,I958; Lipsky et al.,I955) and if, in the long term, a steady state is established then this reduced synthesis must be balanced by a decrease in the elimination of cholesterol, especially if hypercholesterolaemia develops. The results reported here show a decrease in the amount of cholesterol excreted in the bile and confirm the longer term studies of Eriksonn(1957b) and of Thompson and Vars(I953) both of which showed reductions in vivo in the amount of cholesterol excreted by hypothyroid rats.

The specific radioactivity of biliary cholesterol did not change during the perfusion period, but the values were reduced in comparison to the normal liver perfusions. This probably reflects the lower values for the specific radioactivity of liver unesterified cholesterol in livers taken from thyroidectomised animals. It is consistent with the suggestion of Byers and Friedman (I952) that the excretion of biliary cholesterol is a function of hepatic synthesis.

The specific radioactivities of the liver cholesterol esters in livers from hypothyroid and control rats were similar. Schweppe and Jungman(I969) demonstrated that increased cholesterol ester synthesis took place in vitro

when rat liver microsomes were exposed to L-thyroxine. It seems likely therefore that a reduction in the level of the active hormone might inhibit intracellular esterification. This was not found in the study reported here. The effect reported by Schweppe and Jungman (1969) may not represent a real effect operative in the intact cell because of the absence of co-factors derived from other cellular organelles.

The lower specific radioactivity found for the unesterified cholesterol in the liver is reflected in the curves describing changes in the specific radioactivity of cholesterol in the blood compartments. Thus the plasma unesterified cholesterol curve was significantly lower ($p = < 0.001$) in perfusions of livers from thyroidectomised animals.

The plasma esterified cholesterol also showed a difference from the normal or control experiments ($p = < 0.02$) even though the specific radioactivities of the liver esters were similar in both sets of experiments. This suggests that the exchange of esterified cholesterol between liver and plasma is at least partially controlled by thyroid hormone since one would have expected statistically similar plasma ester curves when the specific radioactivities of the liver esters were similar in test and control experiments. This concept was supported when comparing the slopes of the regression lines (cholesterol/time) for unesterified and esterified cholesterol in the plasma during perfusion. When perfusing livers obtained from thyroidectomised animals the regression lines describing the change in plasma cholesterol concentration with time diverge for ester and unesterified cholesterol. The ester cholesterol regression line had the lower slope. This divergence was not found in the normal or control perfusions.

In the experiments using thyroidectomised rats the biosynthesis of unesterified cholesterol was inhibited

by the lack of thyroid hormone (Table 24). No such effect was noted on the biosynthesis of cholesterol esters since the specific radioactivity values of hepatic ester cholesterol in both test and control experiments were similar. This corresponds to the divergence of the specific radioactivity of unesterified and esterified cholesterol in the livers of thyroidectomised animals when compared to the control group. This would suggest that thyroid hormone acts solely on the hepatic biosynthesis of unesterified cholesterol and not on intrahepatic esterification.

In the study reported here, not only was the specific radioactivity of cholesterol in all compartments lower in perfusions of livers from hypothyroid rats, (with the exception of ester cholesterol in the liver) but also the radioactivity recovered was only about 65% of the active isomer. This implies that the thyroid hormone has a direct effect on the synthesis of cholesterol at some point past the major rate-limiting step of conversion of HMGCoA to mevalonate, but there is no published evidence available to support this suggestion. Alternatively the hormone may have an effect on the permeability to the plasma membrane of the hepatocyte and prevent the uptake of mevalonate at low hormone concentrations. Fletcher and Myant (1958) found that there was no reduction in the rate of cholesterol synthesis from mevalonate in the livers taken from hypothyroid animals, although an inhibition was noted using acetate as precursor. Their study was not directly comparable because these authors used tissue slices suspended in a synthetic medium and membrane transport characteristics would not be representative of <u>in vivo</u> conditions.

The concept of a change in permeability is supported by simulations with the computer model. For example, a reduction in the value of k_m (the rate constant controlling the synthesis of cholesterol) simulates a condition such that at 180min after the beginning of a perfusion not all the radioactivity is incorporated into cholesterol Fig. 58. In the simulation an adjustment of k_m to smaller values allowed a better fit to the experimental data and the model predicted that 18% of the mevalonate would not be utilised. Although this figure did not match the experimentally determined loss of radioactivity, nevertheless it indicated that under-utilisation of mevalonate could account for the low recoveries.

