

THE EFFECT OF DIETARY LIPIDS
ON THE
SERUM CHOLESTEROL CONCENTRATION OF THE RAT

A THESIS

presented for the Degree of

DOCTOR OF PHILOSOPHY

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To
Boronia

" It is vain to do with more
what can be done with less."

William of Occam
1290 - 1349

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PART I

INTRODUCTION

SECTION 1

THE PROBLEM

The original investigation which led to the chain of events of which the present thesis is a small part, was a survey of the incidence of ischaemic heart disease among the different racial groups in Cape Town, conducted by Bronte-Stewart, Keys and Brock (1955). Among the results arising out of this work was the discovery that highly significant differences in the mean total cholesterol level exist between the three racial groups, with the Bantu having the lowest, the Europeans the highest serum cholesterol and the Cape Coloured somewhere in between. These differences correlated with a number of factors among which was the habitual consumption of fat, the intake of the European being more than twice that of the Bantu, with the Coloured again in between. These authors conclude:

"The results of this survey support the hypothesis that the dietary intake of fat influences the level of the serum cholesterol.....and may be one of the major factors influencing the pathogenesis of coronary heart disease. "

A further finding was that within each racial group the serum cholesterol level was highest in those with the highest income and bore a parallel relationship to the intake of foods rich in fat of animal origin, whereas there was no correlation with the intake of vegetable fat. This indicated

that in addition to the quantity of fats consumed, the origin or quality of these fats seemed to have an important bearing on the resulting serum cholesterol level produced.

To investigate this aspect, Bronte-Stewart, Antonis, Bales and Brock (1956) fed a variety of different fats and oils to humans under strictly controlled conditions, studied the effect on the serum cholesterol levels and concluded;

"Animal fats and hydrogenated vegetable fat behaved differently from vegetable and marine fish and mammal oils. The most likely common difference between these fats and oils is the proportion of unsaturated and saturated fatty acids in the fats and oils concerned."

Thus whereas relatively saturated fats such as butter, partially hydrogenated groundnut oil and beef muscle fat raised the serum cholesterol level, unsaturated oils such as sunflower seed oil, groundnut oil and South African Pilchard oil had the opposite effect.

Due to their possible bearing on the pathogenesis of ischaemic heart disease, these interesting findings have triggered off a vast amount of research in all parts of the world, which has on the whole confirmed this work in innumerable studies on animals and man. A considerable portion of this work was, and still is, directed towards attempts to explain why and how different dietary fats produce different serum cholesterol levels. Many interesting experimental facts have been reported and a number of theories have been proposed to account for these facts.

None of these theories, however, bears detailed

and critical examination and many investigators would probably agree that the factors in fats and oils responsible for their serum cholesterol regulating effect are at present (November, 1959) still ill defined, if not completely unknown. It is the object of the present thesis to contribute towards a solution of this problem. This can perhaps be stated more clearly as follows -

What precisely are the chemical or physical properties of different natural triglyceride fats and oils which, when fed to man, produce different serum cholesterol concentrations?

In the following pages, attention will be confined strictly to the solution of this limited objective. Many investigations relating to this subject deal with the effect of different dietary substances on "cholesterol metabolism", i.e. the simultaneous effect is studied on a variety of blood lipids, faecal sterols, liver lipids, cholesterol synthesis, bile acids, etc. There is no doubt that such comprehensive studies yield valuable results. They often suffer from the disadvantage, however, of being difficult to interpret and are consequently wasteful of much ingenious and laborious experimentation. It is felt, therefore, that a precise definition of the relationship between two single variables (i.e. property of dietary fat versus serum cholesterol level) may be of value as a foundation to more comprehensive studies.

The first aim in an investigation of this nature is the creation of the necessary experimental machinery, the scope of which is, of course, determined by the resources available. As the title suggests, the present study is mainly

concerned with experimentation on rats. To substitute rats for humans requires extensive qualification and justification. A major part of the work will therefore be concerned in investigating the rat as an experimental animal and in demonstrating that under certain conditions rats react in a similar fashion to humans as far as the biological mechanism under study is concerned. It is believed that this has been done satisfactorily. It follows that any results and conclusions arrived at from experimentation on rats are likely to throw strong light on the parallel mechanisms under study in humans. An attempt will therefore be made in the concluding parts of this thesis to apply any new knowledge gained on rats to the human sphere. This new knowledge, after integration into a theory (the solubility theory) will then be offered for consideration as a new solution to the problem stated above.

Further remarks concerning the general structure, form of presentation and plan of this investigation will be made in Sections 5 and 8, after the work of some of the more prominent workers in the field has been briefly reviewed, and the results of an exploratory experiment on rats reported. Against this background more detailed discussion will become possible.

SECTION 2THE EFFECT OF DIETARY FATS
ON THE SERUM CHOLESTEROL OF MAN

Although the serum cholesterol regulating effect of dietary fats has been known for a considerable time, it is only comparatively recently that it became evident that fats of different chemical composition and origin behaved differently in this respect. Up to about 1952 the view was prevalent that all dietary fats raised the serum cholesterol and that the extent of this effect was proportional to the amount of fat fed, and was unrelated to the origin of the fats fed. As recently as 1952, Keys discussed the relative merits of different fats in raising the serum cholesterol and came to the conclusion that "there is not the slightest evidence for a difference between animal and vegetable fat."

One of the first indications to the contrary apparently came from Groen et al. (1952) who fed diets rich in different fats to humans and found that animal fats produced higher serum cholesterol levels than vegetable fats. Kinsell et al. (1952) actually produced a lowering of the serum cholesterol level in hospital patients consuming diets rich in vegetable fat. Two years later, Mayer and associates (1954) followed with a report of a greater serum cholesterol

raising effect of lard as compared with vegetable fats consisting of groundnut oil, corn oil and vegetable margarine. Further confirmation of the view that animal fats behaved differently from vegetable fats came from the work of Harding and Stare (1954) who compared the serum cholesterol levels of vegetarians and non-vegetarians and found the latter to have higher values.

Although no agreement had been reached as to whether vegetable fats actually lowered the serum cholesterol level or only produced a moderate raising effect, it became evident that in general they behaved differently from animal fats. That this different behaviour is not due to the small cholesterol content commonly found in such fats as butter, beef, tallow, lard, etc. has been shown by many workers, notably Messinger et al. (1956) and can therefore be assumed to be of little importance.

It is against this background that Bronte-Stewart and his collaborators (1956) took one step further by demonstrating that certain chemical properties of fats such as total unsaturation, linoleic acid content or related properties such as the content of essential fatty acids correlated well with the serum cholesterol values as discussed in the previous section. This correlation, furthermore, was independent of the origin of the fats and oils fed, thus superseding the "animal versus vegetable" fat theory. A typical experiment is illustrated in Fig. 1.

Later in the same year, Beveridge, Connel and Mayer (1956, 1957a) studied the properties of butter and

corn oil and reported that whereas α -tocopherol (which is present in corn oil) when added to butter did not abolish the elevating effect of the latter, β -sitosterol when administered in doses much in excess of those present in corn oil did produce a highly significant depression of the serum cholesterol, which equalled that of corn oil itself. These workers then went on to study the molecularly distilled fractions from both fats and found the lightest fraction from butter to have the most potent elevating effect whereas the same fraction from corn oil had the most depressing effect. They noted that these active fractions were associated in both cases with high percentages of unsaponifiable matter consisting of much digitonin-precipitable, Lieberman-Burchard positive sterols. As a result of this they suggested that the composition of the unsaponifiable matter may be partly responsible for the potent depressing effect of corn oil. It must be pointed out, however, that some of these differences could not be shown to be statistically significant.

It is at about this point in time (March, 1957) that the present investigation was initiated. Much material has been published since. Some of this will be discussed in more detail in relation to the findings of the present study. The more widely held views at present can, however, be summarised as follows:

Kinsell and Sinclair (1957) believe that the hypocholesterolaemic action of unsaturated oils is due to

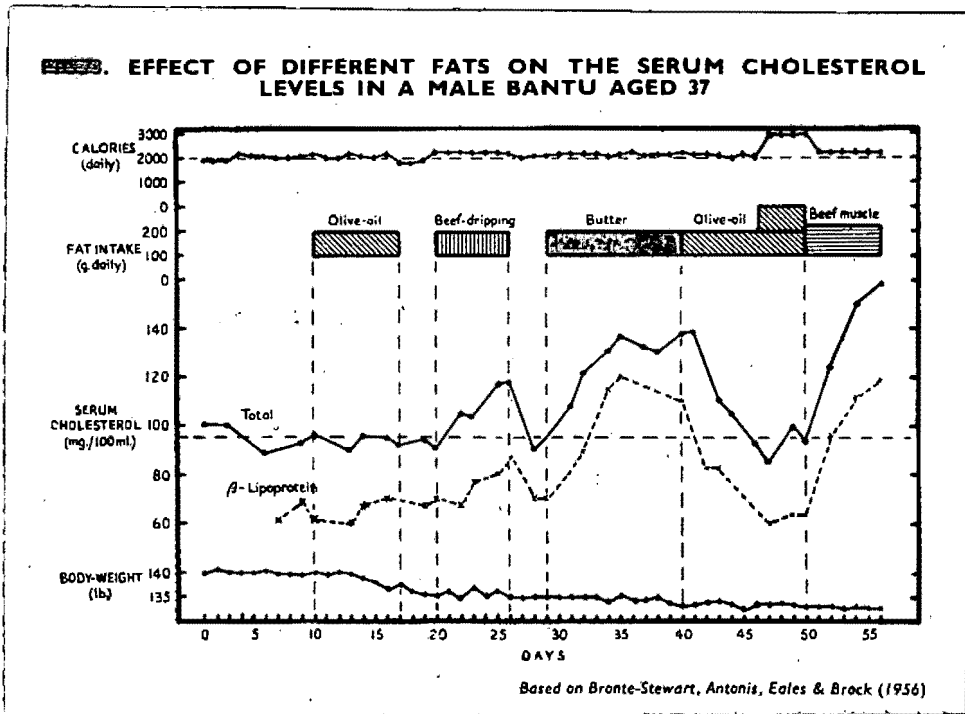


Fig. 1 Example of experiment by Bronte-Stewart, Antonis, Eales and Brock, 1956.

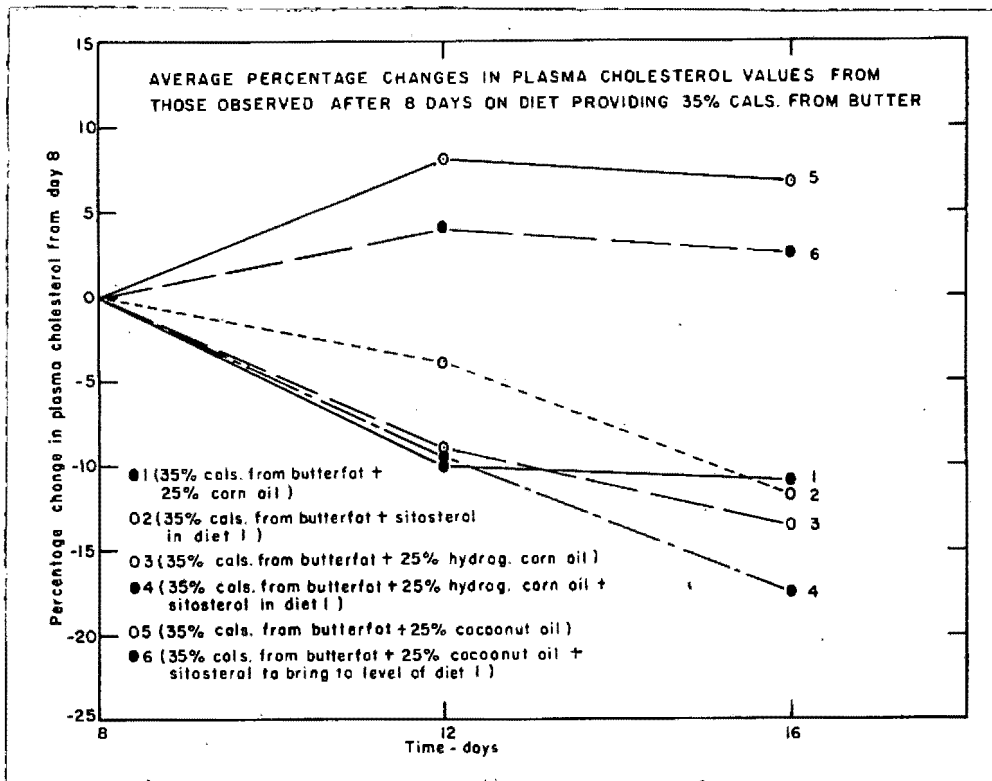


Fig. 2 Example of experiment by Beveridge, Connell, Mayer and Haupt, 1958.

the presence of so-called "essential fatty acids", i.e. linoleic acid, linolenic acid and arachidonic acid. Kinsell, Michaels, Friskey and Splitter (1958) concluded after the feeding of purified ethyl and glycerol esters to healthy and unhealthy humans that "the major active ingredient in various vegetable fats responsible for lowering the plasma cholesterol is linolenic acid."

One of the major criticisms against this view has been made by Ahrens et al. (1959a) who pointed out that fish oils which are the most potent cholesterol lowering oils known are practically devoid of essential fatty acids. Even more recently, Keys, Anderson and Grande (1959) actually reported an increase in serum cholesterol after administering a preparation of arachidonic acid to human subjects. Ahrens and collaborators (1957), after conducting one of the most comprehensive investigations into the properties of a large number of fats and oils, expressed the opinion that the total unsaturation as measured by the iodine value constitutes the best correlation obtainable to date. They admit, however, that there are serious discrepancies which will be evident from the inspection of Fig. 3.

Fig. 3/

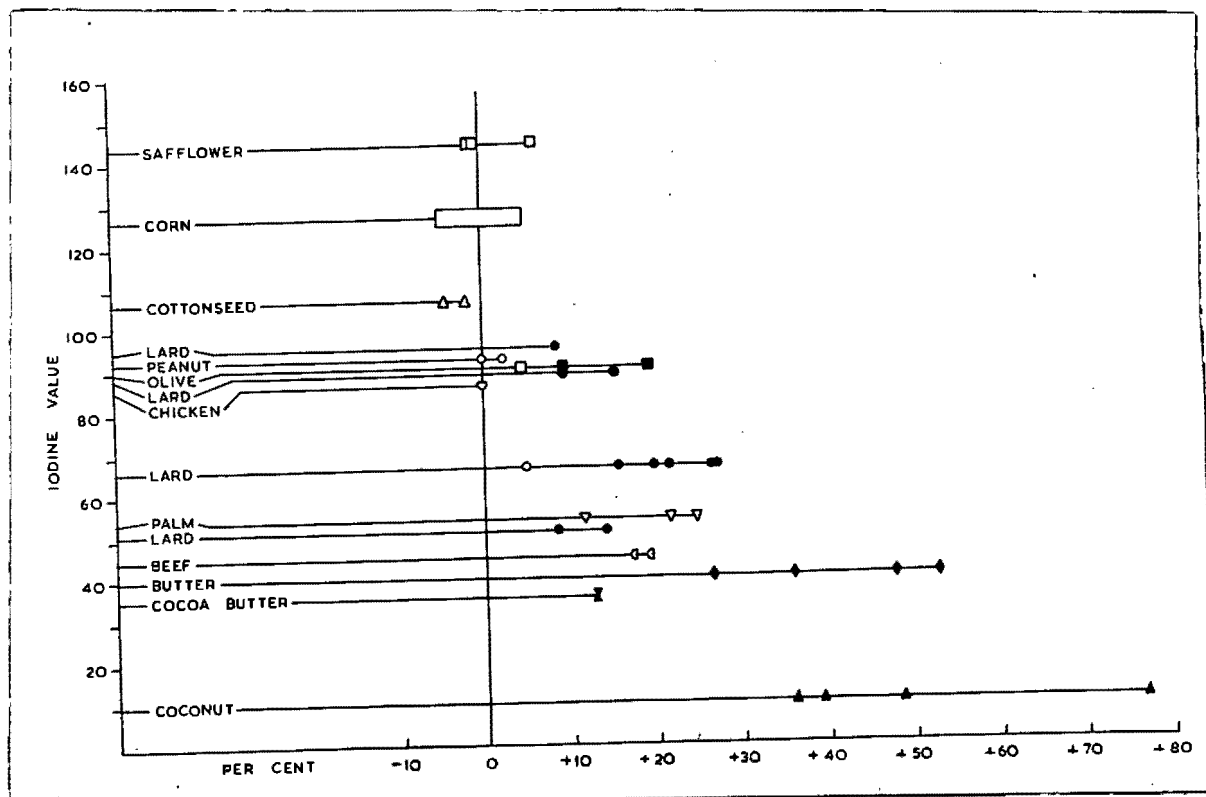


Fig. 3: Relationship between iodine values of dietary fats and serum cholesterol levels, expressed as percentage differences from base-lines established during ingestion of corn oil.

Open bar at I.V. 126 = estimated reproducibility of corn oil base-line (from Ahrens et al., 1957)

Keys, Anderson and Grande (1957, 1958). This group claims to be able to predict changes in serum cholesterol level due to a change in dietary fat from the following formula:

$$\Delta \text{Chol.} = 2.74 \Delta S - 1.31 \Delta P$$

where Chol. = change in serum cholesterol concentration in mg./100 ml S = difference between percent of total calories provided in the two diets from glycerides of saturated fatty

acids and P being the corresponding difference in glycerides of polyunsaturated fats. This formula relegates the mono-unsaturated oleic acid to a completely neutral position, and incidentally breaks down in the case of corn oil which produces lower cholesterol values than predicted while hydrogenation of oils produces values not as high as predicted.

Beveridge et al. (1958a) discussing some further work with butter, coconut oil, natural and hydrogenated corn oil, conclude "There can be no doubt that in the case of corn oil at least, plant sterols such as sitosterol, or some other substance associated with it in the unsaponifiable matter, account for much of this (cholesterol lowering) activity". After finding only a minimal effect with the artificial saturation or hydrogenation of corn oil they further state that "...there is no simple relationship between degree of saturation and the ability of a fat to depress plasma cholesterol levels."

From the above discussion it is evident that our present state of knowledge of the subject is highly unsatisfactory and fragmentary. It can safely be said that the factors in dietary fats and oils which influence the serum cholesterol level are at present still unknown. To venture an intelligent guess as to a possible mechanism by which dietary fats behave as they do, is therefore premature at this stage.

SECTION 3EFFECT OF DIETARY FAT ON THE SERUM CHOLESTEROL
LEVEL OF THE RAT

The amount of work that has been devoted to the study of the effect of dietary fats and cholesterol on the serum cholesterol level of animals is prodigious. No doubt this is due to its growing interest in relation to the pathogenesis of atherosclerosis. Another reason may be the comparative ease with which cholesterol is determined in the laboratory. To give even a brief outline of all animal work is beyond the scope of this study. If attention is mostly confined to experimentation with rats as experimental animals the output of work is still great and the results obtained are bewildering. Fortunately, good reasons can be found for making an even narrower selection of material for closer examination.

In the great majority of animal experiments in which dietary factors are studied in relation to their effects on the serum cholesterol level, the diet itself was supplemented with cholesterol. In the case of the rat this was often further supplemented with other substances such as cholic acid. Thiouracil and other drugs were occasionally added to lower the basal metabolic rate. The purpose of these manipulations was to produce or accentuate effects not normally observed with more natural diets.

There can be no doubt, that in the case of man at least, dietary fats - mostly free from cholesterol - have a

characteristic effect on the serum cholesterol level. It is precisely this effect, however, which is under study and large doses of cholesterol must therefore be excluded from the diet if conclusions arrived at from animal experimentation are to have any application to man. Attention will therefore be focussed mainly on reports of feeding experiments with more natural diets.

It is curious that the classical work of Schettler (1948) is rarely quoted in more modern texts. He fed various animal and plant fats together with a basic diet consisting largely of skim milk to mice. Dog depot fat, lard and tallow clearly raised the blood cholesterol whereas the more unsaturated sesame and olive oils had little influence. Although these results anticipated the effects now accepted for humans, no attempts were made by others to confirm or extend these studies.

The work of Swell et al. (1955) on rats illustrates the very different effects obtainable by feeding a fat (olive oil) under different conditions. Much greater elevating effects on the serum cholesterol level are obtained when cholesterol and sodium taurocholate are included in the diet. In the same year, Shapiro (1955) observed higher serum values with safflower oil than hydrogenated vegetable fat in the unsupplemented diet of rats. This is not in accordance with later human experience.