A further experiment in support of this concept is based upon the work of Percy-Robb (1968). In that study the recovery of radioactivity in bile acids, after addition of (2^{14}C)DL mevalonic acid to the perfusate of an isolated rat liver, was lower in the thyroidectomised animals than in controls, and the author concluded that this was evidence for a reduced specific radioactivity of the intracellular pool of cholesterol which acted as a precursor for bile acid synthesis. This reduction was found in the study reported here, although this applied to all of the intracellular cholesterol and no distinction was made between the specific radioactivity of cholesterol in the intracellular organelles.

The most interesting results from this part of the study are:-

1) There appears to be an inhibition of the synthesis of unesterified cholesterol in the liver, brought about by the lack of thyroid hormone. This effect may not be a direct one and could be related to a lack of substrate (i.e. mevalonic acid) subsequent to a change in the uptake of the precursor into the hepatocyte.

2) The intrahepatic esterification of cholesterol is unaffected by the presence or absence of thyroid hormone.

3) The rate of exchange of ester cholesterol between liver and plasma may be influenced by thyroid secretions.

4) The reduced specific radioactivity curves in the plasma can be accommodated by changes in the rate constant controlling cholesterol synthesis in the liver. It is therefore unlikely that a reduced rate of hepatic synthesis of unesterified cholesterol has any effect on the exchange of cholesterol between plasma and liver.

Effect of CPIB (Atromid-S500) on cholesterol metabolism in the isolated perfused rat liver.

The first reports of large reductions in blood cholesterol concentrations by ethyl chlorophenoxy-isobutyrate (Atromid-S, Clofibrate, CPIB) published by Thorp and Waring (I962) have been confirmed many times in a wide variety of species.

CPIB is known to reduce hepatic biosynthesis of cholesterol from acetate (Avoy et al.,I965) but these authors reported only small reductions (I2%) when using mevalonate as precursor. In contrast Azarnoff et al. (I965) found a reduction of about 60% when using mevalonate as precursor. The discrepancy in the percentage inhibition reported by the two groups of investigators is probably related to the different experimental conditions used. Azarnoff et al. (I965) administered the drug over long time periods and the effect noted by them may be a secondary one. However in the study reported here rats were fed with CPIB in their diet (0.25%) for 2I days and no such large effect was demonstrated; the results confirm those of Avoy et al.(i965).

The specific radioactivity of (^{I4}C)cholesterol derived from mevalonic acid at the end of perfusion was about 20% lower in the CPIB treated livers than in normal liver, both in the short term experiments,i.e. where CPIB was added directly to the perfusate, and in the longer experiments where the rat was given CPIB for three weeks prior to the removal of the liver removal. An even greater inhibition of cholesterol biosynthesis was noted when the precursor was sodium (^{3}H) acetate and a mean value of 85% inhibition was found (Table 25). The interesting feature of these results is that the effect of the drug was very rapid, changes in the rate of appearance of (^{3}H) cholesterol in the plasma being apparent in less than 30min after adding CPIB to the perfusate (Fig.79).

The drug therefore acts very rapidly and on several parts of the biosynthetic chain with a preference for inhibition between acetate and mevalonate, probably at the rate limiting step of the conversion of HMG-CoA to mevalonate. However inhibition may occur before the rate limiting step because Burch and Curran (I969) found increased hepatic acetoacetyl-CoA deacylase activity in CPIB treated rats and postulated that this reduced the mitochondrial concentration of HMG-CoA. However it is possible that a direct inhibitory effect of CPIB on HMG-CoA reductase activity could lead to a compensatory increase in acetyl-CoA reductase activity. The actual point of inhibition is still therefore unresolved.

Kritchevsky et al. (I969) have also reported an effect of CPIB on rat liver mitochondria; there was an increased capacity to oxidise (26-^{14}C) cholesterol to $^{14}CO_2$ after the animals had been fed on a diet containing CPIB for three weeks. This was primarily due to the increased protein content of the liver (Platt and Thorp, I966; Hess et al.,I965) because the effect disappeared when calculated on the basis of the mitochondrial nitrogen content.

The large reduction in the specific radioactivity of unesterified cholesterol derived from sodium (^{3}H) acetate in the livers of CPIB treated rats could be explained by assuming a redistribution of cholesterol into the liver. This would expand the hepatic cholesterol pool and effectively reduce the specific radioactivity of the liver cholesterol. This explanation is unlikely because Azarnoff et al. (I965) showed that the cholesterol content of the liver was reduced in animals treated with CPIB. Additionally Mishkel and Webb (I967) showed that the concentration of cholesterol in the perfusate of an isolated rat liver preparation did not change under the influence of CPIB.

The specific radioactivity of the cholesterol in the perfusate in both types of experiment (i.e. acute and chronic) was slightly lower than in the normal liver perfusion but this was not statistically significant. Azarnoff et al. (I965) also showed that the rate of release of cholesterol into the perfusate of an isolated rat liver after CPIB administration to the whole animal was not significantly different from normal.

In both types of experiment with CPIB the excretion of cholesterol in the bile was much reduced when compared to the excretion in normal liver perfusions. Thus only about I0µg cholesterol per hour were excreted by CPIB treated livers, whereas the normal liver excreted about 50µg/h. This is in keeping with the suggestion of Friedman and Byers (I952) that the biliary excretion of cholesterol is a function of the rate of synthesis of cholesterol in the liver.

However the amount of cholesterol excreted in the bile of CPIB treated livers is not consistent with the observations in thyroidectomised rats where the reduced biliary excretion of cholesterol by the thyroidectomised rat was associated with marked reductions in the specific radioactivity of the hepatic cholesterol pool(Table 24). Only small reductions in the specific radioactivity of hepatic cholesterol in CPIB treated livers (Table 25) were associated with biliary cholesterol concentrations even less than those found in thyroidectomised rats (CPIB treated= I0µg/h, thyroidectomised=I7µg/h). The specific radioactivity of the biliary cholesterol was also lower than in the thyroidectomised rat liver preparation (CPIB treated=II.5nCi/mg, thyroidectomised= I5.2nCi/mg). This might suggest that CPIB acts directly on the actual mechanism of cholesterol excretion into bile as well as exerting an inhibitory effect on cholesterol biosynthesis.

In the steady state the reduced biliary excretion

of cholesterol resulting from the action of the drug would have to be balanced by an even greater reduction in cholesterol biosynthesis noted in the experiments reported here if the hypocholesterolaemic action of the drug is to be adequately explained. It is possible that the effect described by Kritchevsky et al. (I969), i.e. the increased capacity to oxidise the cholesterol side chain would serve to explain the reduced excretion of cholesterol in CPIB treated rats. Not only would the rate of cholesterol biosynthesis be reduced but there would also be an increased catabolism of newly synthesised cholesterol resulting in the low concentrations of biliary cholesterol. This would mean however that bile acid synthesis and excretion may be increased. If that is so then the higher concentrations of bile acids in the intestine would promote cholesterol absorption and effectively shut off hepatic cholesterol synthesis.

The main points arising from this study with CPIB are:

I) CPIB has no obvious effect on the mechanism for the exchange of cholesterol between liver and blood.

2) The action of the drug is very rapid, inhibition of cholesterol synthesis in the liver occurring within thirty minutes of the appearance of the drug in the portal blood.

3) The major point of inhibition of cholesterol synthesis lies at, or before, the rate limiting step of the conversion of HMG-CoA to mevalonic acid. There was however some suggestion that a minor control point exists after mevalonic acid in the biosynthesis.

4) Biliary excretion of cholesterol was reduced by the administration of CPIB. It is suggested that this is not entirely due to an inhibition of hepatic cholesterol synthesis but may arise by a direct effect on the secretion of cholesterol into the bile.

Conclusion.

Liver perfusion can be considered as an acceptable method for examining the equilibria for cholesterol which exist between the liver and the perfusing blood.

Mevalonic acid, a cholesterol precursor, is taken up into the hepatocyte and synthesised into cholesterol under first order kinetics. It is suggested that the uptake of 'acetate' and synthesis of cholesterol from this precursor may occur more rapidly than for mevalonate. The synthesis can be stimulated by prior biliary drainage and inhibition of synthesis occurs as a result of thyroidectomy or administration of CPIB. The action of the drug is very rapid, inhibition occurring before the rate limiting step within thirty minutes of administration. Inhibition of cholesterol synthesis as a result of thyroidectomy has been shown to occur at some point past the rate limiting step in the synthesis.

The newly synthesised cholesterol can be found in three intracellular pools, namely the soluble fraction, the nuclear fraction and a pool consisting of the combined mitochondrial and microsomal fractions. The intracellular cholesterol is then distributed between various anatomical and biochemical compartments of the blood and liver system. The turnover of unesterified cholesterol between plasma and erythrocytes occurs the most rapidly. This is followed by the exchange of either unesterified or esterified plasma cholesterol with their counterparts in the liver. The exchange between unesterified cholesterol and ester cholesterol in the plasma and within the liver takes place much more slowly. Transfer of cholesterol into the bile, relative to exchanges taking place elsewhere in the blood and liver system, is so slow that it is difficult to provide a reliable estimate of the rate.

When the transfer between compartments is considered on the basis of how much cholesterol moves per minute then the exchange between unesterified plasma cholesterol and that in the erythrocytes is again foremost. The smallest transfer as might be expected is from the unesterified cholesterol pool in the liver into the bile.