One year later Olson, Jablonski and Taylor (1956) fed cholesterol-free diets to rats but added various amounts of methionine and choline to the rations. All serum lipids were depressed but"this effect was not influenced by level

or kind (lard, corn oil, butter) of dietary fat." Similar results with natural diets were obtained by Okey and Leyman (1957), who were unable to find consistent differences between total cholesterol levels in coconut oil and cottonseed oil-fed animals. In a later study (Okey et al., 1959), these workers extended their investigations to other fats which were fed with and without cholesterol supplement for seven weeks after weaning. Again the serum cholesterol levels produced by 13 different saturated and unsaturated fats showed no correlation with results obtained on humans. The same results were, for example, obtained with corn and coconut oil.

Grunbaum, Geary, Grande, Anderson and Glick (1957) actually observed higher serum values with sunflowerseed oil as compared with butter when fed to rats on a cholesterol-free Purina chow diet. The animals of Aftergood, Deuel and Alfin-Slater (1957) required 24 weeks' feeding under similar conditions to demonstrate a significant difference in effect between dietary lard and cottonseed oil, the latter oil producing the lower values. Diller, Woods and Harvey (1957) failed to demonstrate any influence of hydrogenation on the effects of corn and safflower oil in the diets of rats.

More recently Best, Lucas, Patterson and Ridout (1958) studied the effects of ten different edible oils and fats on total hepatic lipids and serum cholesterol in the presence of various amounts of dietary choline and concluded that "...no fat or oil produced a level of serum cholesterol that differed significantly from that produced by any other".

Nath, Wiener, Harper and Elvehjem (1959) developed

a diet containing 25% of hydrogenated coconut oil, 1% of cholesterol and 0.5% of cholic acid. They claimed that this diet is "suitable for the study of nutritional factors affecting serum cholesterol concentration in the rat". These workers do not state, however, exactly what nutritional factors are to be studied under these artificial conditions. Neither dietary cholesterol nor fats are apparently envisaged. The former is included in the basic diet whereas of the latter it is said that "the variations in the fat level of the diet had little effect on the serum cholesterol concentration".

Even a brief review of the subject is incomplete without referring to the extensive work on rats of Hegsted and his associates. Although these investigators fed cholesterol, choline and cholic acid along with fats in the diet their results are nevertheless worth noting. They have recently (Hegsted, Gotsis, Stare and Worcester, 1959) summarized their findings in the form of an equation.

$$\log. \text{ serum chol.} = -0.00648S + 0.02105M - 0.02415P + 2.4836$$

This relates the serum cholesterol level in mg./100 ml with the percentage of saturated fatty acids (S), mono-unsaturated fatty acids (M) and poly-unsaturated acids (P) in the diet. This equation suggests that the mono-unsaturated acid raises the serum cholesterol level while saturated and poly-saturated acids reduce it. Unfortunately these authors proceed to qualify this by disclosing that other equations can be derived which give as good a "fit" as this equation. It is thus not easy to see what conclusions can be drawn from this work. Nor is

the relationship of these findings to the human sphere clear. Thus comparison of this equation with that derived by Keys et al. from work on humans (see Section 2) reveals that opposite effects are attributed to saturated fats when fed to rat and man.

It would appear from the above brief review of the literature that rats are not suitable experimental animals for the study of fats and oils in relation to their different effects on the serum cholesterol level of man.

SECTION 4A PILOT EXPERIMENT ON RATSA. Introduction

From the brief literature survey given in Section 3, any attempt to reproduce with rats the effects of dietary fats on the serum cholesterol of humans would seem to be doomed to failure. An attempt was made, nevertheless, for the following reasons:

1. Rats are omnivorous animals which thrive on a variety of diets and are easy to keep.
2. Unlike rabbits or birds, they do not easily respond to dietary cholesterol treatment and are thus more like humans in this respect.
3. The visualised fractionation of oils in the laboratory with a view to isolating a hypercholesterolaemic concentrate or substance is likely to yield only small quantities of oil. If statistically valid results are to be obtained, a minimum of six test animals per dietary group would be desirable. Six 200 gm. rats on a 20 per cent fat diet would consume about 130 gm. of fat in a test period of, say, 7 days. This would seem to be a reasonable quantity of fat to handle in the laboratory.
4. The amount of blood obtainable from the tail of rats without strain is approximately 0.2 to 0.5 ml or 0.1 to 0.2 ml of serum. The cholesterol in this quantity

can be estimated with suitably modified conventional methods, obviating the use of ultra-micromethods requiring expensive equipment.

With these overwhelming advantages in mind it would appear that a re-examination of the rat as an experimental animal is worth while.

B. Experimental

In the present pilot experiment the effect of two dietary fats and a fat-free diet on the serum cholesterol concentration of rats has been explored. Coconut fat and sunflower seed oil were chosen as test fats. The serum cholesterol raising properties of the former and lowering properties of the latter appear to be well documented from human experiments.

Method: An approximately seven months' old colony of 24 male white albino rats, previously subsisting on standard laboratory chow was divided at the beginning of the experiment into four dietary groups of six rats each. Group I was offered an essentially fat-free diet while the remaining groups received 16 per cent of combinations of coconut fat and sunflower seed oil in the diet. Details of the diets are given in Table Ia. In addition, a mineral and vitamin supplement was given to all groups. The daily ration per rat was 20 gm. for Groups II to IV and an isocaloric 23.2 gm. for Group I. The animals were kept on their experimental

diets for 33 days after which the ad.lib. laboratory chow was restored to them for a further 18 days' observation.

TABLE 1a

	Dietary groups			
	I	Parts by weight		
		II	III	IV
Skim milk powder	60	60	60	60
Potato starch	46	14	14	14
Laboratory chow	10	10	10	10
Coconut fat	0	16	12	8
Sunflower seed oil	0	0	4	8

The animals were bled from the tail at the beginning of the experiment, and after 24, 33 and 51 days. Serum cholesterols were estimated according to the method of Zlatkis, Zak and Boyle (1953), which was adapted for this single experiment to cope with the very small amounts of serum available.

Results

The results are tabulated in Table 1b and illustrated in Fig. 4. Only part of the food offered to the animals was accepted for the first few days, resulting in an initial loss in weight of up to 17 per cent in some cases. Subsequently the full rations were consumed with consequent steady gain in weight. Group I on a fat-low diet produced a considerably lower weight curve than the other groups. The growth of these groups was remarkably uniform, suggesting equal absorption of coconut and sunflower seed oils.

TABLE Ib - (Experiment 1)

MEAN SERUM CHOLESTEROL CONCENTRATIONS IN MG./100 ML OF GROUPS OF SIX
RATS ON COMBINATIONS OF COCONUT FAT AND SUNFLOWER SEED OIL DIETS

GROUP	DIET	DAYS ON DIET			
		0	24	33 ^{xx}	51
1	"Fat free"	84.2 ± 8.8 ^x	113.8 ± 3.5 ^x	92.2 ± 5.2	115.0 ± 3.6
2	16% coconut fat	85.2 ± 9.8	141.6 ± 6.9	139.0 ± 11.7	126.1 ± 6.7
3	12% coconut fat 4% sunflower seed oil	85.0 ± 5.3	116.5 ± 4.8	110.3 ± 3.9	110.7 ± 7.8
4	8% coconut fat 8% sunflower seed oil	79.3 ± 4.0	112.2 ± 5.4	101.1 ± 7.1	113.8 ± 8.0

^x standard error of mean ^{xx} experimental diets were replaced by laboratory chow after this day.

Mean Percentage Changes in Weight

GROUP		DAYS ON DIET				
		0	12	18	28	33 ^{xx} 57
1	0	-15.4	-17.6	-17.2	-15.2	-2.5
2	0	-10.2	- 9.6	- 3.2	- 1.4	+4.0
3	0	- 9.8	- 7.5	- 1.5	0.0	+6.3
4	0	-11.6	- 9.2	- 3.2	- 2.0	+4.0

The mean serum cholesterol levels of all groups rose considerably above their starting values during the first three weeks. Pure coconut fat produced the greatest elevation of 67 per cent whereas the quarter- and half-substitution of coconut fat by sunflower seed oil resulted in rises of only 37 to 42 per cent. The differential gain of approximately 30 per cent, due to the pure coconut fat over all other diets was statistically significant at the 2 per cent level, or less. After another two weeks a tendency for these differences to increase was noted. The reverse happened, when the experimental diets were withdrawn for a further 18 days and replaced by the original laboratory chow.

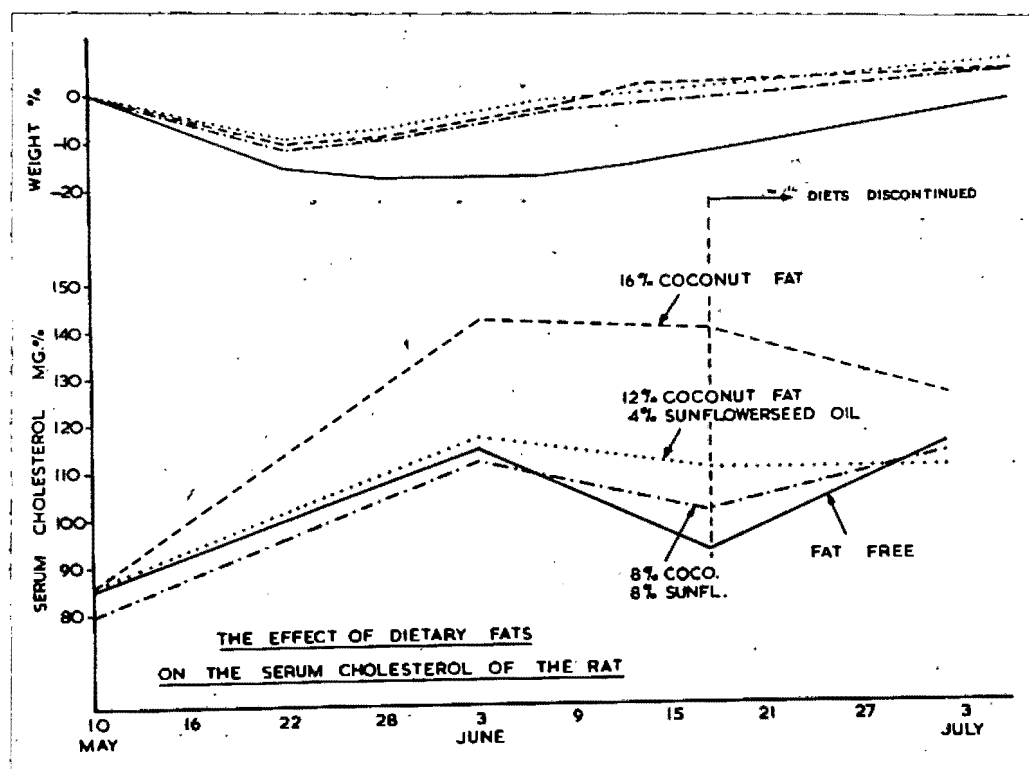


Fig. 4. The effect on the serum cholesterol level of the partial replacement of coconut fat by sunflower seed oil. (Experiment I)

Conclusions: Although by no means a model of experimentation, this pilot study has proved encouraging. Lack of previous experience with animal work fortunately proved to be no undue handicap in effecting the various manipulations. Serum cholesterols were estimated with an improvised method, with improvised apparatus. The animal colony was far from uniform with regard to age and weight. Yet the substitution of only four per cent of coconut fat by sunflower seed oil in the diet of the animals was reflected by an easily detectable difference in the serum cholesterol level.

SECTION 5METHOD OF APPROACH

Before embarking on a definite course of action it may be permissible to record a few leisurely reflections concerning the plan of investigation and mode of approach to the problem stated in Section 1. This may conceivably be attacked in a number of ways, depending on the inclinations of the investigator. The physiologist might be inclined to favour, as a first step, the elucidation of the mechanism whereby dietary fats - any fats - and cholesterol are absorbed from the intestine and thereafter influence the cholesterol metabolism as a whole. The biochemist might prefer to start by studying the effects of a large number of different fats on the serum cholesterol concentration under strictly controlled experimental conditions in the hope of relating these effects to a substance known to be present or absent in these oils from existing analytical data.

The chemist, on the other hand, is likely to neglect biological mechanisms altogether and direct his whole attention to the chemistry of the oils themselves. To him an experimental animal is a tool only, a kind of indicator or reagent which enables him to recognise, isolate and identify a compound having certain specified biological properties. He will thus probably prefer to tackle the systematic fractionation of one single oil to track down a specific compound suspected of being

present in this substance.

It is the last of these alternative approaches that has been adopted in the present study. It is also the method that has been least explored by others in seeking an answer to the same questions.

It is difficult at the outset to predict which of these policies is most likely to lead to a solution of the problem. The truth, of course, is that no particular branch of science or approach has a priority in this respect. The course taken by the present study is, in fact, an excellent example of the sterility of a "sectionalist" outlook. As will be seen, the third approach led to a promising start. It will also become evident, however, especially from the later parts of this thesis that this line of investigation eventually led to an impasse from which only the physiological approach could rescue it.

The encouraging results obtained from the pilot experiment with rats (Experiment 1), if confirmed and consolidated, afford an excellent opportunity to develop this animal as a sensitive chemical "reagent" in the above sense. If successful, this will create the preconditions for embarking on the chemist's approach, namely the chemical or physical fractionation of an oil to isolate or concentrate therefrom serum cholesterol regulating substances, assuming, of course, that such entities do, in fact, exist.

PART II

METHODS

INVESTIGATION OF THE RAT

AS AN

EXPERIMENTAL ANIMAL

SECTION 6

THE DEVELOPMENT OF AN ACCURATE METHOD FOR THE TOTAL SERUM CHOLESTEROL DETERMINATION IN 0.2 ML OF BLOOD

A search through the medical and chemical abstracts reveals the existence of a bewildering and ever faster growing profusion of methods for the estimation of cholesterol. Many of these methods are advertised as being simple, rapid and reliable. On closer inspection and especially on trial in the laboratory, however, the frustrated newcomer finds that the accuracy of most methods is inversely proportional to their speed and the amount of trouble taken to ensure good results.

A compromise has to be found. Since the determination of total serum cholesterol of rats forms the basic analytical procedure in this thesis, careful attention has been given to the selection and development of a suitable method. In addition to the obviously desirable attributes, this method must be capable of being scaled down to compensate for the small quantity of serum available and the comparatively low serum cholesterol concentration of rats. The latter ranges normally between 45 and 70 mg./100 ml.

A. Methods based on the Liebermann-Burchard reaction

The Liebermann-Burchard reaction (1885 - 1889), i.e. the production of a green colour when cholesterol is

reacted with H_2SO_4 in acetic anhydride is the basis of most classical methods. It is also the basis of a method due to Abell, Levy, Brodie and Kendall (1952), which has been practised with success in our laboratory for some years. It was discarded as a basis for the development of a micromethod on account of the relatively low intensity and stability of the colour produced by the Liebermann-Burchard reaction. It must be admitted that some of the tempting short-cut methods offered more recently, influenced this decision.

B. The p-toluene sulphonic acid method

This method, first developed by Pearson et al. (1953), together with its variations, e.g. Turner and Eales (1957), has the advantage of producing equal colour intensities with both free cholesterol and its esters and does not, therefore, require a saponification step. It has been given a thorough trial but was eventually abandoned for the following reasons:

1. The handling of minute volumes of liquid, made necessary by the low colour intensity produced by the p-toluene sulphonic acid reaction and the small volume of serum available ($25\mu\text{l}$) necessitated the use of micro absorption cells in a Beckman Ultraviolet Spectrophotometer. This was uneconomical as a routine method.
2. No less than five different pipettes are used in the method described by Pearson. This required the construction of a set of five micro constriction pipettes with capacities ranging from 0.025 ml upwards, an extremely laborious task even after considerable skill had been acquired in the glass-blowing technique used. A single breakage of these delicate pipettes caused a delay of some days.

3. Reproducibility was poor in spite of the elaborate equipment used.

C. The ferric chloride method

The reaction of cholesterol with ferric chloride has first been used by Zlatkis, Zak and Boyle (1953) to determine serum cholesterol concentrations. This method depends on a measurement at 560 m μ of the colour intensity produced when serum dissolved in glacial acetic acid is treated directly with a colour reagent consisting of a solution of FeCl₃ in concentrated sulphuric acid. The following advantages render this method attractive:

1. Extreme simplicity. Like the p-toluene sulphonic acid method, no previous extraction of the cholesterol from the serum is required.
2. Equimolecular concentrations of cholesterol and cholesterol esters yield equal colour intensities, thus obviating the saponification of the esters.
3. The purple colour is relatively stable.
4. The colour intensity produced is appreciably higher than that due to either the Liebermann-Burchard or p-toluene sulphonic acid reactions.

The last point in particular decided the adoption of this method as a starting point for scaling-down operations. Following the direction given by Zlatkis et al. (1953), but scaling down solvent and reagent volumes to a half and serum volume to a quarter (0.025 ml) from a constriction micropipette), values for human sera were obtained which were from 60 to 70% higher than when determined by the Abell method. Obviously

substances other than cholesterol contribute to the colour production. This is in agreement with the high values found by Best et al. (1954) with this method. Poor reproducibility was also noted. The obvious advantages of this method, however, warranted further efforts at development.

D. The further refinement of the FeCl_3 method

An obstacle in the above procedure is the high viscosity of the colour reagent due to the concentrated sulphuric acid. This renders the quantitative dispensing from small pipettes uncertain. A related factor is the sluggish and inadequate mixing of the viscous reagent with the acetic acid-serum solution. Local overheating in the resulting exothermic reaction is difficult to avoid. For these reasons, Furst and Lange (1954) criticised this method. It was felt, however, that a potentially promising reaction cannot be rejected for what are hardly insurmountable difficulties. One of these difficulties was met by the use of all-glass syringe pipettes. A detailed description of these pipettes appears in the next Section. The mixing problem was overcome by bubbling a stream of air into the reaction mixture from a fine capillary glass tube and was operated by means of a hand rubber bellows. The intermittent air stream was cushioned by intermediary passage through an empty winchester bottle. These modifications improved the reproducibility of the method without eliminating the comparatively high cholesterol values obtained with it. The

latter defect was realised by Zak et al. (1954) who first filtered off the serum proteins obtained by precipitation with a 50/50 solution of alcohol - acetone. They then applied the colour reagent to the dried alcohol - acetone extract dissolved in glacial acetic acid. These authors report extensive recovery experiments to illustrate the accuracy of their method. It is seldom realised, however, that good recoveries often give more information on the skill of the operator than on the original amount of cholesterol present in a given sample.

In the hands of the author the scaled-down method of Zak et al. still produced results consistently higher than those obtained by the Abell method. Attempts were made to find more selective fat solvents. Using the method of Zak et al. but employing different solvent systems, the following cholesterol concentrations were found for a serum with an "Abell" cholesterol value of 152 mg.

TABLE 2

The efficiency of different solvents on the extraction of cholesterol from serum

		mg./100 ml	
Hot acetone/ethanol	1:1	181	184
Cold acetone/ethanol	1:1	186	184
Chloroform-methanol	1:1	198	188
Ethanol-ether	4:1	185	182
Methylal		183	182

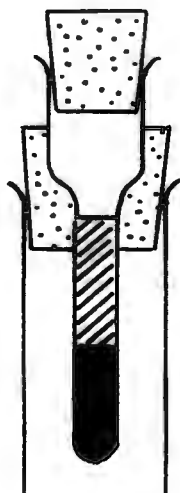
The equal efficiency of hot or cold acetone-ethanol as extraction media confirms the views of Mertens and Albers (1953). These results lend no support to the often held view

of the alleged superiority of one particular common fat solvent over others, for the extraction of cholesterol from serum.

On searching for a reason for the residual discrepancy between the Zak and Abell methods, it was realised that in the latter all saponifiable material is removed by saponification. That the saponifiable lipid contributes to the colour production was proved by determining "cholesterol" in a sample of fatty acids from which it had previously been removed by saponification and extraction of the unsaponifiable matter. Small positive readings were obtained. Substituting the solvent extraction of lipids directly from serum by the extraction of unsaponifiable matter from the saponified serum failed to eliminate the discrepancy. The saponification procedure had other very desirable advantages however. It solved the problem of removing solvent precipitated proteins by filtration or centrifugation, thus increasing the reproducibility of the method. As will be shown below, it also correlated well with the Abell method and was therefore finally adopted for subsequent work on rats. The final form of the method is described in the next Section.

SECTION 7A MICRO-METHOD FOR THE DETERMINATION OF TOTAL
SERUM CHOLESTEROL IN 0.2 ml OF BLOODA. ExperimentalPreparation of sample

The rats are caged in tight fitting cylindrical wire mesh containers stoppered at both ends by means of rubber bungs, one of which is provided with a notch to allow the tails to protrude. The latter are suspended in water at $38 - 39^{\circ}\text{C}$ for 20 minutes. After careful drying, about 2 mm. is cut from the tip of the tails and the cage is clamped vertically in a retort stand. The blood is gently "milked", drop by drop, into micro-centrifuge tubes. The wounded tails are treated with Friar's Balsam, which has on no occasion failed to effect satisfactory healing within a few days.