The newly synthesised cholesterol exchanges with the cholesterol in the blood in preference to being degraded to form bile acids. It is likely that the increased excretion of bile acids,found when hepatic cholesterol synthesis is increased, causes low blood bile acid concentrations and as a consequence reduces the exchange of cholesterol between plasma and erythrocytes.

The amount of cholesterol excreted in the bile appears to be dependent upon the rate of synthesis of hepatic cholesterol. Reduction in the rate of synthesis causes the biliary excretion of cholesterol to fall. The low concentrations of cholesterol found in the bile when hepatic cholesterol synthesis was reduced by administration of CPIB may also arise by a direct effect of the drug on the secretion of cholesterol into the bile. The low biliary cholesterol concentration foumd when hepatic synthesis of cholesterol was stimulated by prior biliary drainage is probably due to a diversion of newly synthesised cholesterol to produce bile acids.

In the rat the plasma cholesterol esters are probably derived mainly from the liver since the activity of LCAT was low as shown by constant specific radio-activity values for the plasma esters in the <u>in vitro</u> experiments. Stimulation or inhibition of the synthesis of unesterified cholesterol has little effect on the intrahepatic esterification of cholesterol. It is suggested that cholesterol esters exchange between liver and blood by a different mechanism than for unesterified cholesterol.

In conclusion these studies indicate that changes in the rate of synthesis of cholesterol in the liver,

induced by biliary drainage, thyroidectomy or administration of CPIB, have little effect on the exchange of cholesterol between liver and blood.

Further Studies suggested by the results published in this thesis.

These can be conveniently divided into two areas, firstly relating to cholesterol metabolism and secondly to the use of compartmental analysis as a diagnostic aid using information obtained by analysis of patient specimens.

Cholesterol

1) Since the liver accounts for the major proportion of cholesterol synthesised de novo then the rate constants determined for the exchange of cholesterol in the blood and liver system should be usable in deriving a quantitative model of cholesterol exchanges occurring in the whole animal. Thus if radioactive mevalonic acid is administered intravenously to a rat the specific radioactivity curves associated with the blood cholesterol compartments would be of a different form to those derived from the isolated blood and liver system. Any differences found could be ascribed to the synthesis of cholesterol by extrahepatic sources. A model could therefore be constructed for whole body cholesterol equilibria using the rate constants determined in the more restricted model (Blood and liver).

Subsequently it might be possible to administer radioactive cholesterol by mouth and construct a model to allow for intestinal absorption as well. In this short term type of experiment only exchanges occurring within the cholesterol pool with the most rapid turnover could be considered (Goodman and Noble, 1968).

2) The perfusion experiments in normal, bile drained and thyroidectomised rats should be repeated using natural lipoproteins containing labelled cholesterol to confirm the results shown in the main body of the thesis.

3) There was some suggestion in the experiments using livers from bile drained animals that the exchange of cholesterol between plasma and erythrocytes was disturbed. It would be of interest to examine the effect of different bile acids at different concentrations on the exchange of cholesterol between plasma and erythrocytes in vitro.

4) Weiss and Dietschy (1969) have suggested that the feedback control of cholesterol synthesis in the liver is due to chylomicrons originating in the intestinal cell. It would be of some interest to examine the effect of other fractions of the blood which contain cholesterol on the rate of synthesis of cholesterol in the liver. For example, using an isolated liver preparation, high concentrations of the different lipoprotein fractions in the perfusate may affect synthesis rates to varying degrees.

5) In an animal fed on a high cholesterol diet the rate of cholesterol synthesis in the liver falls. This is consistent with the model proposed by Weiss and Dietschy (1969). It does not however explain why the synthesis rate also falls in the fasted animal. Here the reduced absorption of cholesterol is associated with fewer chylomicrons entering the lymphatic system and thus should produce and increased cholesterol synthesis rate in the liver. It is possible that this effect may be

due to a diminished supply of substrate precursor. This area needs further study.

6) Myant and Eder (1960) showed that an increased excretion of bile acids in the bile is associated with increased synthesis of cholesterol in the liver. The primary event is still not clear. In rats fed on a high cholesterol diet the rate of cholesterol synthesis in the liver is reduced. At the same time there is an increased excretion of bile acids to prevent the development of a hypercholesterolaemic state. There is some divergence therefore between the reduced rate of synthesis of cholesterol in the liver and the increased excretion of bile acids since one would have expected the synthesis of bile acids to fall when the rate of cholesterol synthesis fell.

It is considered that endogenous cholesterol is the initial precursor of bile acids (Percy-Robb and Boyd, 1970; Ogura et al., 1971) and the results of the study reported here suggest that it is cholesterol which is synthesised de novo which is preferentially used. If this is correct then in the animal fed cholesterol the liver must at some point change from the utilisation of endogenous cholesterol to that of exogenous cholesterol for the formation of bile acids. This would be necessary to prevent the development of a hypercholesterolaemia.

The question of substrate preference in the production of bile acids could be examined using the isolated liver preparation. For example if the cholesterol in the liver is labelled with (^{14}C) prior to perfusion then the bile acids excreted will be similarly labelled. Addition of substantial quantities of (^{3}H) labelled cholesterol in natural lipoproteins to the perfusate should lead to a progressive increase in the ratio of (^{3}H)/(^{14}C) in the bile acids excreted.

7) Further intracellular studies should be performed in livers taken from bile drained and thyroidectomised animals to examine the effect of stimulation or inhibition of cholesterol synthesis on the comparative rates of movement of cholesterol within the cell.

Compartmental Analysis.

In laboratories providing an analytical service for a hospital increasing use is being made of mathematical and statistical methods to increase the usefulness of the service to the clinician. For example, multi-dimensional csaling, discriminant function analysis, cluster analysis and multiple and partial correlation are techniques which can categorise disease groups and may lead to new definitions of disease processes.

Compartmental analysis has been used spasmodically in the past in attempts to obtain information about control mechanisms in the homeostasis of biochemical constituents of the blood, e.g. Ackerman et al.(I969) described a model of glucose regulation in normal and diabetic subjects; Billing et al.(I964) used this technique in examining the hepatic transport of bilirubin and Ching-Chung and Urquhart (I969) established a model for the adrenocortical secretion of cortisol.

Any test performed on a patient where a number of datum points are produced over some time period can be expanded on a theoretical basis to produce a model which would fit the observed data. Provided this model is sensibly chosen using a priori information then it may be possible to delineate more precisely the pathological lesion. This would eventually provide a better basis for therapeutic intervention.

Tests commonly carried out in clinical laboratories where a continuous variable is generated are shown in Table 27.

Table 27.

	Test.	Target Organ.	Variable.
I)	Pentagastrin stimulation	Gastric mucosa	H^+
2)	ACTH stimulation	Adrenal cortex	Cortisol
3)	Bromsulphthalein excretion	Liver	Concentration of BSP in blood.
4)	Glucose tolerance.	Pancreas(insulin) Intestine(absortion)	Blood glucose.
5)	Xylose absorption.	Intestine(absorption) Kidney(excretion)	Blood and urine xylose.
6)	Calcium infusion	Parathyroids	Blood calcium.
7)	Inulin clearance	Kidney	Blood inulin.
8)	Thyroxine ^{131}I infusion.	Thyroid	Blood ^{131}I
9)	Elimination and metabolism of drugs.	Kidney and liver.	Blood drug levels.

Application of compartmental analysis in some of these tests might be potentially useful to the physician.

Appendix.

I) Determination of plasma glucose.

0.Iml of rat plasma was diluted with I.4 ml of distilled water and the concentration of glucose determined by a method using glucose oxidase (Morley et al., I968). The method had a coefficient of variation of 5% between analytical runs.

2) Determination of plasma urea.

50µl of rat plasma were diluted to I.Oml with distilled water and the concentration of urea in the plasma determined using the method described by Rigby and Hull(I964). The method had a standard deviation of ±2.0mg/I00ml over a range of values between I0-95mg/I00ml.

3) Determination of blood haematocrit.

Approximately 0.05ml of blood was collected in a heparinised capillary tube (Type D55I, Radiometer, Copenhagen) and one end sealed by melting the glass in a low Bunsen flame. After cooling, the tube was centrifuged at I000g for I0min. The height of the erythrocyte column was compared with that of the whole blood column and the percentage of erythrocytes present was calculated. The method had a standard deviation of I.0% over a range of values between 20-50%.

4) Determination of enzyme activity.

a) Cytochrome oxidase.

This activity was measured by the method of Cooperstein and Lazarrow(I95I) using a substrate (cytochrome c) concentration of $2.4 \times I0^{-5}$M. The assays were performed on a Gilford recording spectrophotometer. The specimens were diluted to produce an adequate response on the spectrophotometer and the test was measured against a blank in which the cytochrome c was completely oxidised by the addition of two crystals of potassium

ferricyanide. The enzyme activity was measured for the first 90 seconds of reaction in which time period first order kinetics were evident. The activity was expressed as the absorbance change/minute/total volume of the fraction being tested.

b) Glucose-6-phosphatase.

The assay procedure for this enzyme has been described by King(I965) and was followed exactly as described. The amount of enzyme from each separated fraction was adjusted to obtain an absorbance measurement in the region of the standard used. The results were calculated as the mass (µg) of phosphorous released from the substrate, glucose-6-phosphate, per total volume of the fraction being tested.