Fig. 5

The micro-centrifuge tubes are specially made for this purpose and hold approximately 0.4 ml of blood in the narrow shank of 4 mm. I.D. In Fig. 5 a tube, mounted in a centrifuge container, is illustrated. After spinning at 3,000 r.p.m. for 10 minutes, the supernatant serum is transferred to stoppered micro-test tubes constructed from 5 mm I.D. glass tubing. In this condition the serum is stored below 0°C , ready for analysis.

Reagents

1. Sulphuric acid A.R. (Merck)
2. Glacial acetic acid A.R. (Merck)
3. 33% aqueous KOH solution
4. Iron stock solution. 6.0 gm. of FeCl_3 anh. and 4 ml of distilled water made up to 100 ml with glacial acetic acid. Store below 0°C and thaw when required.
5. Petroleum ether, 60/80 $^\circ\text{C}$.
6. Cholesterol standard. 200 mg. re-crystallised dried cholesterol in 100 ml of glacial acetic acid solution.

Procedure

Thoroughly stir the serum contained in micro-tubes by means of glass rod drawn out to a thin point. Using a constriction micro-pipette (see below) with a capacity of 25 to 35 μl , transfer serum accurately to the bottom of 5 ml glass-stoppered (B 10) test tube. Add exactly 0.5 ml of alcoholic KOH stock solution, made up freshly by diluting 2 ml of KOH stock solution to 30 ml with absolute alcohol.

Saponify for one hour in waterbath at 40°C . After cooling, add exactly 0.5 ml of distilled water followed by 3.0 ml of petroleum ether. Replace glass stopper and shake thoroughly for one minute. Thirty tubes are conveniently shaken simultaneously in a test tube rack. The tubes are held in place by pressing a foam rubber lined board against the stoppers.

Allow to settle, then transfer 2.0 ml of supernatant petroleum ether layer to micro colorimeter tubes. Blow to

dryness in waterbath at 60°C with stream of warm air. To the dried residue add 1.5 ml of glacial acetic acid from all-glass syringe pipette (see below). Continue heating in waterbath at 60°C until unsaponifiable matter has dissolved. This takes about 3 to 5 minutes. Allow to cool.

From a second all-glass syringe pipette add 1.0 ml colour reagent. This is made up freshly by diluting 1 ml of iron stock solution to 100 ml with concentrated sulphuric acid. Immediately after adding the colour reagent, bubble a gentle even stream (see below) of air through the solution by means of a capillary tube to mix. Measure absorbance in colorimeter at 560 m μ within one hour.

A distilled water blank and cholesterol standard, dispensed from the same micro pipette, receive identical treatment.

Notes on Apparatus



An enlarged close-up photograph of a micro-constriction pipette constructed by the author is shown in Fig. 6. Some experience is necessary to get the volume within the required range (36.2 μ l in specimen shown). Some practice was also required to prevent the constriction from collapsing altogether when the required minute opening was obtained. These constriction pipettes are operated by mouth by means of a suction tube and deliver a very accurate and reproducible volume.

Fig. 6 Micro-constriction pipette.

The hot-air manifold-water bath combination was made as illustrated in Fig. 7. from which its construction is evident. This apparatus was also found convenient for the evaporation of chromatographic fractions.

A set of all-glass syringe pipettes is made by sacrificing a pyrex syringe, from the barrel of which a length of capillary tubing is drawn. Three inch portions of the latter are sealed to the outlet of similar intact syringes of the same make.



Fig. 7. Hot air manifold-waterbath combination.

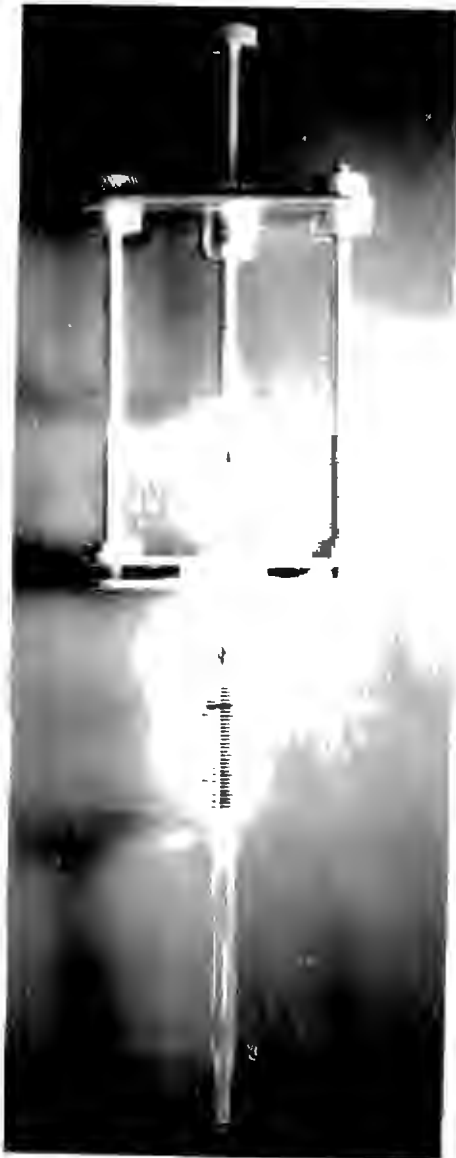


Fig. 8 Adjustable
all-glass syringe pipette

These are then mounted in fittings which allow the plungers to travel a fixed but adjustable predetermined distance (see Fig. 8). The syringe and fitting in turn are held in a retort clamp in a vertical position, making their operation with one hand possible. An accurate, fast and highly reproducible delivery of small volumes of viscous liquid is thus possible which is incidentally also independent of the room temperature. The air stream for mixing the colour reagent was produced by a hand rubber bellows. The pulsating air flow produced by the latter was cushioned by intermediary passage through an empty winchester bottle, thus producing a continuous flow from the capillary.

B. Results

The present method has been in use for over two years, in the course of which over 2,500 serum samples have been successfully analysed in duplicate. Twelve sera in duplicate are conveniently included in one batch and can be estimated by one experienced operator in six hours. Details of experimental results are given in Table 3.

TABLE 3 (See p.37)

A Standard error between duplicate readings			B Standard Deviation		C Coefficient of Correlation		
Sample No.			Sample a	Sample b	Sample No.	L.B. (x)	FeCl ₃ (y)
1	59	60	195	253	1	158	186
2	54	54	197	255	2	150	173
3	47	48	201	259	3	95	112
4	51	54	194	260	4	91	109
5	59	61	197	260	5	111	134
6	63	65	195	262	6	119	134
7	53	54	198	257	7	128	151
8	69	71	200	257	8	130	160
9	31	33	202	256	9	120	155
10	37	38	199	261	10	116	145
11	48	49	200	257	11	133	156
12	47	46	202	256	12	249	287
13	58	61	199	261	13	172	205
14	44	43	S.D. = 2.7 S.D. = 2.9		14	182	223
15	44	43			15	264	320
16	44	42			16	133	163
17	56	56			17	209	255
18	65	64			18	225	270
19	46	45			19	221	264
20	46	45			20	249	296
21	54	55			21	120	140
22	44	43			22	150	180
23	48	46			23	223	262
24	54	53			24	178	220
S E = 1.1					r = 0.997		
$S E = \sqrt{\frac{\sum \Delta^2}{2R}}$			$S D = \sqrt{\frac{\sum x^2 - \frac{(\sum x)^2}{n}}{n - 1}}$		$r = \frac{n \sum xy - \sum x \sum y}{\sqrt{n \sum x^2 - (\sum x)^2} \sqrt{n \sum y^2 - (\sum y)^2}}$		

The standard error between duplicate readings on 24 different rat sera determined on different days was 1.1 mg./100 ml (Table 3A).

The mean and standard deviation of 10 successive determinations on different days over a period of one month of the same human serum was 197.8 ± 2.7 mg./100 ml. Individual values ranged from 194 to 202 mg./100 ml. For a different sample of serum analysed 10 times on different days these figures were respectively 258.0 ± 2.9 (253 - 262). (See Table 3B).

To determine the relationship between the values obtained by the present procedure and those obtained by the now classical marco-method of Abell et al. (1952), the cholesterol content of 24 different human sera were determined by both methods. The results (Table 3C) are summarised in a scattergraph reproduced in Fig. 9. From this it is seen that the correlation ($r = 0.997$) between the present method and that due to Abell et al. is excellent.

The equation for interconversion is:

$$y = 1.182x + 2.139$$

where $y = \text{FeCl}_3$ value and $x = \text{L.B. value}$.

Fig. 9/

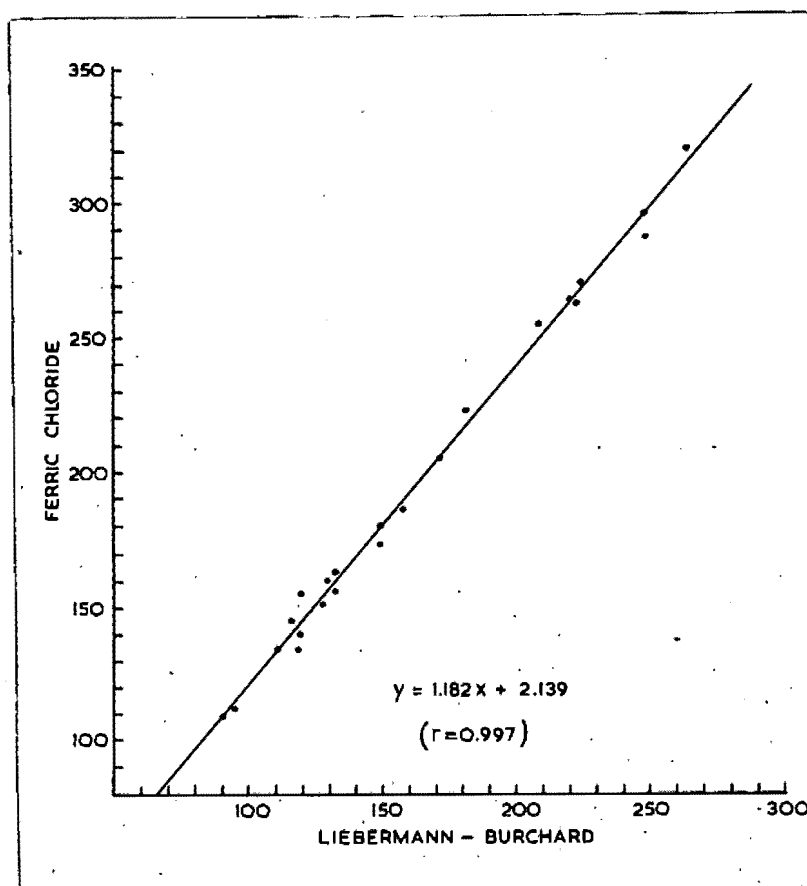


Fig. 9 Relationship between the serum cholesterol concentration as determined by the micro ferric chloride method and the Liebermann-Burchard reaction as described by Abell et al. (1952).

C. Discussion

A difficulty encountered in most methods employing the ferric chloride sulphuric acid reagent is the sluggish and inaccurate dispensation of the viscous colour reagent. This difficulty, accentuated by the small volumes employed in the present case, was overcome by the use of special all-glass syringes. The accurate measurement of volumes of serum of the order of 20 to 40 μ l is even more important. Unless this can be done successfully, care in subsequent manipulations is obviously not fully effective. The micro-constriction

pipette was found to be capable of delivering highly reproducible volumes of liquid.

The saponification step is not theoretically necessary, since equimolecular amounts of free and esterified cholesterol have been shown by Zlatkis et al. (1953) and Zak et al. (1954) to produce equal colour intensities with the ferric chloride reagent. The saponification step, adapted from the Abell method has, however, been included to remove the saponifiable matter. This makes a small but significant contribution to the total absorbance. A further great advantage is the neat separation of proteins it affords. Neither filtration nor centrifugation are required.

A similar comparison between the ferric chloride reaction and the Liebermann-Burchard reaction as described respectively by Zak et al. on the one hand and Mertens and Albers (1953) and Colman and McPhee (1956) on the other, has been made by Smit and Weymeyer (1957). Their reported regression equation, after interchanging variables to make it comparable is:

$$y = 1.196x - 11.29$$

with a correlation coefficient of 0.917. Except for the constant term which is larger in their case the two equations are in good agreement.

It is believed that the excellent reproducibility obtained with the present micro method is attributable largely to the special attention that has been paid to the experimental

details attending the accurate measurement and handling of small volumes of liquid. While no originality is claimed for any of the apparatus used here, no reports could be found where similar apparatus have been described for the micro estimation of cholesterol in serum.

SECTION 8DESIGN OF ANIMAL EXPERIMENTSA. Choice of experimental diet

The choice of the non-fatty part of the experimental diets would be a comparatively simple matter if it were known that proteins, carbohydrates, minerals and vitamins played no role in the regulation of the serum cholesterol concentration. Unfortunately, this is not the case. After spending a day or two in the library one might, in fact, be tempted to conclude that it is difficult to find a chemical substance known to dietitians which has not been claimed by someone, at some time, to influence the serum cholesterol of some species in one way or another.

For the present purpose these various influences are of little concern except in so far as they might interfere with the mechanism by which dietary triglyceride fats as such undoubtedly exert their effects. In the absence of this knowledge, the a priori choice of a suitable basic diet necessarily involves some guesswork.

The basic diet listed in Table 4 has been chosen as a compromise between the following desirable characteristics -

1. Minimum number of ingredients
2. Nutritional adequacy
3. Easily procurable components at reasonable cost
4. Keeping quality at room temperature
5. Acceptability
6. Uniformity of components from batch to batch
7. Negligible fat content
8. Probable absence of components likely to affect the serum cholesterol level seriously. The importance of excluding cholesterol, cholic acid, thiouracil, etc., has already been stressed in Section 3.

TABLE 4BASIC FAT-FREE COMPONENT OF EXPERIMENTAL DIETS

	<u>Parts by weight</u>
Skim milk powder	66
Dehydrated lucerne	8
Brewer's yeast	2
De-fatted sunflower seed husks	3.5
Salt mixture (NaCl containing 0.1% FeSO ₄ · 7H ₂ O and 0.01% CuSO ₄ · 5H ₂ O	0.5
	80.0
To complete experimental diet add fat under test	
	20.0
Total	
	100.0

Although deficiency symptoms are not likely to arise in short term experiments, this diet was found to meet the requirements of rats in all the more important vitamins and minerals with the possible exception of iron and copper which were added as a supplement in the salt mixture. This basic

diet, together with fat, did in fact promote satisfactory growth as will be shown in a long term experiment (No.3) with an experimental butter diet. The growth curve in this instance (Fig. 12) closely resembles a similar curve described by Weeks (1957) to illustrate normal growth in rats.

It is more important to meet a possible criticism that serum cholesterol levels may be influenced by essential fatty acid deficiency. The linoleic acid content of the most saturated fat fed, i.e. coconut fat, however, assured the animals of a minimum daily intake of 50 mg., an entirely adequate quantity. Aaes-Jørgensen et al. (1958) moreover, have since shown that this deficiency is very difficult to induce in adult rats.

B. Experimental Design

Management of rats

With few exceptions, the animal experiments to be described in these pages follow the same pattern. The pilot experiment described in Section 4 served as the basis for the development of a standard experimental design which was as follows:-

The animals used for each experiment consisted of 24 white male albino rats reared on laboratory chow. They were selected from a larger group for uniformity of weight and appearance. At between two and three months of age, the colony was split up randomly into four groups of six rats each. The animals were individually marked and housed in

12 cages, two rats to each cage. The distribution of the four dietary groups among the 12 cages followed a definite systematic pattern. The first group was assigned to Cages 1, 5 and 9; the second group to Cages 2, 6 and 10, etc. This served to randomise possible variables arising out of unequal distribution of light and temperature and the considerable time interval between bleeding the rats in the first cage in the early morning and those in the last cage in the afternoon.

The animals were bled as described in Section 7 at the beginning of the experiment while still on a laboratory chow diet and thereafter at weekly intervals. Great care was taken not to exceed the minimum necessary quantity (0.2 to 0.3 ml) of blood drawn for analysis. Recently, Coleman and Beveridge (1959) have likewise drawn attention to the importance of this point. The normal duration of a routine experiment was two or three weeks, after which the colony was discarded.

Unless otherwise stated, the experimental diets were fed ad. lib. and consisted of 80 per cent of the fat-free basic diet described under A above and 20 per cent of the lipids to be tested. All animals were weighed at weekly intervals during the test period.

Presentation of results

Weights and serum cholesterol concentrations on the experimental diets containing the oils under test were recorded as percentage changes from the initial values, i.e. those obtaining immediately before splitting up the colony into the experimental dietary groups, while still on a laboratory chow diet. Means were calculated from the individual percentage

change of each animal. For statistical convenience the initial values were taken to be 100 per cent.

The method of working with changes in serum concentration rather than with absolute values produces better statistical "p" values by smoothing out disturbing effects due to abnormally high or low serum concentrations which some individual animals have in their normal state.

The statistical methods and formula used were those described by Bancroft (1957).

"t" values were calculated from the formula

$$t = \frac{\bar{x} - \bar{y}}{s} \sqrt{\frac{n_1 n_2}{n_1 + n_2}}$$

where s, the standard deviation for small samples is derived from

$$s = \sqrt{\frac{\left(\sum x^2 - \frac{(\sum x)^2}{n_1}\right) + \left(\sum y^2 - \frac{(\sum y)^2}{n_2}\right)}{n_1 + n_2 - 2}}$$

"p" values, i.e. the probability that the difference in the change in serum cholesterol concentration of any two groups of rats due to two different diets are identical were computed and are recorded with each table of results. Variations from mean values are recorded as standard error of the mean:

$$S.E. = \frac{s}{\sqrt{n}}$$

Regression lines and coefficients of correlation were likewise computed as directed by Bancroft.

SECTION 9THE EFFECT OF VARIOUS FATS AND OILS ON THE
SERUM CHOLESTEROL CONCENTRATION OF RATS

Although the results of the pilot experiment reported in Section 4 are encouraging, the demonstration of the rat as a suitable experimental animal requires confirmation. It will be remembered that the object of this study is to contribute to an understanding of the effects of dietary fats and oils on the cholesterol level of humans. The interest in rats extends only as far as it can be shown that these animals can be induced to imitate these effects. How well can they do this? In the following three experiments, rats are given a more rigorous test under what are hoped to be improved conditions, and using the improved experimental techniques described above.

A. The effect of replacing coconut oil by sunflower seed oil
(Experiment 2)

Experimental

From human studies it is known that of all the common oils, dietary coconut oil produces most probably the highest serum cholesterol levels. Assuming that rats behave similarly, all animals were placed on the experimental diet containing coconut oil (see Section 8) for 17 days in order to produce uniformly high serum cholesterol levels. (It is seen that in the present experiment the "standard experimental design" had not yet become fully established but the deviations

therefrom are fully described).

After this preliminary treatment the colony was split up into four dietary groups, one of which was left unchanged as control. The remaining three groups received a quarter, half, and total replacement of the coconut oil by sunflower seed oil (S.S.O.). The coconut oil was alkali refined, bleached and steam-deodorised and had an iodine value of 9.2, whereas the S.S.O. was a crude product of iodine value 132 and linoleic acid content of 65 per cent (alkali isomerisation method of Herb and Riemenschneider, 1953), which had been expressed from the seeds without further treatment.

After 36 days the S.S.O. replacements were discontinued and the animals kept for a further 27 days on the experimental coconut oil diet. Serum cholesterols were determined after 9, 36 and 63 days.

Results

The results are tabulated in Table 5 and illustrated in Fig. 10. It is seen that only 9 days after the introduction of the S.S.O. in the diets there was a progressive drop in the serum cholesterol level with increasing proportions of S.S.O. replacements. Although in this instance the effect of one quarter substitution of coconut fat by S.S.O. could not be shown to be significant, the half and total replacements produced depressions of approximately 20 per cent and 33 per cent respectively at 3 per cent and less than 1 per cent. After a further 27 days on the experimental diets these statistical figures deteriorated. It is of interest, however, that the

serum values had not yet converged after the subsequent 27 day period in which the S.S.O. replacements had been discontinued.