5) Determination of the amount of haemolysis in a perfusate sample.

20µl of plasma and whole blood were pipetted into separate 5ml aliquots of distilled water. The whole blood haemolysate was centrifuged and diluted to give an absorbance of about I.0 at 4I0nm. The plasma and whole blood dilutions were then measured at 4I0nm and the percentage haemolysis calculated.

$$\%\text{haemolysis} = \frac{\text{Absorbance of plasma} \times I00}{\text{Absorbance of whole blood} \times \text{dilution}}$$

6) Rats.

The experiments reported in this study were carried out on adult female rats of the Wistar strain. These were bred and maintained in the Animal House, Department of Biochemistry.

In all of the studies reported here the rats were fed on a diet consisting of:-

70% wholemeal flour
25% skimmed milk
5% brewers yeast Oliver and Boyd(I958)

Supplements to the diet are described in the main text.

7) Radioactive materials.

a) (2-^{14}C)DL mevalonic acid lactone.

This was available in benzene solution which was evaporated by warming at 50°C. The residue was dissolved in acetone and stored at -20°C.

Specific radioactivity.....I0.3 mCi/mM
.....79μCi/μg

Molecular weight.....I30

Radiochemical purity by paper chromatography in n-butanol:formic acid:water (77:I0:I3).....97%

Radiochemical purity by thin layer chromatography on silica gel in
a) benzene:ethyl acetate (I:I).....99%
b) benzene:ethyl alcohol (I:I).....99%

b) Sodium (^{3}H)acetate.

The freeze dried material supplied in glass ampoules was dissolved in 0.9% saline and stored at 4°C.

Specific radioactivity (using TRR.I. n-hexadecane--I,2-T as reference material).....500mCi/mM
.....6.ImCi/mg

Molecular weight(anhydrous).....82

Radiochemical purity by gas liquid chromatography.....98%

Both substances were supplied by the Radiochemical Centre, Amersham, Buckinghamshire.

c) The scintillator for liquid scintillation counting was supplied by Nuclear Enterprises Ltd. Sighthill, Edinburgh and contained 2-5 diphenyloxazole and I-4 bis(2(4-methyl-5-phenyloxazolyl))benzene dissolved with naphthalene in the solvent toluene.

8) Chemicals.

All chemicals used throughout this study were ANALAR grade.

9) Statistical tests.

a) Linear regression.

The constants in the equation

$$y = mx + c$$

were determined using the following formulae.

$$m = \frac{N\Sigma xy - (\Sigma y)(\Sigma x)}{N\Sigma x^2 - (\Sigma x)^2}$$

$$c = \frac{\Sigma y - b\Sigma x}{N}$$

N = number of variables

x,y = variables.

b) Significance tests.

In comparing two sets of curves, two methods were available:-

i) A 't' value is calculated at each time point using an unpaired 't' test, and the 't' values obtained for N sampling points averaged to obtain a common overall 't' value for the comparison of the two sets of curves. This procedure is suspect because the data at successive time points on the same curve are dependent.

ii) The mean value for the specific radioactivity at each time point for one set of experimental curves is considered to be paired with the mean values obtained for another set of curves at similar sampling times. This set of paired values were then used to derive a 't' value comparing both sets of experimental curves.

$$D = x - y$$

$$d^2 = (D - \bar{D})^2 \qquad \text{where } \bar{D} = \frac{\Sigma x}{N} - \frac{\Sigma y}{N}$$

$$S_D^2 = \frac{d^2}{N-I} \qquad \text{and} \qquad S_{\bar{D}}^2 = \frac{S_D^2}{N}$$

$$S_{\bar{D}} = \sqrt{\frac{S_D^2}{N}}$$

$$t = \frac{D}{S}$$

All calculations were performed on an Olivetti Programma IOI desk top computer.

IO) Technique of manual curve fitting. (Fig.85.)

Fitting of the best curve to the experimental data was performed as follows:-

a) a mean value $\bar{x}$ was calculated at each time interval for curves with an equal number of datum points at each time interval. The mean values were plotted on a graph with $\bar{x}_{I \cdots n}$ on the vertical axis and time(t)on the horizontal axis. A linear interpolation between successive $\bar{x}_{I \cdots n}$ values was constructed and the distance between co-ordinates $(\bar{x}_I, t_I), (\bar{x}_2, t_2)$ measured and divided into two equal parts. The mid-point was called $y_{I \cdots n}$ and these new co-ordinates used to construct a curve. If the curve was still not smooth then $y_{I \cdots n}$ was called $x_{I \cdots n}$ and the process repeated until a smooth curve obtained.

b) if the curve had an unequal number of datum points at each time interval, then the distance given by the linear interpolation between successive $x_{I \cdots n}$ values was divided in proportion to the number of values at each time point. For example, if $\bar{x}_I$ is the average of six datum points and x_2 the average of nine datum points, then the line joining $\bar{x}_I$ and $\bar{x}_2$ was divide into fifteen parts and the new value y_I placed at 6/I5 units from $\bar{x}_2$.