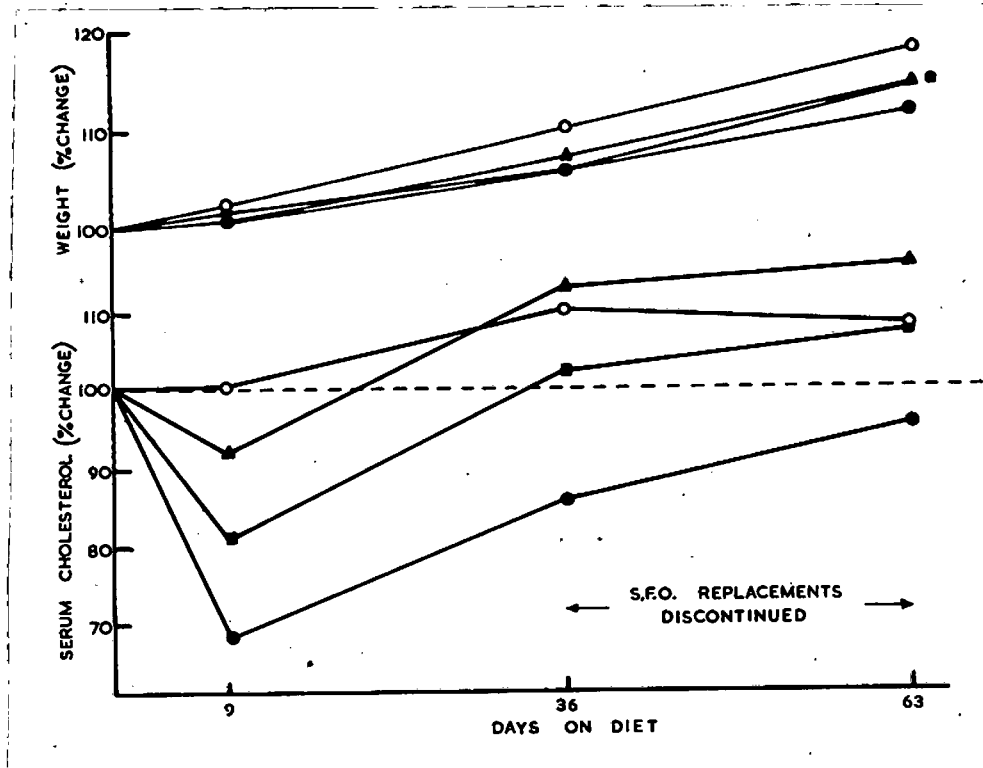


Fig. 10. The hypocholesterolaemic effect of various concentrations of S.S.O. in coconut oil (Experiment 2).

- 20% coconut fat
- ▲— 15% coconut fat, 5% S.S.O.
- 10% coconut fat, 10% S.S.O.
- 20% S.S.O.

Comment: When this experiment had been completed, it was realised that not much is gained by pre-feeding the colony with coconut oil. No doubt the serum cholesterol "lowering" action of S.S.O. could have been as well detected without special pre-feeding if considered relative to a coconut

TABLE 5 - (Experiment 2)

MEAN PERCENTAGE CHANGES IN SERUM CHOLESTEROL CONCENTRATION OF GROUPS OF SIX RATS AFTER 20% OF COCONUT FAT IN THE EXPERIMENTAL DIET IS SUCCESSIVELY REPLACED BY SUNFLOWER SEED OIL (S.S.O) THE REPLACEMENTS BEING WITHDRAWN AFTER 36 DAYS
(STARTING VALUE = 100%)

GROUP		9	36	63
		Days	Days	Days
I	20% Coconut fat (diet unchanged)	102.0 ± 6.4* (102.5)**	110.2 ± 5.3 (110.0)	108.2 ± 3.4 (117.8)
II	15% Coconut fat 5% S.S.O.	91.8 ± 5.8 (100.9)	113.1 ± 1.2 (106.7)	116.0 ± 3.3 (114.0)
III	10% Coconut fat 10% S.S.O.	81.0 ± 4.4 (101.8)	102.3 ± 5.5 (105.9)	107.9 ± 7.4 (114.0)
IV	20% S.S.O.	68.6 ± 4.0 (100.7)	86.0 ± 4.2 (105.9)	95.4 ± 4.2 (111.5)

Significance of differences (p - values)

I - II		0.30	0.90	0.50
I - III		0.03	0.40	0.90
I - IV		0.01	0.01	0.05

* = Standard error of mean

** = Mean percentage changes in weight of rats

control diet. The previous dietary history is of little concern. It was also learned from this experiment that nothing is probably gained by prolonging these tests beyond two or three weeks. With these points in mind the final standard experimental design was adopted.

B. The effect of butter fat, beef tallow, groundnut oil and fish oil (Experiment 3)

Experimental

Of the many oils tested on humans there is fair agreement that butter, and to a lesser extent beef tallow, raise the serum cholesterol, whereas highly unsaturated fish oils have the opposite effect. There is some controversy concerning "semi-unsaturated" oils such as groundnut oil. In the present experiment, the following four representatives of these three classes of oils were tested on rats in a standard experiment of three weeks' duration (see Section 8).

<u>Group</u>		<u>Iodine value</u>
I	Butter fat	31.9
II	S.A. Pilchard oil (sardina ? oscellata capensis)	182.1
III	Beef tallow ^{lanys}	39.6
IV	Arachis oil	95.6

After the experiment proper, the butter and fish groups were maintained for a further 28 weeks on their experimental diets to study long term effects

Results (see Table 6 and Figs. 11 and 12).

The results show that after only seven days on the experimental diets each group attained a characteristic serum cholesterol level which was maintained for three weeks. The butter and fish oil groups maintained their levels essentially unchanged for the duration of the experiment, i.e. 31 weeks. This fact will be found useful when discussing the relation between dietary fats and cholesterol metabolism in Section 26.

From the statistical figures supplied with Table 6 it is seen that the differences between these group averages stand on firm foundations in spite of the small number of rats used.

The small differences in the growth rates show no correlation with the serum cholesterol levels up to 9 weeks. This did not apply after this time, however. The fish group ceased to grow after some months as is illustrated in Fig. 12 and did in fact begin to lose weight after a further period of time. The question of whether this is due to an essential fatty acid deficiency or some other cause will not be pursued since this would deviate from the straight and narrow path adopted in this work.

Figs. 11 & 12/

TABLE 6

MEAN PERCENTAGE CHANGES IN SERUM CHOLESTEROL CONCENTRATION OF GROUPS OF SIX RATS AFTER CHANGING TO EXPERIMENTAL DIETS CONTAINING 20% OF DIFFERENT FATS (STARTING VALUES = 100%)

GROUP	DIET	WEEKS ON DIET					
		Iodine value	1	2	3	9	31
I	Butter	31.9	127.5 $\pm$ 8.1* (100.9) **	119.7 $\pm$ 8.3 (109.9)	116.3 $\pm$ 5.6 (112.8)	117.6 $\pm$ 7.0 (137.6)	113.9 $\pm$ 9.5 (157.9)
II	Fish Oil (S.Afr. Pilchard)	182.1	73.4 $\pm$ 4.5 (98.6)	66.0 $\pm$ 4.5 (107.4)	69.2 $\pm$ 5.1 (108.8)	74.8 $\pm$ 5.0 (133.0)	82.1 $\pm$ 4.4 (135.3)
III	Beef	39.6	115.4 $\pm$ 3.3 (96.2)	109.5 $\pm$ 2.8 (103.2)	113.2 $\pm$ 4.9 (108.3)	-	-
IV	Groundnut oil	95.6	101.1 $\pm$ 5.8 (97.7)	101.1 $\pm$ 4.4 (104.9)	98.1 $\pm$ 5.2 (109.9)	-	-
Significance of differences (p - values)							
I - II			0.001	0.001	0.001	0.001	0.01
I - III			0.20	0.20	0.60	-	-
I - IV			0.03	0.03	0.03	-	-
II - III			0.001	0.001	0.001	-	-
II - IV			0.01	0.001	0.01	-	-
III - IV			0.06	0.20	0.05	-	-

* = Standard error of mean ** = Mean percentage changes in weight

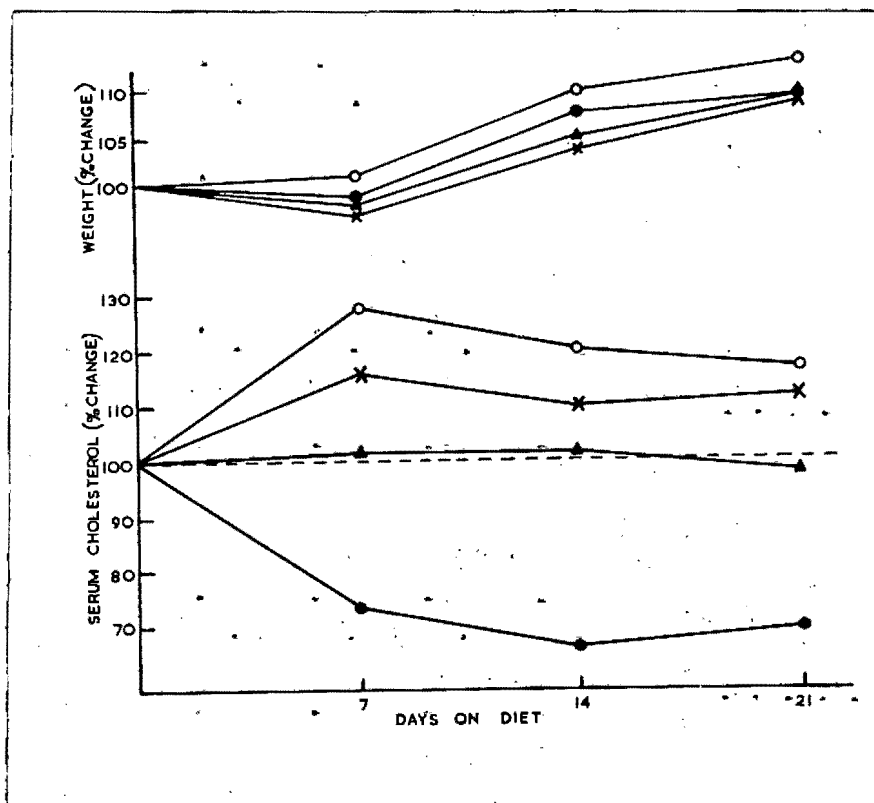


Fig. 11 The effect on the serum cholesterol level of various fats (Experiment 3)

- Butter fat
- ×— Beef tallow
- ▲— Groundnut oil
- Fish oil (S.A. Pilchard)

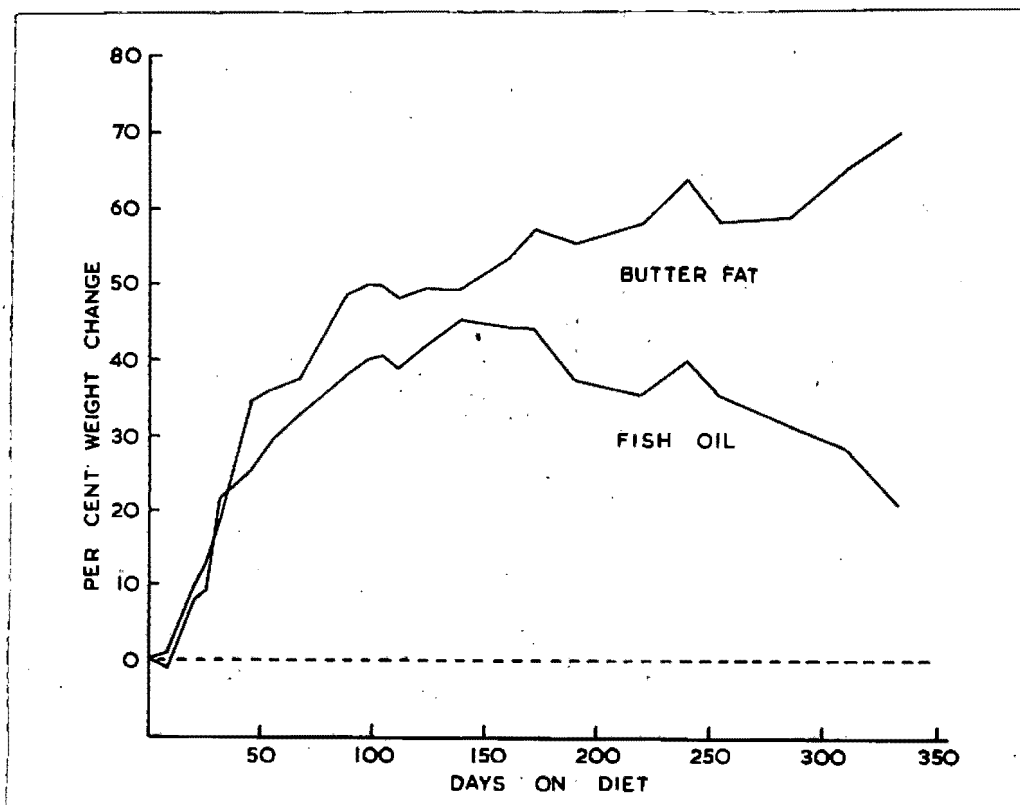


Fig. 12 Growth curve of rats on diets containing butter fat and fish oil, expressed as per cent increase from weight at two months of age (part of Experiment 3).

**C. The effect of small quantities of dietary cholesterol
(Experiment 4)**

There is a good deal of evidence that in man the effect of different oils on the serum cholesterol level is largely independent of the small amounts of cholesterol associated with animal fats such as butter and beef tallow. This aspect was therefore studied in the rat.

Experimental

The standard experimental design was followed.

The diets were:-

- | | |
|---------|--|
| Group I | Experimental coconut diet (20% coconut fat) |
| II | Experimental S.S.O. diet (20% S.S.O.). |
| III | As for II plus 0.2% cholesterol (1% of oil) added in crystalline form to diet. |
| IV | As for II plus 0.2% cholesterol (1% of oil) in dissolved form in S.S.O. |

The animals were bled at the beginning of the experiment and after one, two and three weeks on the experimental diets.

Results (see Table 7 and Fig. 13)

Neither form of cholesterol administration with S.S.O. could be shown to produce effects which differed significantly from that of pure S.S.O. in the diet. The experimental coconut diet, on the other hand, produced serum cholesterol levels which were very significantly higher. It is noteworthy that higher serum cholesterol levels were found in this experiment than in those of Experiment 2. This is attributed to the different basic levels from which these changes were calculated, i.e. experimental coconut diet in Experiment 2 and laboratory chow in the present one.

Fig. 13/

TABLE 7

MEAN PERCENTAGE CHANGES IN SERUM CHOLESTEROL CONCENTRATION OF GROUPS OF SIX RATS AFTER CHANGING
TO EXPERIMENTAL DIETS CONTAINING 20% OF DIFFERENT FATS
(STARTING VALUES = 100%)

GROUP	DIET	WEEKS ON DIET		
		1	2	3
I	20% Coconut fat	131.8 $\pm$ 3.4* (100.4)**	121.6 $\pm$ 3.5 (105.3)	126.0 $\pm$ 5.6 (110.7)
II	20% Sunflower seed oil (S.S.O.)	110.8 $\pm$ 5.2 (101.8)	97.0 $\pm$ 3.5 (106.3)	102.3 $\pm$ 4.5 (111.3)
III	20% S.S.O., 0.2% dry cholesterol (1% of oil)	120.1 $\pm$ 4.5 (99.5)	101.5 $\pm$ 3.6 (104.3)	105.3 $\pm$ 4.8 (111.2)
IV	20% S.S.O. containing 1% dissolved cholesterol	114.1 $\pm$ 6.4 (102.2)	93.1 $\pm$ 4.5 (107.6)	97.3 $\pm$ 5.3 (113.0)
Significance of differences (p - values)				
I-II		0.01	0.001	0.01
I-III		0.06	0.01	0.02
I-IV		0.03	0.001	0.01
II-III		0.20	0.40	0.70
II-IV		0.70	0.60	0.50
III-IV		0.50	0.20	0.30

* = Standard error of mean

** = Percentage change in weight

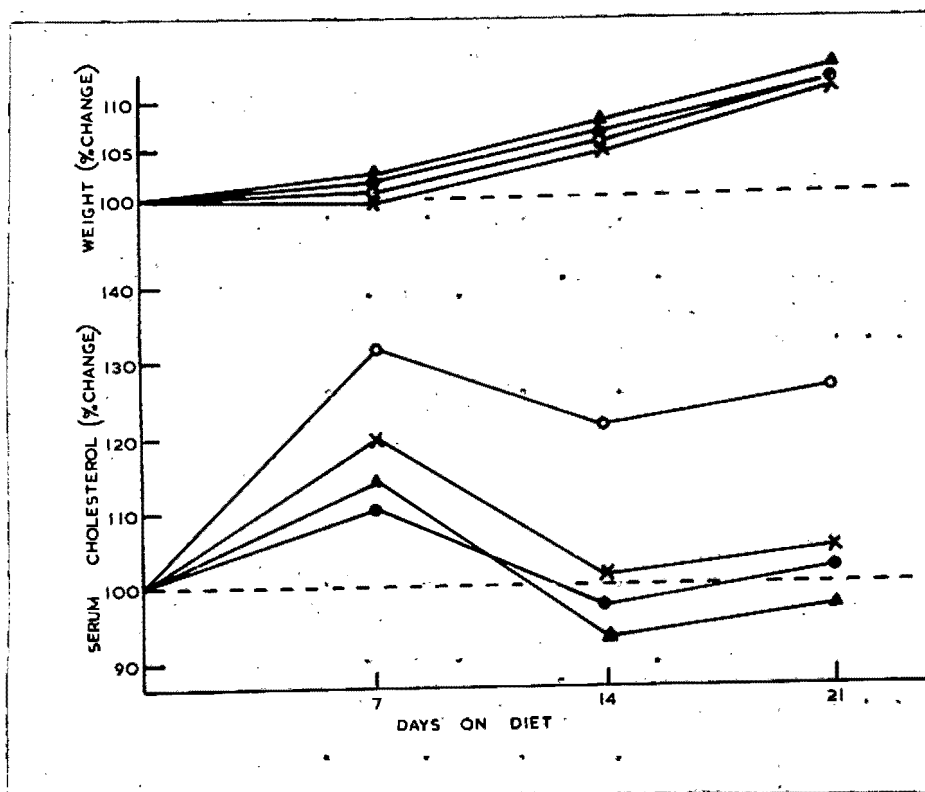


Fig. 13 The effect of small quantities of cholesterol (Experiment 4)

- 20% coconut fat.
- 20% S.S.O.
- x— 20% S.S.O. plus 0.2% chol. (crystalline)
- ▲— 20% S.S.O. plus 0.2% chol. (dissolved in S.S.O.).

D. Discussion

In the present pilot study, using a simple and inexpensive diet, devoid of artificial additives, no difficulty was experienced in demonstrating significant differences between the effects of some saturated and unsaturated fats on the serum cholesterol level. In one case (Experiment 3) these differences

were maintained essentially unchanged for 31 weeks. The serum cholesterol concentrations produced in rats with six dietary oils showed similarities with those produced in humans with the same oils. Thus, in Experiment 3, butter produced the highest levels, followed by beef tallow and groundnut oil and ending with fish oil at the bottom of the scale. These oils were placed in the same order by Ahrens et al. (1957) who fed the same oils to humans. The high and low levels produced respectively by coconut oil and sunflower seed oil are likewise well established from human experimentation (e.g. Bronte-Stewart et al., 1956).

These findings are not in agreement with similar studies on rats, discussed in Section 3 above. A report by Funch, Nielson and Dam (1960), appearing at the time of writing is likewise at variance with the present results. A rise in serum cholesterol is described after changing from margarines and butter to maize oil in the diet of rats.

The choice of suitable compounds for the non-fatty part of the experimental diet may be responsible for obtaining the present effects. That this has a bearing on the subject becomes evident from the work of Portman, Lawry and Bruno (1956), who find that dietary corn starch results in lower cholesterol levels than glucose in mice. Similarly, different proteins (Olson, Jablonski and Taylor, 1957) and vitamins (Hsu and Chow, 1957) are claimed to have an influence.

It may be significant in this respect that the early success of Schettler's work on mice (see Section 3) was

likewise achieved with skim milk as the basic non-fatty dietary component.

It was stressed in Section 3 that the omission from the diet of cholesterol, cholic acid, thiouracil, etc. is of prime importance if the effect of triglyceride fats, and fats alone, are to be studied. The present series of experiments has shown that these additives are not required to imitate human experiments with rats.

One of the problems encountered in some of the earlier experiments (Experiments 2, 5 and 6) was the tendency of the serum cholesterol to escape from the initial influence of the different dietary fats. A similar phenomenon has been described recently by Seskind and his co-workers (1959) who observed that the "...elevation (of serum cholesterol) appears transient for the less saturated oils but is more sustained for highly saturated fats." The reason for this is not clear. A possible explanation is that temporary lipaemia may result from excessive bleeding. The elimination of this trouble in later experiments by improved cholesterol determination methods and consequently milder bleeding techniques tends to support this explanation. In a given experiment, however, this difficulty does not vitiate the discrimination between oils and their fractions, which is the objective. To counter this and other difficulties it was decided to carry at least one, but preferably two control groups (experimental coconut and S.S.O. diets) through each experiment. Since, without exception, coconut oil produced significantly higher serum cholesterol levels than S.S.O., the effect of

the unknown oil fractions could then be assessed in relation to these controls.