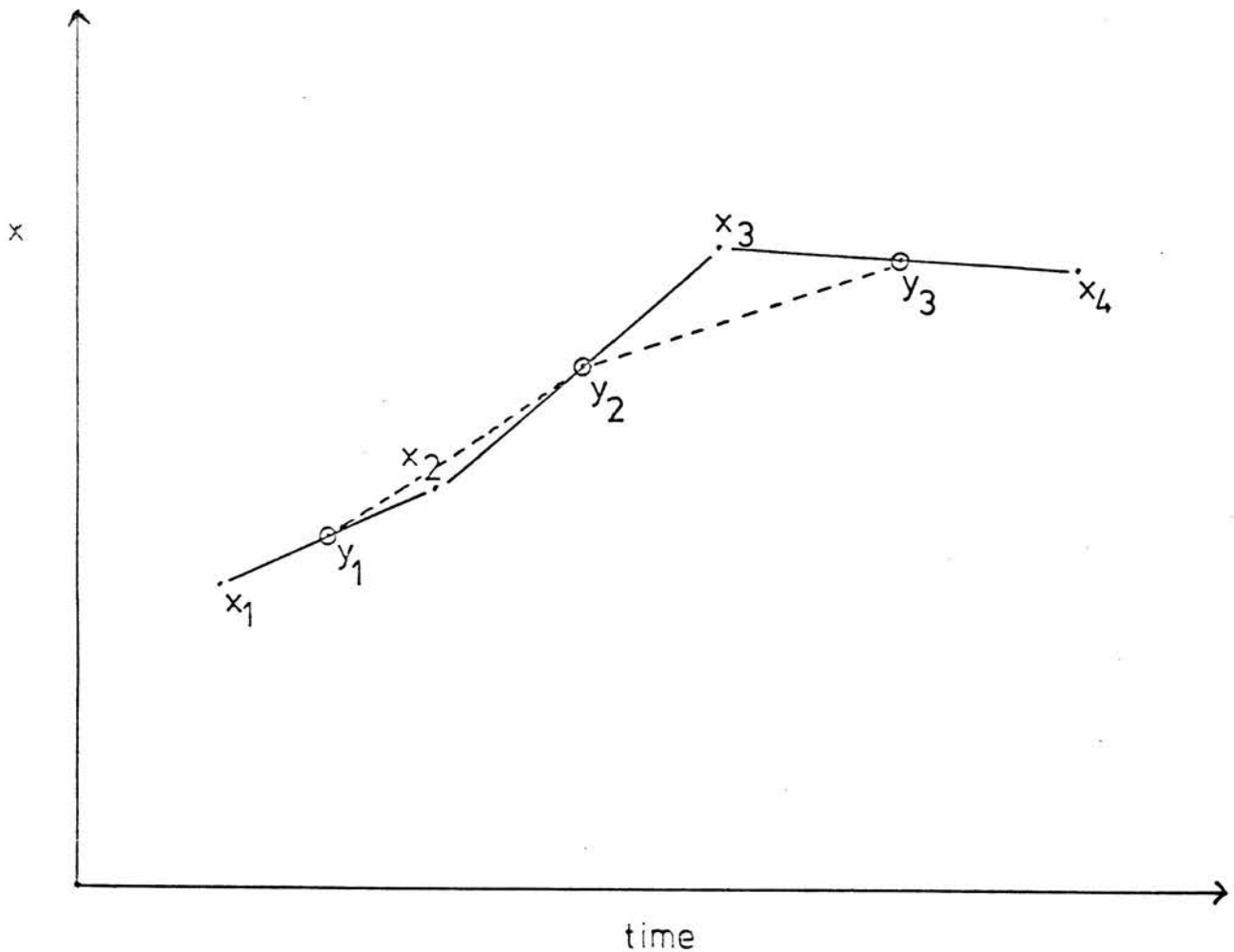


Fig.85 Technique of manual curve fitting.

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Acknowledgements.

To my wife Janet — for typing this thesis through its many phases of production. Especially for her patience with the odd hours of work which a study of this type entails.

To Dr.I.W.Percy-Robb
Senior Lecturer
Dept.Clinical Chem. — who as my immediate supervisor I wish to thank for the introduction to this area of research. We have spent many hours in interesting and stimulating discussion, not necessarily restricted to the content of this thesis.