E. Conclusions from Part II

It was felt at this stage that the experimental routine machinery had been brought to a state of sufficient refinement for its practical application to the main problem, without fear of breakdown. Although the lipid metabolism of rats may differ in some important respects from that of humans, conditions have been created under which the serum cholesterol regulating properties of different oils may be similar for both species. If such is the case, any clear understanding of the lipid metabolism of the rat is not immediately essential for the rat to be used as a test animal for the study of these oils by fractionation or other techniques. The sensitivity of the present rat assay method can best be judged from its application. In Experiment 12 (Section 14), for example, significantly different effects ($P = 0.001$) relative to a standard fat have been demonstrated with as little as 35 gms. of oil after seven days' feeding. Furthermore, it can be predicted with confidence from the results of Experiment 4 that under the conditions of these experiments any manipulation of the serum cholesterol of the rat by dietary fats is unlikely to be materially affected by such small quantities of cholesterol as are present in most common animal fats.

P A R T III

THE HYPOCHOLESTEROLAEMIC EFFECT

OF SUNFLOWER SEED OIL

SECTION 10INTRODUCTION

Careful thought has been given to the selection of a particular fat or oil for closer examination. Preference was given to a typical cholesterol-lowering oil since this appeared to hold out the prospect of isolating from such an oil a substance which might by itself be capable of depressing the serum cholesterol level. If sufficiently potent in this respect, the practical value of this hypothetical substance X might be considerable.

Of the existing number of oils of known hypocholesterolaemic properties, sunflower seed oil (S.S.O.) was selected for detailed study for the following reasons:

1. The effect of sunflower seed oil on humans is well established (e.g. Bronte-Stewart et al., 1956, Williams and Thomas, 1957, Gordon and Brock, 1958).
2. The fatty acid composition of sunflower seed oil is very simple, consisting of four fatty acids only. Should a fractionation of the fatty acids become necessary this fact is important.
3. The various intermediary products and by-products from the extraction and expression of the seeds to the refined oil were obtainable from a local refinery.
4. The author had some experience in the technology of this oil.

The chemistry of sunflower seed oil

The oil expressed from the seeds of the sunflower plant (*Helianthus annuus*) contains four fatty acids only, namely palmitic, stearic, oleic and linoleic acids. The relative proportions of these acids in the oil varies widely according to the climate in which the parent plants are grown. Tropical climates favour the production of more saturated acids whereas the reverse holds for more temperate regions. Variations of linoleic acid content from the extremes of 30 to 75 per cent are encountered. From the author's experience, South African grown plants yield oils with a linoleic acid content ranging from 57 to 66 per cent as determined by alkali-isomerisation techniques (Am. Oil Chemists' Soc., 1953). With the modern methods of gas-liquid partition chromatography used in Section 13, considerably higher values were obtained. This is in agreement with the work of Craig and Murty (1959). These investigators likewise found that the older analysis of S.S.O. had to be revised, and report the following figures for some oils ranging in iodine value between 138.8 and 141.6:

<u>Fatty acid</u>	<u>% Composition</u>
Palmitic	6.6 - 7.2
Stearic	3.7 - 4.1
Oleic	15.5 - 16.2
Linoleic	73.9 - 78.3

Like most vegetable seed oils, crude S.S.O. contains small

quantities of phosphatides. These are, however, normally removed in the refining process. Less is known of the composition of the unsaponifiable matter, but sitosterols, carotenes, squalene and tocopherols have been reported. These will be considered in more detail in Section 14.

The chemical constants and full analysis of a sunflower seed oil used in these tests will be found in Table IIb of Section 13.

SECTION 11THE EFFECT OF WHOLE SUNFLOWER SEEDS
(Experiment 5)

Sunflower seed oil (S.S.O.) is obtained industrially by the expression or solvent extraction of kernels which in most cases are only very incompletely separated from husks. Crude S.S.O. in fact, develops a cloudiness on standing which can be traced to a wax, ceryl cerotate, originating from the husks (Barentner, 1923). Any claim to a systematic search for hypocholesterolaemic substances should, therefore, include not only the husk, but also the non-fatty protein portion of the seeds. In the present experiment, the relative serum cholesterol depressing properties of husks, expressed oil and the protein fraction of the whole seeds were tested.

Diets

Due to the nature of the substances to be tested, the diets for this experiment deviated somewhat from the standard pattern, and were as follows:

- Group I : Experimental coconut diet (see Section 8).
- Group II : 10 per cent of coconut fat of experimental coconut diet replaced by crude S.S.O.
- Group III: 10 per cent of coconut fat of experimental coconut diet replaced by crude S.S.O. given in the form of kernels.
- Group IV : As for Group I plus 5 per cent of finely ground sunflower seed husks.

The Group III rations necessitated an adjustment of the skim milk component to keep the diets isonitrogenous. The resulting

deficiency of carbohydrate was made up with potato starch.

Other departures from the standard experimental design were as follows:

1. All animals were pre-fed with experimental coconut diet for 15 days before the beginning of the experiment.
2. To ensure equal consumption of these heterogeneous diets, rations were restricted to 20 gm. per day, per rat.
3. Husks were excluded from the diets of Groups I, II, and III.

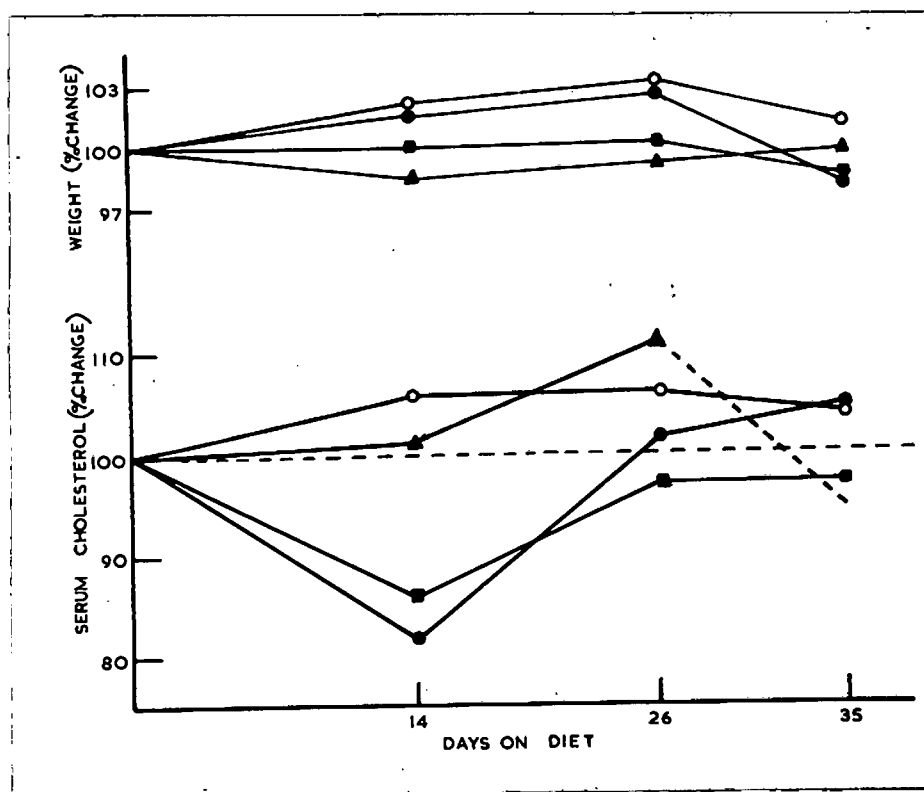


Fig. 14 The effect of various fractions from whole sunflower seeds (Experiment 5).

- Experimental coconut diet (20% coco.)
- 10% coco., 10% S.S.O. (pure oil)
- 10% coco., 10% S.S.O. (as kernels)
- ▲— 20% coco., 5% husks.

TABLE 8 - (Experiment 5)

MEAN PERCENTAGE CHANGES IN SERUM CHOLESTEROL CONCENTRATION OF GROUPS OF SIX RATS AFTER THEIR
EXPERIMENTAL DIET* CONTAINING 20% COCONUT FAT IS MODIFIED AS SHOWN
(STARTING VALUE = 100%)

GROUP	DIET	DAYS ON DIET		
		14	26	35
I	20% Coconut fat (diet unchanged)	105.8 $\pm$ 3.7 ^{**} (101.5) ^{**}	105.8 $\pm$ 3.7 (102.3)	103.3 $\pm$ 3.8 (100.8)
II	10% Coconut fat 10% S.S.O. (pure oil)	81.4 $\pm$ 4.2 (102.2)	101.7 $\pm$ 4.9 (102.9)	104.4 $\pm$ 3.1 (104.4)
III	10% Coconut fat 10% S.S.O. (given as kernels)	85.8 $\pm$ 2.7 (100.0)	96.6 $\pm$ 5.1 (100.0)	96.6 $\pm$ 2.7 (98.1)
IV	20% Coconut fat 5% Sunflower seed husks	100.9 $\pm$ 3.2 (98.4)	111.1 $\pm$ 3.6 ^{***} (99.0)	94.4 $\pm$ 3.6 (99.3)
Significance of differences (p - values)				
I-II		0.01	0.50	0.50
I-III		0.01	0.20	0.20
I-IV		0.50	0.30	0.10
II-III		0.50	0.50	0.10

* = Husks omitted from Basic Diets of Groups I - III

$\pm$ = Standard error of mean ** = Percentage changes in weight of rats *** = Changed to Diet II from 26th day.

Results (See Table 8 and Fig. 14)

After 14 days on the test diets there was a mean drop of 16 per cent in the serum cholesterol due to the unsaturated oil diets of Groups II and III which were not significantly different from each other ($P = 0.50$) but significantly different ($P = 0.01$) from the control Group I and the husk Group IV, the latter two again being similar.

As after 26 days differences tended to disappear, it was decided to switch the husk group to 10 per cent S.S.O. (as for Group II) for reassurance as to the sensitivity of the animals at that stage. The consequent drop of 15 per cent confirms the initial observations. The dotted line in the graph represents the mean value of this secondary drop.

It is concluded from this experiment that no appreciable cholesterol depressing substances are present in the whole sunflower seed other than those due to the oil itself. The transient nature of the response of the rat exhibited under the conditions of this experiment does not, it is believed, detract from the validity of this conclusion.

SECTION 12THE CHEMICAL FRACTIONATION OF SUNFLOWER SEED OIL

The next step in the study of the serum cholesterol lowering property of S.S.O. is to separate the latter into its two basic parts, i.e. fatty acids or triglycerides and the unsaponifiable matter (U.M.) and examine each fraction separately. Although the unsaponifiable matter constitutes only approximately 1 per cent of the whole oil, an "active" substance, if such exists, might conceivably reside in this fraction.

A. Preparation of fractions

The starting material was an alkali refined, bleached, steam-deodorised and cold filtered product with an iodine value of 133 and linoleic acid content of 62 per cent (alkali - isomerisation). To ensure maximum protection of labile unsaturated substances from isomerisation and oxidation the following mild technique was developed to separate the unsaponifiable matter:

930 gm. of oil to which 2 gm. of hydroquinone had been added as antioxidant, was shaken vigorously with one volume of 5 N. KOH and two volumes of freshly distilled (over zinc powder and KOH) ethanol.

Under these conditions, saponification was complete in a few minutes at room temperature. The conventional and possibly harmful refluxing of the saponification mixture for hours at



Fig. 15 Apparatus for the extraction of unsaponifiable matter.

elevated temperatures was thus eliminated.

The resulting soap solution was diluted immediately to 15 litres with distilled water and extracted with freshly distilled diethyl ether in an apparatus adapted from an extractor described by Hilditch (1956). The diethyl ether was kept free from peroxides by continuous distillation over alkaline pyrogallol (see Fig. 15).

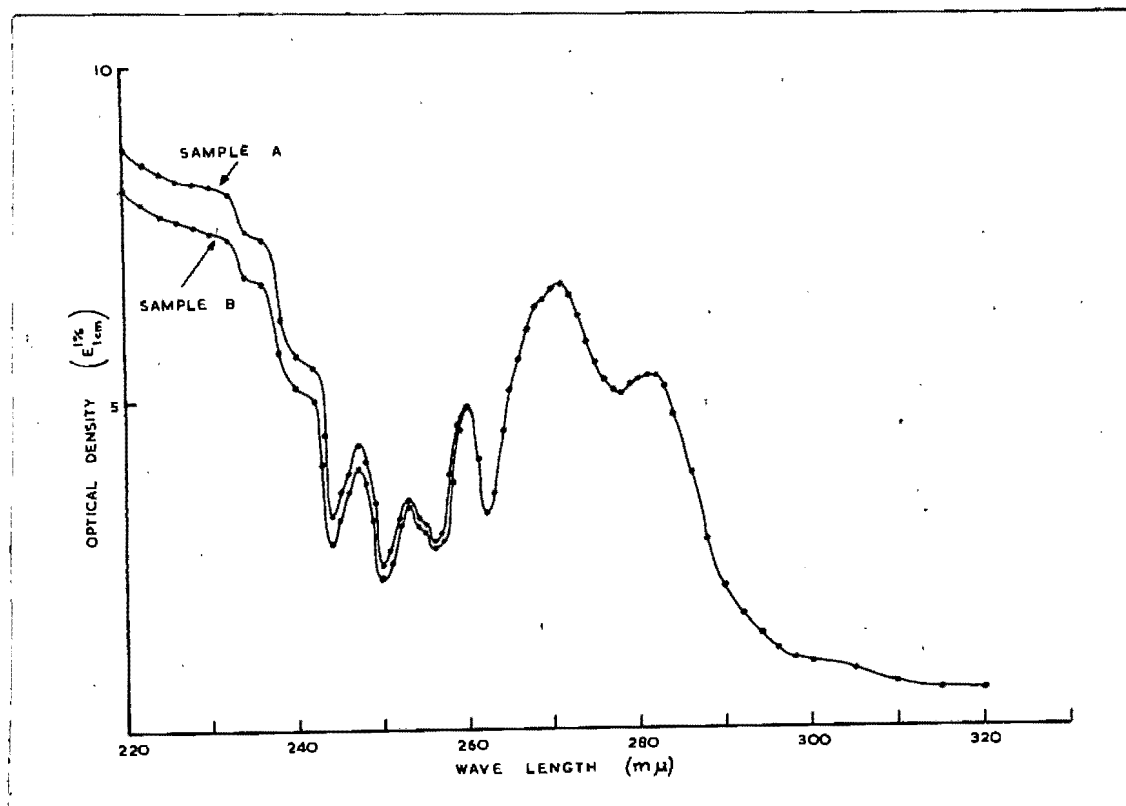


Fig. 16 Ultra-violet absorption spectra of ethyl esters of S.S.O. fatty acids (see text).

After 36 hours of continuous extraction time, 96.5 per cent of the 1.05 per cent of U.M. originally present in the oil was thus removed. The ethereal extract was freed from pyrogallol and hydroquinone by repeated partition with 0.5 N KOH and distilled water. The ether was finally removed

by distillation and vacuum.

The potassium soaps were converted via the free acids to the ethyl esters in 97 per cent yield following the procedure described by Hilditch.

A second batch of ethyl esters were prepared as above but without removal of the U.M. The iodine values of the ethyl esters (122.5 and 123.3) agreed within experimental error with the theoretical value calculated from the iodine and saponification values of the original oil, demonstrating no appreciable deterioration of the acids. That the extraction step caused no structural changes in the oil was further confirmed by the negligible difference found in the absorption spectra in the ultra-violet region of the two samples, i.e. the extracted (sample A) and unextracted (sample B) esters. The two spectra are illustrated in Fig. 16.

B. The effect of saponifiable and unsaponifiable matter (Experiment 6)

Experimental

The two fractions prepared as described above were used to prepare the following experimental diets.

Group I. 20% coconut fat (experimental coconut diet).

- II. 10% coconut fat, 10% S.S.O., ethyl esters, U.M. removed.
- III. 20% coconut fat containing 2% (0.4% of whole diet) of U.M. separated from S.S.O., in dissolved form.
- IV. 10% coconut fat, 10% S.S.O., ethyl esters, U.M. not removed.

It will be noticed that in Group III the quantity of U.S. fed with coconut fat is approximately twice the concentration in which it is present in S.S.O. This was done to accentuate possible effects.

Results (see Table 9 and Fig. 17)

As on previous occasions the experimental coconut control diet produced the highest serum cholesterol, whereas the crude S.S.O. esters (U.S. not removed) produced the lowest. Extremely interesting, however, is the low cholesterol curve produced by Group III, receiving S.S.O. unsaponifiables. This group closely resembled the behaviour of Group IV and was, in fact, statistically indistinguishable from it, but significantly lower than Group I after nine and sixteen days ($P = 0.01$ and 0.04). In view of this result it is not surprising that Group II receiving purified S.S.O. esters, i.e. substantially free from U.M., occupied a position intermediate between the Groups I and IV. As in Experiment 5, under the conditions which obtained here, the responses of the rats to these diets appeared to be of a transient nature.

Fig.17/

TABLE 9 - (Experiment 6)

MEAN PERCENTAGE CHANGES IN SERUM CHOLESTEROL CONCENTRATION OF GROUPS OF SIX RATS AFTER CHANGING
FROM A LABORATORY CHOW DIET TO EXPERIMENTAL DIET CONTAINING THE FAT PREPARATIONS LISTED
(STARTING VALUE = 100%)

GROUP	DIET	DAYS ON DIET		
		8	16	23
I	20% Coconut fat	125.0 $\pm$ 6.0 ^x (97.0) ^{xx}	122.5 $\pm$ 4.6 (101.6)	111.7 $\pm$ 3.3 (99.5)
II	10% Coconut fat 10% S.A.O. esters unsap. removed	105.8 $\pm$ 5.4 (99.5)	104.0 $\pm$ 4.3 (106.5)	103.6 $\pm$ 2.9 (108.7)
III	20% Coconut fat 0.4% unsap. from S.S.O.	96.8 $\pm$ 5.6 (96.8)	107.4 $\pm$ 4.2 (100.8)	114.0 $\pm$ 5.2 (103.5)
IV	10% Coconut fat 10% S.S.O. esters unsap. <u>not</u> removed	90.9 $\pm$ 5.0 (96.5)	101.5 $\pm$ 3.6 (98.5)	104.1 $\pm$ 6.7 (101.0)

Significance of difference (p - values)

I - II	0.05	0.02	All N.S.
I - III	0.01	0.04	
I - IV	0.01	0.01	
II - IV	0.07	0.70	
III - IV	0.50	0.40	

x = Standard error of mean

xx = Mean percentage changes in weight

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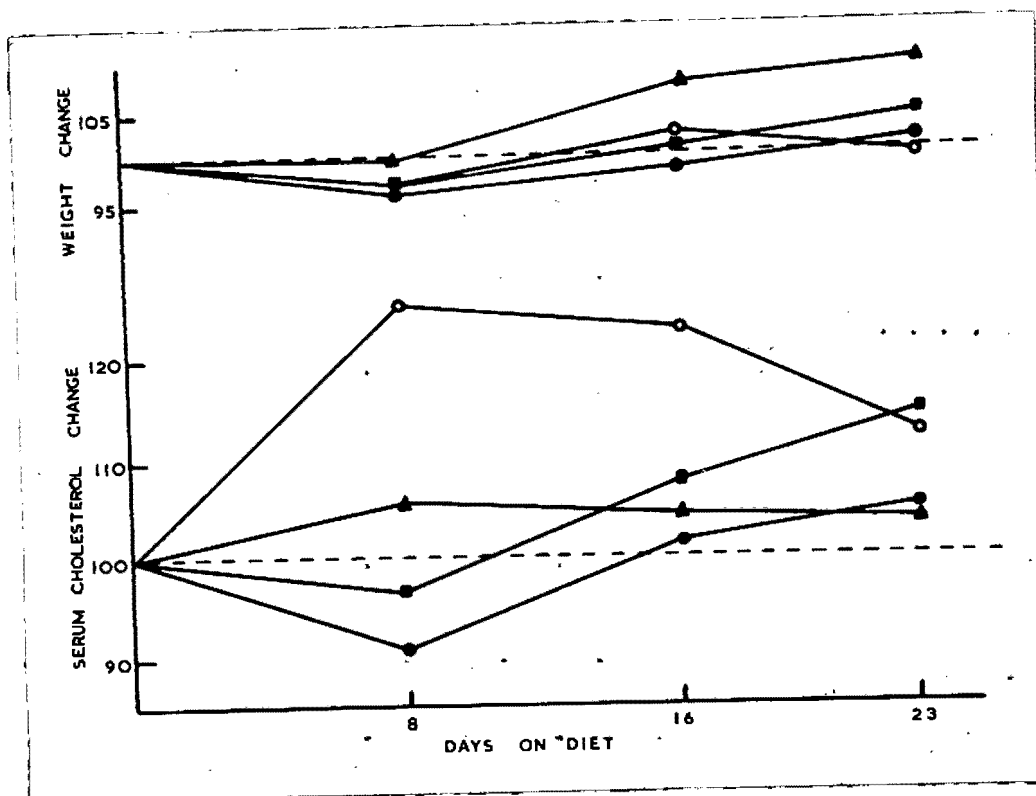


Fig. 17 The effect of the unsaponifiable matter from S.S.O. - (Experiment 6).

- 20% coconut fat
- ▲— 10% coconut fat + 10% S.S.O. esters
U.M. matter removed
- 20% coconut fat plus 0.4% U.M. from
S.S.O.
- 10% coconut fat + 10% S.S.O. esters,
U.M. not removed.

C. The effect of interchanging the unsaponifiable matters of coconut and sunflower seed oils - (Experiment 7)

If the serum cholesterol regulating substances in fats and oils reside in the U.M., it should be possible to reverse the effect of coconut and sunflower seed oils by a total interchange of unsaponifiable matters between the two

oils, in the proportion of their natural occurrence. This was attempted here.

Experimental

Using the techniques described above, the following four fractions were prepared and tested on rats:

1. Crude ethyl esters from coconut fatty acids, i.e. without the removal of its own U.M.
2. Purified ethyl esters from coconut fatty acids containing 1.05% U.M. from S.S.O.
3. Crude ethyl esters from S.S.O. fatty acids.
4. Purified ethyl esters from S.S.O. fatty acids containing 0.34% of U.M. from coconut fat.

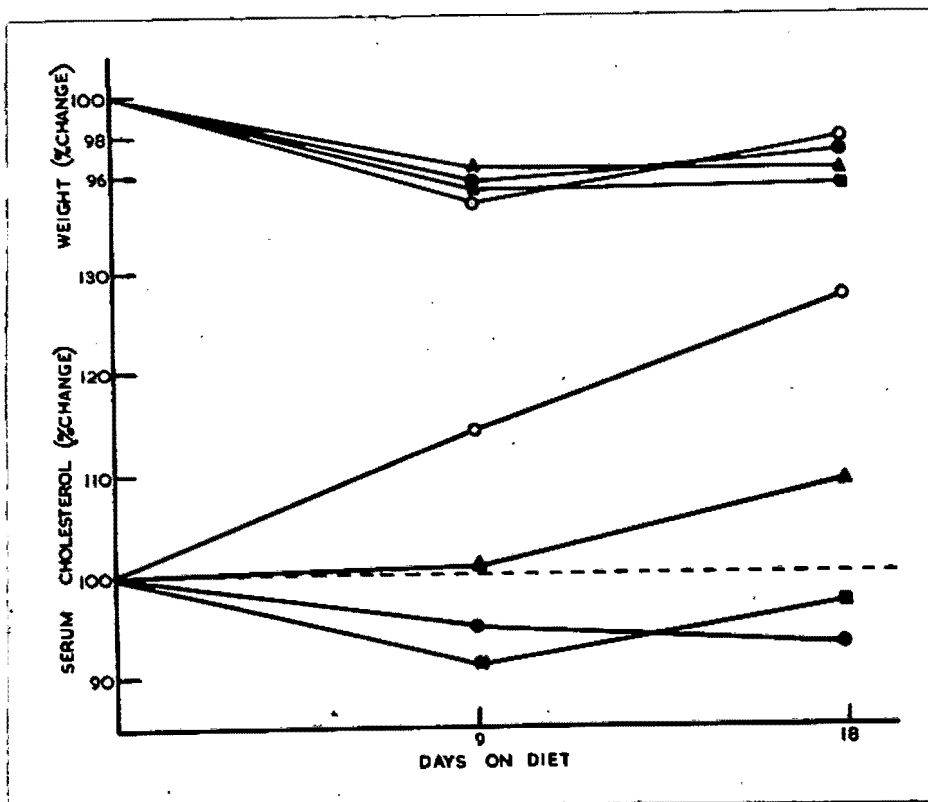


Fig. 18 The effect of interchanging the unsaponifiable matter between coconut and sunflower seed oil ethyl esters - (Experiment 7).

- Coconut esters + coco. U.S.
- ▲— Coconut esters + S.S.O. U.S.
- S.S.O. esters + coco. U.S.
- S.S.O. esters + S.S.O. U.S.

Since insufficient quantities of material were available, these fractions were fed as 15 per cent only in the basic fat-free diet.

Results (see Table 10 and Fig. 18).

The replacement of the U.M. of S.S.O. by that of coconut fat had no demonstrable effect. The reverse procedure, however, i.e. the exchange of U.M. of coconut fat by that of S.S.O. resulted in a complete repression of the serum cholesterol raising property of the crude coconut fraction after 9 days and about 60 per cent repression after 18 days' feeding. These effects were statistically secured respectively at two and three per cent. The results of the present experiment (like those of Experiment 6) suggest that the feeding of fatty acids in the form of ethyl esters instead of triglycerides does not materially change their characteristic effects.

D. Discussion

It can be concluded from the results of Experiments 6 and 7 that there is little doubt that the unsaponifiable matter of sunflower seed oil is somehow implicated in the serum regulating property of this oil. Exactly to what extent this is the case it is difficult to say at this stage. It appears that the U.M. is probably not the only responsible factor since it was possible to demonstrate a partial reversal only, of the effects of two oils by the interchange of their unsaponifiable matters. In the later parts of this work (Part V) an explanation will be given for these observations.

TABLE 10 - (Experiment 7)

MEAN PERCENTAGE CHANGES IN SERUM CHOLESTEROL CONCENTRATION OF GROUPS OF SIX RATS AFTER CHANGING FROM A LABORATORY CHOW DIET TO EXPERIMENTAL DIET CONTAINING ETHYL ESTERS OF COCONUT OIL AND SUNFLOWER SEED OIL FATTY ACIDS WITH INTERCHANGED UNSAPONIFIABLE MATTERS
(STARTING VALUES = 100%)

GROUP	DIET	DAYS ON DIET	
		9	18
I	15% Coco. esters + coco. U.S.	114.3 $\pm$ 2.9* (94.7)**	127.0 $\pm$ 5.1 (97.7)
II	15% Coco. esters + S.S.O. U.S.	100.7 $\pm$ 3.8 (96.3)	109.3 $\pm$ 3.8 (96.0)
III	15% S.S.O. esters + S.S.O. U.S.	94.6 $\pm$ 3.0 (95.6)	93.0 $\pm$ 3.1 (96.9)
IV	15% S.S.O. esters + coco. U.S.	91.1 $\pm$ 1.4 (95.3)	97.2 $\pm$ 3.2 (95.3)
Significance of differences (p - values)			
I-II		0.02	0.03
II-III		0.20	0.01
II-IV		0.05	0.04
III-IV		0.30	0.40

* = Standard error of mean
** = Mean percentage changes of weight

Coco. = coconut fat
S.S.O. = sunflower seed oil
U.S. = unsaponifiable matter

Jones, Reiss and Huffman (1956) were among the first to draw attention to factors other than the fatty acid composition of triglycerides. They fed corn oil and an equivalent amount of corn oil in the form of corn germ to cholesterol fed chicks and found the corn germ to be considerably more active as a serum cholesterol depressant, suggesting the presence of additional factors in the germ other than the oil itself. This principle did not apply to the whole sunflower seed and the oil expressed therefrom and tested with rats as in Experiment 5. Jones and his associates also rearranged the proportions of fatty acids present in cottonseed oil to simulate the composition of corn oil without being able to reproduce the depressing effect of the latter. They conclude "It seems unlikely that this difference.....could be explained by differences in fatty acid composition."

Experimenting on the human scale with a variety of different oils, Armstrong et al. (1957) came to similar conclusions: "...the cholesterol depressant action of corn oil towards humans was not fully accounted for by its degree of unsaturation or content of 'essential' fatty acids." The unsaponifiable fraction of this oil was later investigated by Grande, Anderson and Keys (1958), who fed 0.88 gm. of corn oil U.M. daily to man and recorded a mean decrease of only 3.2 mg. of cholesterol per 100 ml of serum, which was not statistically significant.

Ahrens and associates (1957), after a similar study (see Section 2) find no difference in the activity of

corn oil after reducing the unsaponifiable matter from 1.2 to 0.2 per cent, admitting, however, that their "...readiness to accept the unsaturation hypothesis as firmly proven has been tempered by the realisation that none of the experiments performed here or elsewhere has ruled out the possibility that the factors sought may lie in the non-glyceride portion of the fed fats."

The work of Beveridge and his group with humans has been discussed in Section 2 and elsewhere. Although the present studies were conducted independently from, and approximately parallel in time to those of these investigators, similarities of experimental design, results and interpretation are evident. This applies more particularly to the importance attached in both studies to the role of the non-glyceride fraction.

It may be objected that the chemical separation of saponifiable and unsaponifiable matter constitutes a drastic and perhaps illegitimate interference with the properties of natural oils. This difficulty does not arise if the composition of the unsaponifiable fraction of sunflower seed oil is changed by physical means.

SECTION 13THE PHYSICAL FRACTIONATION OF SUNFLOWER SEED OILA. The liquid propane segregation of sunflower seed oils

The physical fractionation of triglycerides is difficult on account of the small differences in physical properties between triglyceride species found in most common oils. A comparatively crude separation into more saturated and unsaturated fractions can be effected by low-temperature crystallisation in its various modifications. Of the more efficient methods, however, only molecular distillation and segregation in liquid propane have been used on a large scale. The latter process is of comparatively recent origin and is still little known in the oil industry. Only a brief description will be given since it has been fully described among others, by Mettikow (1948), Passino (1949) and Stubbs (1951).

The separation of different triglycerides by liquid propane depends on their selective solubility in this liquified gas, at temperatures near its critical point. Propane, a gas at normal temperatures and pressure, is easily liquified by compression to about 10 atmospheres. At its critical temperature of 95.6°C it has a critical pressure of 43 atmospheres (632 lbs./sq.inch). The solubility of glycerides at a given concentration, i.e. a given propane to oil ratio, is a function of the temperature. Its solubility decreases with increasing temperature and becomes more and more selective with respect

to molecular weight as the temperatures approaches the critical temperature of 95.6°C . In general, molecules of lower molecular weight and unsaturation have a higher solubility, and vice versa.

In practice the efficiency of this simple separation process increases many times in a fractionation tower in which both a temperature and concentration gradient exists. The oil, premixed with propane at a suitable temperature is pumped under high pressure into the tower somewhere near its middle. Equilibration of the constituents between two liquid phases takes place at each point along the length of the tower, the heavier phase dropping to the bottom. The process resembles in some respects ordinary fractional distillation under reflux except that in this case two liquid phases are present and the highest temperature is maintained at the top. The different fractions are bled off at appropriate points along the tower, the high molecular weight components near the bottom, and vice versa. The propane is flashed off and reliquified for further use.

The liquid propane segregation process (Solexol process) was first applied for the concentration of vitamin A from fish liver oil by Marine Oil Refiners of Africa Ltd. at Simon's Town. Vitamin A and its esters, with a comparatively low molecular weight (286) concentrates together with the greater part of the unsaponifiable matter in the light (overhead) fraction. The rest of the unsaponifiable matter tends to concentrate in the heaviest (bottom) fraction.

While the concentration of unsaponifiable matter is

relatively easy, the fractionation of the triglycerides themselves is more difficult on account of the smaller differences of molecular weight obtaining between different triglycerides. Fish oil, however, consisting of esters of fatty acids with widely different chain lengths can be segregated into high and low molecular weight fractions which will incidentally also be respectively of high and low unsaturation.

Attempts to segregate sunflower seed oil into high and low iodine value fractions meet with difficulty. This will be evident from the following table. This summarizes the results obtained in the laboratory by the low-temperature fractional crystallisation in diethyl ether (down to -70°C) of sunflower seed oil according to the method of Hilditch (1950).

TABLE 11a

CHEMICAL CONSTANTS OF GLYCERIDE FRACTIONS OBTAINED BY LOW-TEMPERATURE FRACTIONAL CRYSTALLISATION OF SUNFLOWER SEED OIL *

Glyceride fraction	Weight (gm.)	Linoleic acid %	Iodine value	Sapon. equiv.	Glycerides % mol.
A	13.6	36.2	78.8	291.0	6.9
B	38.1	53.3	111.2	291.0	19.3
C	33.2	61.5	133.4	293.5	16.6
D	20.9	70.5	142.8	291.0	10.6
E	60.0	72.2	144.1	293.0	30.0
F	18.2	68.2	147.7	297.5	9.0
G	16.0	65.8	155.0	310.5	7.6
Total	200.0	64.0	133.8	293.0	100.0

While the iodine values of the fractions range from 78.8 to 155 the mean molecular weights of fatty acids as reflected in the saponification equivalents show comparatively little change.

* These results were obtained by the author in the research laboratories of Marine Oil Refiners of Africa Ltd., and are reproduced from Laboratory Report No. 356, dated October 27, 1953.

The glyceride composition of sunflower seed oil can be calculated from these data. This showed that the distribution of fatty acids in the glyceride molecule follows closely Hilditch's law of even distribution. This is another unfavourable factor against the successful propane segregation of sunflower seed oil. These theoretical considerations were fully borne out in practice. Even under the most selective plant conditions, very little effective fractionation of glycerides was achieved.

With regard to the unsaponifiable matter, however, the situation is different. In the case of vitamin A bearing fish liver oils, for example, fractions consisting of over 70 per cent of unsaponifiable matter can be obtained under optimum conditions. Both from theoretical considerations and past experience, the propane segregation of sunflower seed oil would be expected to yield fractions of uniform glyceride composition, but differing in both quantity and composition of unsaponifiable matter.

Through the generosity of Marine Oil Refiners of Africa Ltd., a liquid propane segregation pilot plant, together with operating staff, was made available for experimentation. Ninety gallons of sunflower seed oil were also donated for this purpose.

The sunflower seed oil was fractionated in liquid propane under comparatively unselective conditions, i.e. comparatively low temperature (165 - 175°F) and low propane to oil ratio (40 : 1). This was done to minimise effective fractionation of triglycerides while still allowing the easier fractionation of unsaponifiable matter to take place. Three

sunflower seed oil fractions were obtained; a light (A), intermediate (B), and heavy (C) fraction comprising respectively 35%, 57% and 8% of the charge.

An impromptu attempt was also made to use the propane plant for the large scale extraction of unsaponifiable matter from aqueous alkaline saponified sunflower seed oil. While the saponification of 45 gallons of sunflower seed oil according to the method described in Section 12 proved comparatively easy, the "unsap." extracted by liquid propane could hardly be recognised as such. It consisted mostly of mineral grease, pipe sealing compound and other unidentified constituents. These were leached out from various parts of the plant by the alcoholic and strongly alkaline soap solution. The experiment, performed with unsuitable equipment, was written off as a fiasco.

B. Composition of propane fractions

The results of the chemical analysis of the three fractions and the original sunflower seed oil are set out in Table 11b. The determination of the usual chemical constants followed conventional lines. Sterols were determined by precipitation of the digitonide from 95% ethanol. The fatty acid composition was determined by gas-liquid chromatography of the methyl esters. The latter were prepared as described in Section 12. The stationary phase was 15% Glutarate-ethylene glycol polyester supported on 80-100 mesh celite. The 200 cm. by 0.5 cm. column was operated at 217°C under an argon gas pressure of 52 lbs./sq.inch. The instrument was a Barber-Colman gas

TABLE 11b
(Experiments 8 & 9)

Chemical Composition of Liquid Propane
Segregated Sunflower Seed Oil Fractions

	Original	Fractions		
		A (Overh.)	B (Interm.)	C (Bottom)
% Free fatty acids (as oleic)	2.36	3.33	0.67	2.39
Iodine value (Wijs)	132.6	131.0	133.8	131.3
Saponification value	189.0	191.3	188.2	185.5
% Composition of fatty acids *				
Palmitic	5.7	5.9	6.6	4.5
Stearic	2.1	3.9	4.3	3.6
Oleic	17.5	18.3	16.9	15.7
Linoleic	74.8	71.9	72.2	76.2
% Unsaponifiable matter	1.08	1.52	0.64	1.19
% Digitonin precipit- able sterols	0.403	0.705	0.210	0.096
% Carotenes (as β -carotene)	0.011	trace	trace	0.23

* determined by gas chromatography

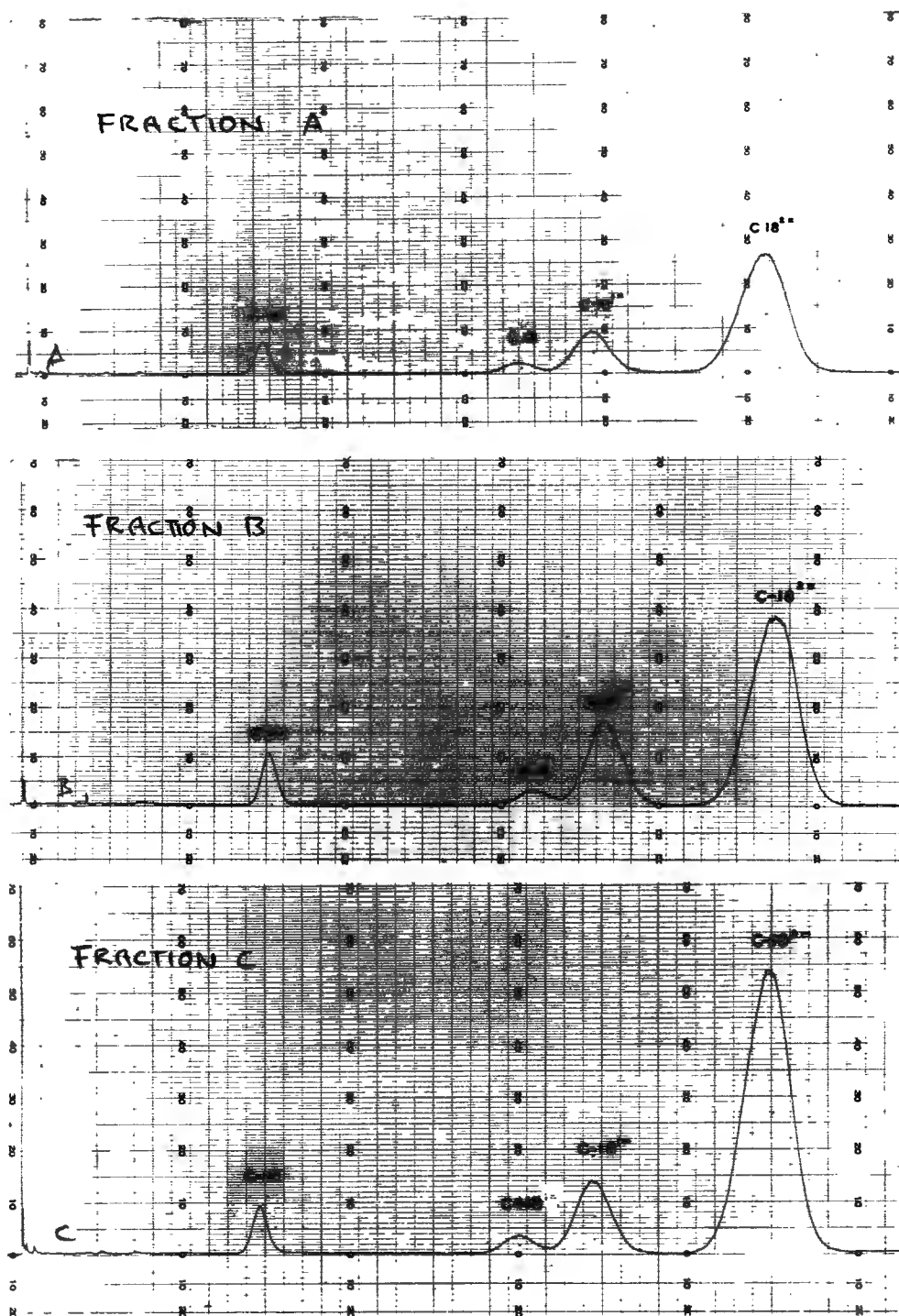


Fig. 19a

Gas-liquid chromatograms of methyl esters from liquid propane segregated sunflower seed oil fractions.