To Prof.L.G.Whitby.
Dept.Clinical Chem. — who provided not only the time and laboratory space in which to complete this work but who also remained a constant source of encouragement.

To Mr.D.E.M.Taylor
Senior Lecturer
Dept.Physiology. — for tutelage in the use of an analogue computer. His enthusiasm led to many late working nights.

To Dr.R.Prescott.
Lecturer.
Dept.Social Med. — for an introduction to the concept and construction of mathematical models. In particular I would like to acknowledge his help in the initial formulation of the model presented here.

To Dr.G.L.Atkins.
Lecturer.
Dept.Biochemistry. — who allowed the use of his curve fitting program.

To Profs.R.B.Fisher & G.S.Boyd. — for use of the animal house facilities in the department of biochemistry.

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Compartmental Analysis of the Distribution of Cholesterol in the Isolated Perfused Rat Liver

By L. B. Roberts and I. W. Percy-Robb. (*University Department of Clinical Chemistry, Royal Infirmary, Edinburgh EH3 9YW, U.K.*)

The rate of cholesterol turnover has been described as conforming to a two-compartmental system consisting of one pool that turns over rapidly and a second pool with a low turnover rate (Goodman & Noble, 1968). The pool with the higher turnover rate is associated with the blood, liver and intestine. We have performed experiments with the isolated perfused rat liver preparation to examine transfer rates of cholesterol between whole blood, liver and bile.

Livers from rats of the Wistar strain were perfused by a method essentially as described by Percy-Robb & Boyd (1970). The liver cholesterol was labelled by adding a single pulse of DL-[$^{14}C_2$]-mevalonate to the perfusate and the time-course of the rates of appearance of radioactive cholesterol in erythrocytes, plasma, liver and bile were determined. Model simulation was performed on an EAL PACE TR48 analogue computer and estimates of the transfer rates were determined by 'Eye-fit' of the experimental results to the simulated curves displayed on a trace oscilloscope.

On a theoretical basis there are at least six compartments in this system, namely plasma esterified and unesterified cholesterol, liver esterified and unesterified cholesterol, and cholesterol in erythrocytes and bile. The system is, however, probably more complex, since individual pools of cholesterol may exist in different lipoprotein classes (Roheim, Haft, White, Gidez & Eder, 1963). We have fragmented the model to determine transfer rates in a closed plasma and erythrocyte system and applied these rates to the larger model. The larger transfer rates (min^{-1}) were associated with the exchange between plasma unesterified and plasma esterified cholesterol, and between plasma unesterified and erythrocyte cholesterol, these being an order of magnitude greater than the lowest rates of exchange between liver unesterified and plasma unesterified cholesterol and between liver unesterified and liver ester cholesterol. The unidirectional flow of cholesterol into the bile has a very low transfer rate when, cholesterol is considered alone, but is much larger if one considers the transfer in the form of bile acids (Kay & Entenman, 1961; Percy-Robb & Boyd, 1970).

This work is supported by a grant from The Secretary of State for Scotland (MRS/19/C95).

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Proceedings of the Biochemical Society

Edinburgh. July 1971
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3.7

Kinetics of Cholesterol Exchange between Liver and Blood

ROBERTS, L.B. and PERCY-ROBB, I.W.
Department of Clinical Chemistry, The Royal Infirmary, Edinburgh, Scotland.

Cholesterol is synthesised in all nucleated mammalian cells and kinetic data in vivo (1) suggest that the most rapid equilibrium is between liver and plasma cholesterol.

The object of this study was to obtain quantitative estimates of the rates of cholesterol transfer between the various biochemical and anatomical compartments associated with liver, blood, and bile. These experiments were carried out using an isolated, perfused rat liver preparation. (2)

A single pulse of DL (C2) mevalonic acid was added to the whole blood perfusate and the rates of appearance of radioactive, esterified and unesterified cholesterol in erythrocytes, plasma, liver, and bile were determined. A mathematical model was set up to describe the distribution of newly synthesised cholesterol between these compartments.

This six compartment model was simulated on an E.A.L. PACE TR48 analog computer and the rate constants between the compartments determined. The experimental data could be fitted by a computer simulation which indicated reciprocal transfer of esterified and unesterified cholesterol between the liver and plasma compartments.

Using the rate constants in conjunction with previously determined pool sizes it was possible to calculate absolute rates of transfer of cholesterol between the compartments. The exchange of cholesterol between plasma and erythrocytes was of major quantitative importance, with the smallest rates occurring between the plasma esterified and unesterified compartments.

Studies of rate constants have been made in liver systems in which the rates of cholesterol degradation have been increased by prior biliary drainage.

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The Scandinavian Journal of Clinical and Laboratory Investigations.

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