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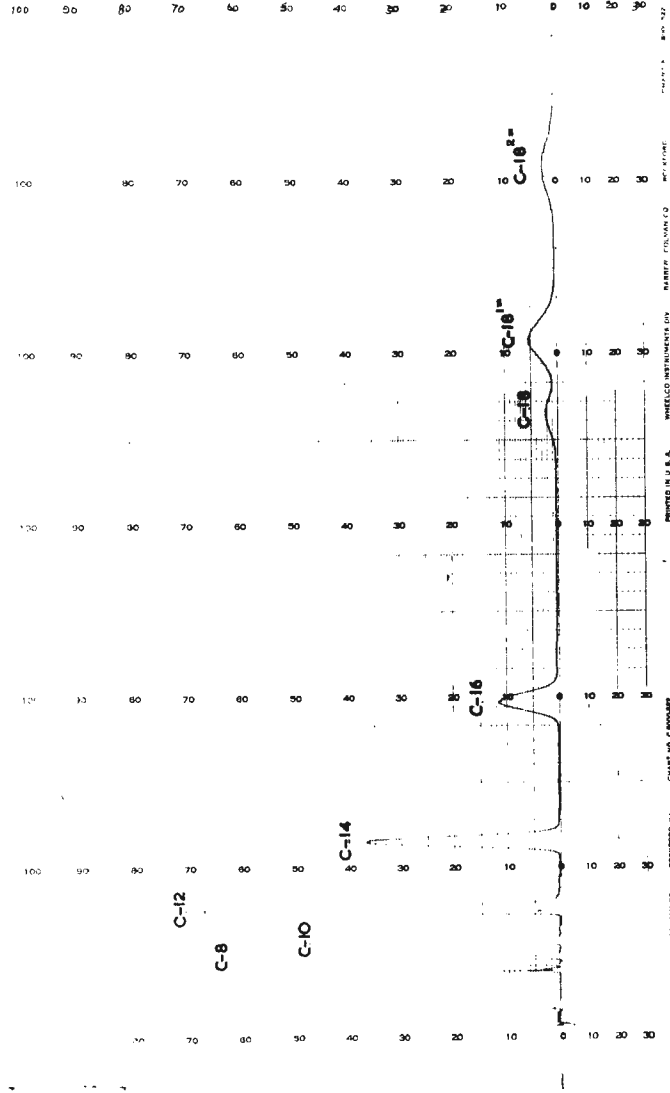
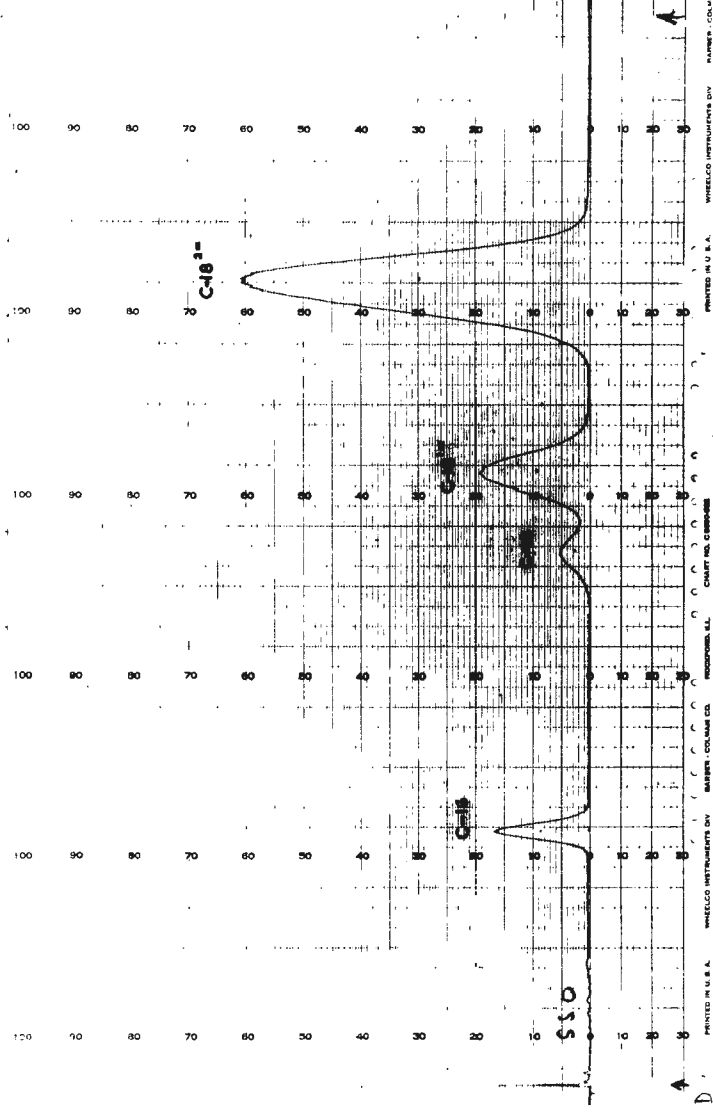


Fig. 19b

Gas-liquid chromatograms of methyl esters from sunflower seed oil (above) and coconut oil (below).

chromatograph. The setting up of the instrument and the supervision of the analysis by Drs. S. Lipsky and J. Lovelock is reflected in the neat chromatograms obtained. These are reproduced in Figs. 19a and 19b.

In Fig. 19c the absorption spectra in the visible region of the unsaponifiable matter of the sunflower seed oil fractions in cyclohexane are reproduced. The presence of a

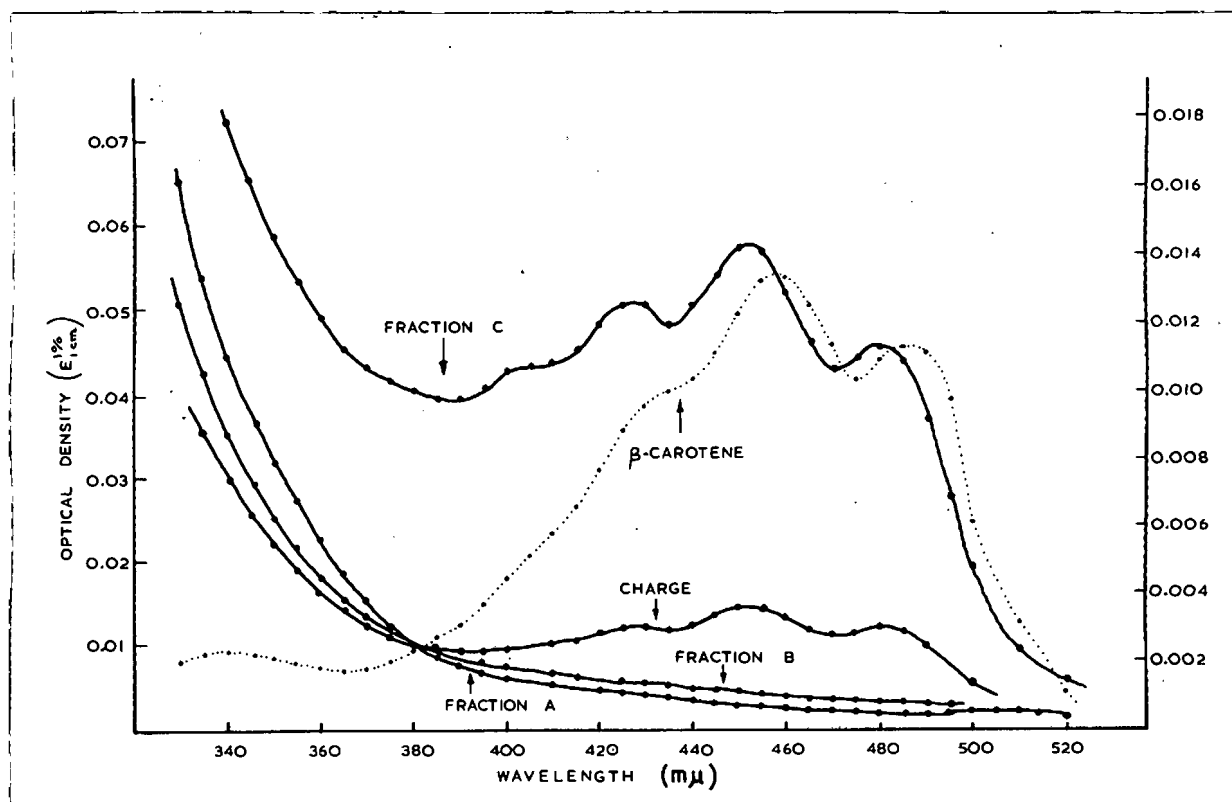


Fig. 19c

Absorption spectra of the unsaponifiable matter from liquid propane segregated sunflower seed oil fractions in cyclohexane. Fraction A, B & charge: refer to right scale
 Fraction C: refer to left scale
 β -carotene: arbitrary scale
 Extinction coefficients are calculated from concentration in whole oil.

carotenoid pigment and its approximately twenty-fold concentration in the heavy fraction (fraction C) is unmistakable. The determination of the absorption curve of a pure sample of β -carotene in the same solvent (dotted line on arbitrary scale) did not correspond to that of the oil fractions. The latter probably contained a mixture of α , β and γ carotenes and other chromogenic carotenoids.

An inspection of the results reveals that earlier hopes were fulfilled. Three unique sunflower seed oil fractions have been created. The fatty acid composition of all fractions shows no significant deviation from that of the original oil. This is also reflected in the similarity of the iodine values. The unsaponifiable matter, however, is seen to differ radically, both qualitatively and quantitatively in all fractions. Thus sterols have concentrated in the light fraction whereas carotenes have collected in the "bottoms". It can be reasonably assumed that a similar fate has overtaken other undetermined constituents of the unsaponifiable matter. Effective fractionation of the unsaponifiable matter has been achieved without materially affecting the composition of the saponifiable matter.

C. The effect of various fractions (Experiments 8 and 9)

The three liquid propane segregated sunflower seed oil fractions were tested in the usual way, i.e. 20% of the oils in the experimental diet were fed together with a similar coconut oil control (Experiment 8).

Encouraged by the results thus obtained the experiment

was repeated under more difficult conditions. In the repeat experiment (Experiment 9) only half of the oil in the diet consisted of the test oils, the remainder being coconut oil.

Results and Discussion

The results of Experiment 8 (Fig. 20, Table 12) show that fraction C produces lower serum cholesterol levels than either fraction A or fraction B. There appears to be no significant difference between the two light fractions (A and B). Their properties are intermediate between that of coconut oil and fraction C.

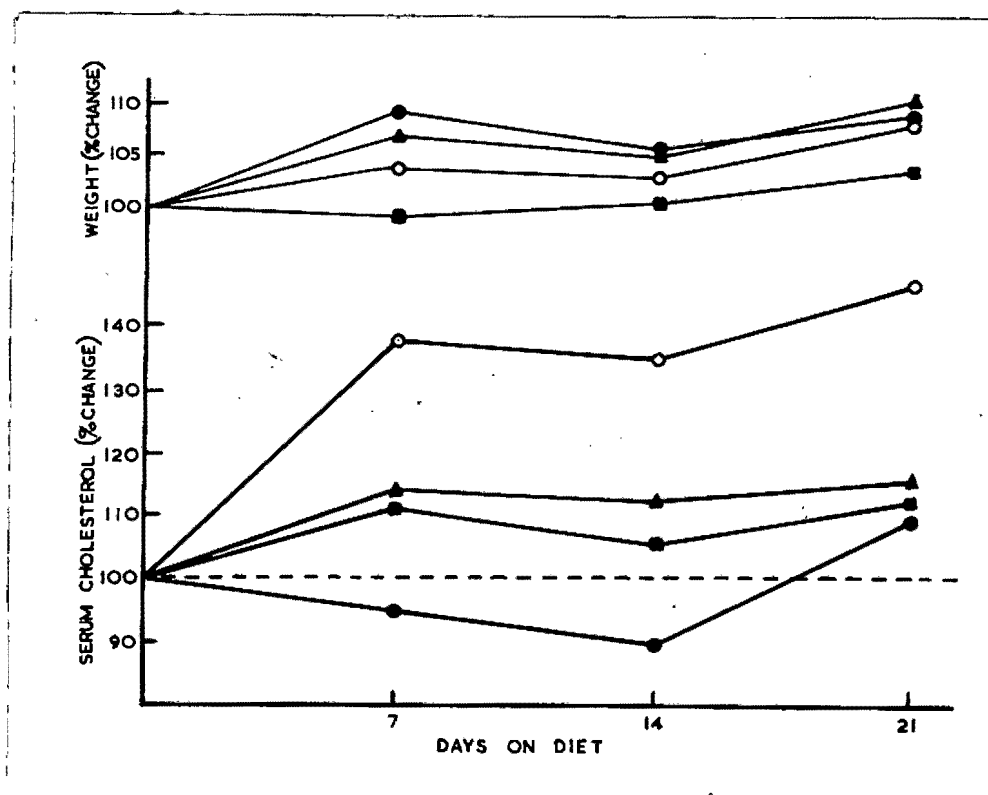


Fig. 20 The effect of liquid propane segregated sunflower seed oil fractions on the serum cholesterol concentration. (Experiment 8)

- coconut oil control
- ▲— fraction A (light)
- fraction B (medium)
- fraction C (heavy)

TABLE 12 - (Experiment 8)

MEAN PERCENTAGE CHANGES IN SERUM CHOLESTEROL CONCENTRATION OF GROUPS OF SIX RATS AFTER TRANSFERRING FROM A LABORATORY CHOW DIET TO EXPERIMENTAL DIET CONTAINING THE SUNFLOWER SEED OIL FRACTIONS LISTED
(STARTING VALUES = 100%)

GROUP	DIET	WEEKS ON DIET		
		1	2	3
I	20% Coconut fat	137.3 $\pm$ 7.5 [±] (103.9) [±]	134.6 $\pm$ 9.7 (102.8)	144.5 $\pm$ 7.1 (107.6)
II	20% Sunflower Seed oil overhead fraction (Fraction A)	113.8 $\pm$ 5.9 (106.8)	112.3 $\pm$ 3.9 (104.9)	115.1 $\pm$ 4.6 (109.9)
III	20% Sunflower Seed oil intermediate fraction (Fraction B)	111.2 $\pm$ 3.8 (109.1)	105.2 $\pm$ 3.3 (105.6)	112.1 $\pm$ 3.4 (108.4)
IV	20% Sunflower Seed oil bottoms fraction (Fraction C)	95.2 $\pm$ 2.7 (99.2)	89.4 $\pm$ 3.1 (100.3)	109.0 $\pm$ 3.5 (103.0)
Significance of differences (p - values)				
I-II		0.03	0.06	0.01
I-III		0.01	0.02	0.01
I-IV		0.001	0.001	0.001
II-III		0.7	0.2	0.6
II-IV		0.02	0.01	0.3
III-IV		0.01	0.02	0.6

± = Standard error of mean

±± = Mean percentage changes in weight of rats

The same trend is apparent in Experiment 9, except that the cholesterol curves in this instance are running closer to the coconut control. This would indeed be expected from the smaller proportion of test oils which were offered to the animals.

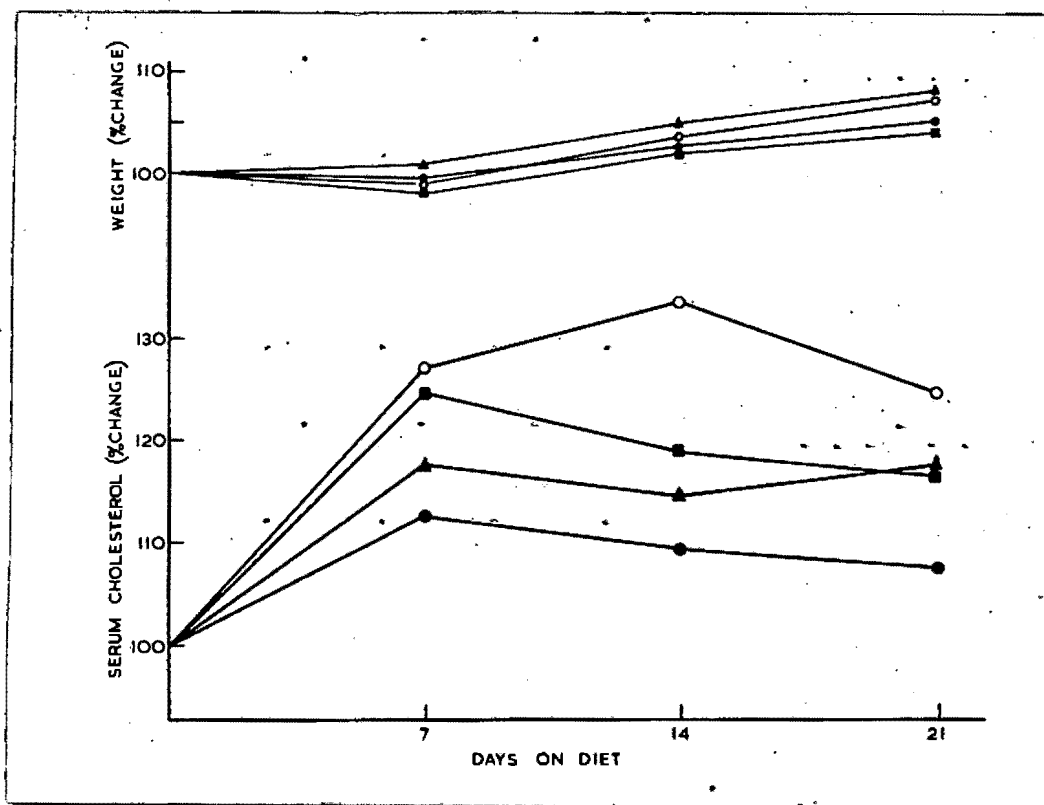


Fig. 21 The effect of liquid propane segregated sunflower seed oil fractions on the serum cholesterol concentration (Experiment 9).

—○— coconut oil control
 —▲— fraction A (light)
 —■— fraction B (medium)
 —●— fraction C (heavy)

} half substitution
 } see text

The difference of the serum cholesterol levels obtained with fraction C as compared with that of the other two, is statistically sound. It is believed that this is the first time that statistically significant differences have been found between the effects of physically fractionated

TABLE 13 - (Experiment 9)

MEAN PERCENTAGE CHANGES IN SERUM CHOLESTEROL CONCENTRATION OF GROUPS OF SIX RATS AFTER TRANSFERRING FROM LABORATORY CHOW DIET TO EXPERIMENTAL DIETS CONTAINING THE SUNFLOWER SEED OIL FRACTIONS LISTED
(STARTING VALUES = 100%)

GROUP	DIET	WEEKS ON DIET		
		1	2	3
I	20% Coconut fat	127.1 $\pm$ 5.2 [#] (98.7) ^{##}	133.7 $\pm$ 5.7 (103.3)	124.6 $\pm$ 4.4 (106.7)
II	10% Coconut fat 10% fraction A (overhead)	118.0 $\pm$ 3.8 (100.8)	115.0 $\pm$ 2.8 (104.6)	117.2 $\pm$ 3.7 (107.6)
III	10% Coconut fat 10% fraction B (interm.)	125.0 $\pm$ 3.3 (97.6)	118.8 $\pm$ 4.1 (101.9)	116.4 $\pm$ 4.1 (103.5)
IV	10% Coconut fat 10% fraction C (bottom)	112.8 $\pm$ 2.7 (99.1)	109.3 $\pm$ 2.7 (102.4)	107.2 $\pm$ 2.3 (104.5)
Significance of difference (p - values)				
I-IV		0.05	0.01	0.01
II-III		0.20	0.90	0.90
II-IV		0.30	0.20	0.05
III-IV		0.02	0.10	0.10

= Standard error of mean

= Percentage change in weight

parts of the same oil. It will be remembered that in the human experiments of Beveridge (see Section 2) the effects reported with molecularly distilled butter and corn oil fractions were not supplied with statistical criteria.

Conclusion

Two important conclusions emerge from Experiments 8 and 9. The first is that the unsaponifiable part of sunflower seed oil when modified by liquid propane segregation also modifies the effect of this oil on the serum cholesterol level. The results of Experiments 6 and 7 (Section 12) with chemically separated unsaponifiable matter have thus been confirmed.

The second conclusion is that there appears to be a limit beyond which it is impossible to modify the properties of sunflower seed oil by manipulating the unsaponifiable matter. It was not possible for example to imitate the extraordinary hypercholesterolaemic effect of coconut oil. This again confirms the partially negative (or positive) result of Experiment 7 (Section 12).

SECTION 14INVESTIGATION OF THE UNSAPONIFIABLE MATTER BY
FEEDING PURE COMPOUNDSA. Effect of β -Sitosterol (Experiment 10)

The results of the experiments described in Sections 12 and 13 leave little doubt that the unsaponifiable matter (U.M.) of Sunflower seed oil is somehow implicated in the cholesterol lowering properties of this oil. To pursue this aspect further, one possible method of approach would be the fractionation of the U.M. itself and the testing of the resulting fractions in the usual way. An easier way may be the testing of such substances as are known to be present in the U.M. of Sunflower seed oil in the form of pure chemical compounds.

The largest single constituent in the U.M. of Sunflower seed oil, in common with many other vegetable oils, is β -sitosterol, which has indeed been shown to produce a depression in serum cholesterol values in humans if administered in massive doses. Inspection of Table 11 (Section 13) reveals that about 0.41% of this sterol is present in Sunflower seed oil. In the present experiment 1% of β -sitosterol has been dissolved in coconut fat and tested together with the pure control coconut fat and Sunflower seed oil groups in the usual way. The β -sitosterol was extracted from a commercial preparation and re-crystallized three times from 95% ethanol. It

probably contains traces of the dihydro derivative. The latter substance is, however, unlikely to interfere, since it has been shown by Siperstein et al. (1953) and Beher et al. (1957) to have cholesterol lowering properties resembling those of the main constituent.

Results

The results revealed by Fig. 22 and Table 14 show clearly that 1% of β -sitosterol in coconut fat had no material effect on the behaviour of this oil, or conversely that the serum cholesterol depressing effect of Sunflower seed oil is unlikely to be due to its β -sitosterol content. It is of interest to note that due to wide fluctuations of cholesterol values in this experiment it required 18 rats per group to produce sound statistical figures.

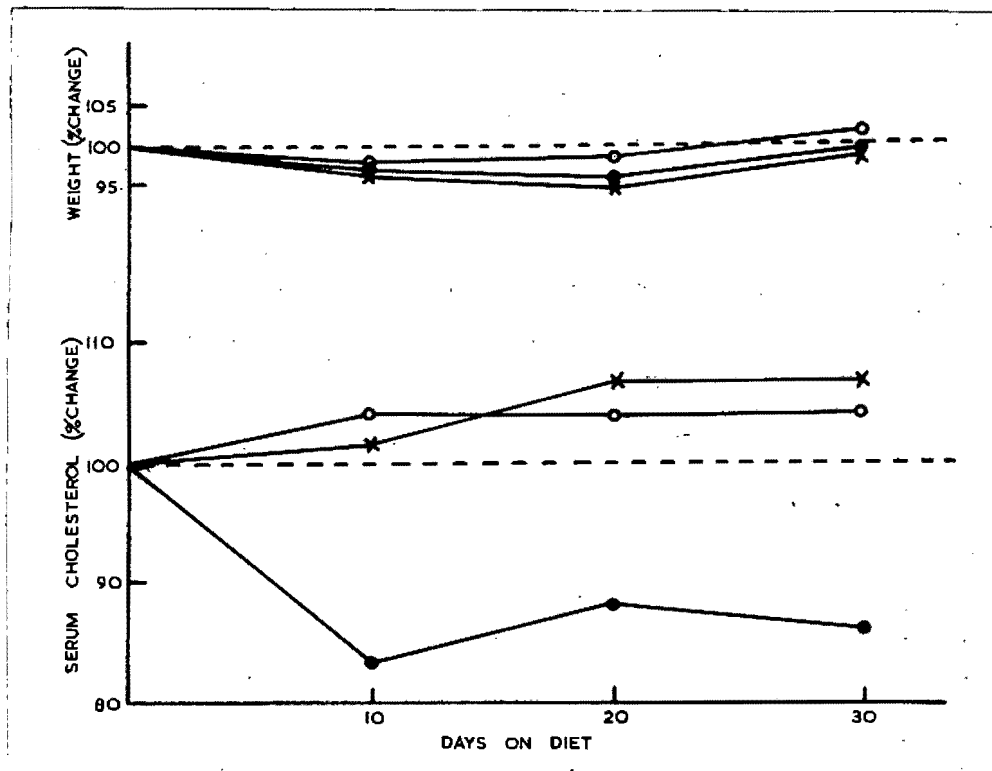


Fig.22. The Effect of 1% β -sitosterol on the Serum Cholesterol Concentration (Experiment 10).
 —○— Coconut oil control
 —×— 1% β -sitosterol dissolved in coconut fat
 —●— Sunflower seed oil control

TABLE 14 - (Experiment 10)

MEAN PERCENTAGE CHANGES IN SERUM CHOLESTEROL CONCENTRATION OF GROUPS OF EIGHTEEN RATS AFTER
CHANGING FROM LABORATORY CHOW DIET TO EXPERIMENTAL DIET CONTAINING 20% OF THE FATS LISTED
(STARTING VALUE = 100%)

GROUP	DIET	DAYS ON DIET		
		10	20	30
I	20% Coconut fat	104.1 $\pm$ 4.5* (95.9)**	103.9 $\pm$ 3.6 (94.8)	104.2 $\pm$ 3.7 (99.4)
II	20% S.S.O.	83.4 $\pm$ 4.8 (97.0)	88.5 $\pm$ 4.2 (96.2)	86.1 $\pm$ 4.1 (98.3)
III	20% Coconut fat containing 1% dissolved β -sitosterol	101.7 $\pm$ 4.8 (97.8)	107.2 $\pm$ 4.4 (98.8)	107.0 $\pm$ 3.2 (101.6)
Significance of differences (p - values)				
I - II		0.01	0.01	0.01
I - III		0.70	0.60	0.60

* = Standard error of mean

** = Mean percentage weight change

B. Effect of Squalene

Another constituent of the unsaponifiable matter common to many unsaturated oils, including fish oils in small amounts, is the highly unsaturated hydrocarbon squalene $C_{30}H_{50}$. This compound was first isolated and identified in Shark Liver Oil by Tsujimoto (1920) and has since been found in Olive Oil by Thorbjarnarson and Drummond (1935) to the extent of 30 to 64% of the unsaponifiable matter and by Fitelson (1943) in most natural triglyceride oils.

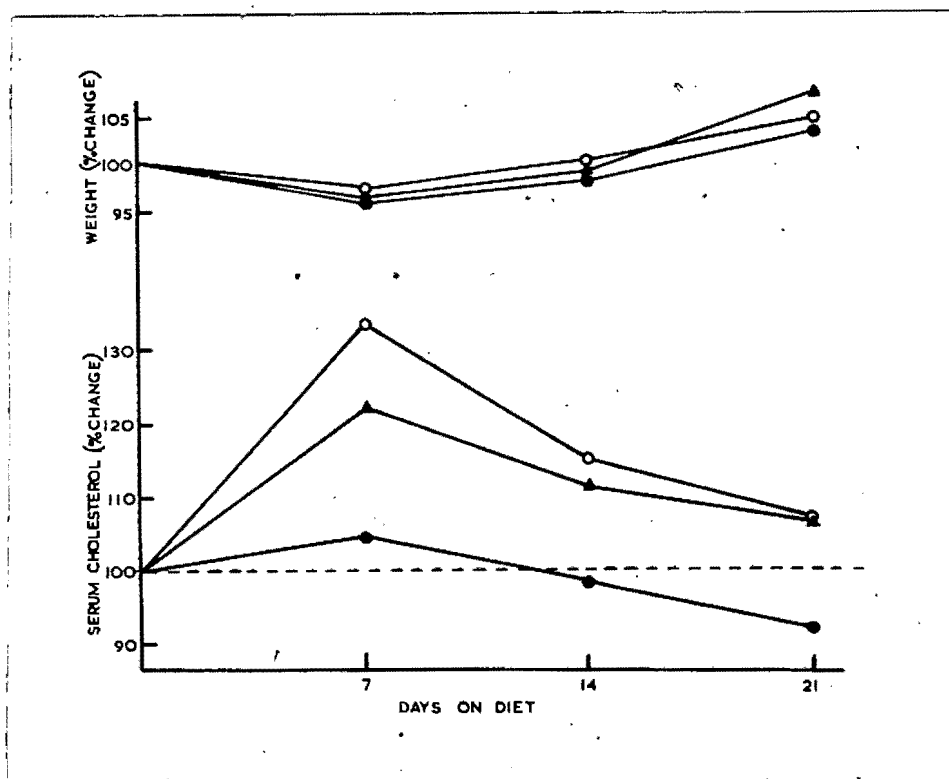


Fig. 23. Effect of 1% Squalene in coconut oil on the Serum Cholesterol level. (Experiment 11)

- coconut oil control
- △— 1% squalene in coconut oil
- sunflower seed control

TABLE 15 (Experiment 11)

MEAN PERCENTAGE CHANGES IN SERUM CHOLESTEROL CONCENTRATION OF GROUPS OF EIGHT RATS AFTER CHANGING
FROM LABORATORY CHOW DIET TO EXPERIMENTAL DIET CONTAINING 20% OF THE FATS LISTED
(STARTING VALUE = 100%)

GROUP	DIETS	WEEKS ON DIET		
		1	2	3
I	20% Coconut fat	133.9 $\pm$ 5.2* (97.2)**	115.1 $\pm$ 4.5 (100.0)	106.7 $\pm$ 5.3 (104.3)
II	20% S.S.O.	104.6 $\pm$ 5.6 (95.8)	98.2 $\pm$ 5.5 (97.7)	91.3 $\pm$ 5.3 (103.0)
III	20% Coconut fat containing 1% Squalene dissolved	122.7 $\pm$ 2.5 (96.6)	111.2 $\pm$ 5.6 (98.4)	106.6 $\pm$ 2.4 (107.1)
Significance of differences (p - values)				
I - II		0.01	0.03	0.05
I - III		0.07	0.6	0.9

* = Standard error of mean

** = Mean percentage weight change

In Sunflower seed oil Fitelson reported a concentration of 12 mg./100 ml. This substance also derives interest from the fact that Langdon and Bloch (1952) believe it to be an intermediary in the synthesis of cholesterol from acetate in the liver of rats. Channon (1926) has demonstrated that squalene is absorbed by this animal, a fact which McGuire and Lipsky (1955) confirmed with humans. In the present experiment squalene was tested in the comparatively massive dose of 1% of the coconut oil in dissolved form.

Results

It is seen from Fig. 23 and Table 15 that the serum cholesterol depressing activity of squalene, if real at all, ($p = 0.07$ after one week) was extremely weak. In the trace quantities present in Sunflower seed oil its effect can no doubt be neglected altogether. It is noteworthy, however, that the squalene curve runs below the coconut control at all times. Larger doses may well have a more pronounced influence.

C. The Combined Effect of β -Sitosterol, β -Carotene, α -Tocopherol and Squalene (Experiment 11).

In this experiment an attempt was made to create an artificial "unsap.", comprising substances known to be present in Sunflower seed oil. In addition to β -sitosterol and squalene, Lange (1950) has reported 70 mg./100 gm.

α -tocopherol in Sunflower seed oil whereas carotenes were identified in the unsaponifiable matter by the author as

reported in Table 10 (Section 13B). α -tocopherol is moderately well absorbed by the rat (Shantz, 1949) whereas Ahmad (1937) showed that β -carotene is almost completely utilized by the rat provided 10% of fat was incorporated in the diet. In the absence of fat this provitamin A is not utilized.

The following amounts were dissolved in coconut fat and tested for serum cholesterol depressing properties: -

β -sitosterol 0.7% ; β -carotene 0.3% ; α -tocopherol 0.2% ; squalene 0.2%, creating 1.4% of artificial "unsap." additional to that already present in coconut oil. Except in the case of β -sitosterol these amounts are necessarily very arbitrary and far in excess of those naturally present in Sunflower seed oil.

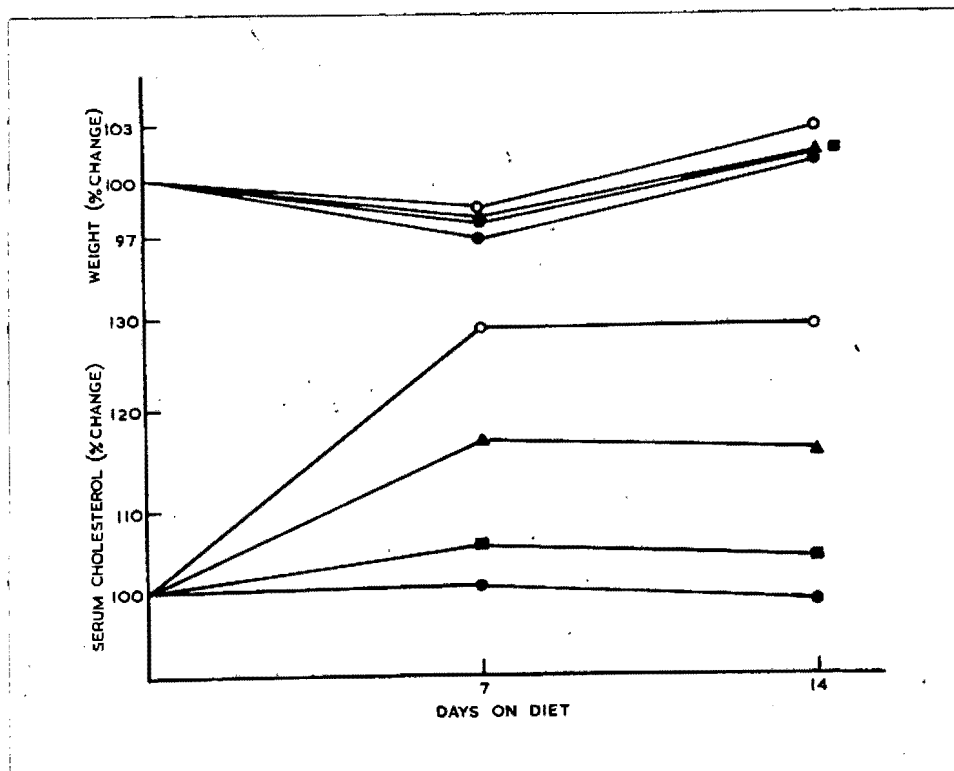


Fig. 24. The Effect of artificial unsaponifiable matter and of the propane segregated "Fraction C" (see Section 13B) on the serum cholesterol level (Experiment 12).

- coconut oil control
- ▲— artificial unsaponifiable matter (See text)
- Fraction "C" from Sunflower seed oil (25% in coconut oil)
- Sunflower seed oil control

TABLE 16 - (Experiment 12)

MEAN PERCENTAGE CHANGES IN SERUM CHOLESTEROL CONCENTRATION OF GROUPS OF SIX RATS AFTER CHANGING
FROM LABORATORY CHOW DIET TO EXPERIMENTAL DIET CONTAINING 20% OF THE FATS LISTED
(STARTING VALUE = 100%)

GROUP	DIET	DAYS ON DIET	
		7	14
I	20% Coconut fat	128.7 $\pm$ 3.7* (98.3)**	128.8 $\pm$ 3.7 (102.5)
II	20% Sunflower Seed Oil	100.7 $\pm$ 4.0 (96.7)	98.7 $\pm$ 3.1 (100.8)
III	20% Coconut fat + artificial unsap. (see text)	116.2 $\pm$ 3.4 (97.8)	114.9 $\pm$ 5.3 (101.2)
IV	15% Coconut fat 5% Propane segregated fraction "C"	105.2 $\pm$ 3.2 (97.5)	103.6 $\pm$ 4.6 (100.8)
Significance of difference (p - values)			
I - II		0.001	0.001
I - III		0.02	0.05
I - IV		0.001	0.001
II - III		0.01	0.02
II - IV		0.40	0.30

* = Standard error of mean

** = Mean percentage weight change

In addition to the above the "strength" of the most active of the liquid propane segregated Sunflower seed oil fractions (Section 13 B) was compared in a blend of 25% in coconut oil (I.V. of blend = 43.4) with pure Sunflower seed oil.

Results

The diet containing the coconut fat with artificial "unsap." produced a serum cholesterol depression relative to pure coconut oil of approximately 10%. Although significant, this depression was far from the 28% produced under similar conditions with Sunflower seed oil.

The Sunflower seed oil fraction C - coconut oil blend - produced values not significantly different from those due to pure Sunflower seed oil.

D. Discussion

In the present series of three experiments it was not possible to track down the cholesterol lowering activity of Sunflower seed oil to any particular compound contained in the unsaponifiable matter. The negative effects obtained by feeding the main constituent, i.e. β -sitosterol in near-natural doses are consistent with the results of Experiments 8 and 9 in Section 13 where the most active propane segregated fraction from Sunflower seed oil contained the lowest amount of digitonin precipitable sterols. When considered against the background of contrary reports from other workers, however, the present findings are surprising.

Peterson (1951) was the first to show that when chicks were fed β -sitosterol along with cholesterol, hypercholesterolaemia did not develop. Pollak (1953) followed with similar studies on rabbits. For the complete suppression of cholesterol-induced hypercholesterolaemia he had to feed these animals the colossal dose of 7 gm. of β -sitosterol per day.

A considerable amount of work has been done with this sterol on rats. Thus Hernandez and co-workers (1953) administered labelled cholesterol to rats and found that the absorption of this compound was reduced by the simultaneous administration of β -sitosterol. Rosenman, Beyers and Friedman (1946), using different experimental conditions failed to confirm this. The addition of either 2% or 10% of Soybean sterols to the diet of rats did not reverse the hypercholesterolaemia produced in these animals by feeding with 2% cholesterol and 1% cholic acid. It is curious, however, that the same investigators obtained positive results with dihydrocholesterol under similar conditions. Alfin-Slater, Wells, Aftergood and Deuel (1954) likewise failed to show effects with rats, feeding 1% of this sterol in the diet with or without cholesterol.

In contradistinction to the above, other workers have reported positive results with β -sitosterol in more recent publications. Among these are Best and Duncan (1957), Herrman (1959) and Nath and Harper (1959) working with rats, and finally, Gordon, Stolzenberg and Cekleniak who used the Mongolian gerbil as an experimental animal. (1959)

In assessing the significance of these results in relation to the present findings it must be pointed out that without exception these investigations were carried out with animals which had been rendered hypercholesterolaemic by the feeding of massive doses of cholesterol and in many cases cholic acid as well. As far as could be ascertained, no lowering of serum cholesterol has been reported with animals on a natural fat-rich diet.

In spite of the negative results obtained with β -sitosterol, in Experiment 10 there is no doubt that under certain artificial conditions this substance does lower the serum cholesterol of rats. The point is made here, however, that β -sitosterol is not believed to be the substance in Sunflower seed oil which is solely responsible for the serum depressing effect of this oil. The case of β -sitosterol may in fact be analogous to that of the cholesterol contained in animal fats. This sterol only produces detectable serum cholesterol raising effects if fed in massive doses when dissolved in oil. This will be shown in subsequent experiments.

Little can be said about the spurious activity found with squalene, as no similar studies are on record. More interesting, however, are the results obtained with the "artificial unsap." in Experiment 12. The significance of the small serum cholesterol depression obtained with this combination of substances will become apparent in Part V.

SECTION 15CONCLUSIONS

One of the primary objectives of this thesis was to find out by means of experimentation on rats why dietary sunflower seed oil produces low serum cholesterol levels. It must be stated at the outset that this objective has so far not been achieved. At the beginning of this work it was believed that the special properties of Sunflower seed oil must be due to chemical substances contained in this oil and that they must inevitably be exposed by some systematic fractionation process or other, much in the same way as an inorganic "spot" is analysed in the chemistry class. The initial success obtained with the unsaponifiable matter in Experiment 6 seemed to confirm these ideas. The search for "substance X" had apparently been narrowed down to the unsaponifiable matter.

The first discordant note in the "unsap." theory was struck by the next experiment (Experiment 7), however, where the total interchange of unsaponifiable matter between coconut oil and Sunflower seed oil did not completely reverse the effects of these oils on the serum cholesterol level. The following two experiments (8 and 9) with liquid propane segregated Sunflower seed oil fractions undoubtedly favoured the "unsap." theory. Experiment 12 with the artificial "unsap." again pointed in the same direction and so did the similar

effects on the serum cholesterol produced by two oils with radically different fatty acid compositions and unsaturation in the same test. It can be concluded, therefore, that if the results obtained in Part III can be relied on, there can be no doubt that the unsaponifiable matter plays a major role in determining the properties of dietary Sunflower seed oil as a serum cholesterol regulating agent.

It also follows from the above that no fatty acid, combination of fatty acids or their compounds in the form of triglycerides in the saponifiable part of the oil, can by themselves account for the whole of the cholesterol depressing action of this oil. This conclusion is in contradiction to the views held at present by the majority of researchers in the field.

There is a disturbing feature about the partial success obtained with squalene and artificial "unsap." of Experiments 11 and 12, the emphasis being on the "partial". Perhaps the indecisive argument of " 'sap.' versus 'unsap.' " can be extended to the unsaponifiable matter itself and one can say that the activity of the "unsap." is not due to any particular chemical compound contained in it but rather to some property of this fraction as a whole. The indications are that this would be a physical property. Thus Weitzel and his co-workers (1956) attributed their success in preventing atherosclerosis in chicks by treatment with Vitamins A and E to the emulsifying powers of these substances. The startling variety of chemical compounds not normally found

in fats, not the least impressive of which is N-(1-methyl-2, 3-di-p-chlorophenyl) - maleamic acid (Sachs et al., 1959), which have been claimed to have hypocholesterolaemic properties which point in the same direction.

The fact that neither the present study nor many similar investigations appear to have shown beyond reasonable doubt that the serum cholesterol regulating properties of oils can be adequately ascribed to any single chemical fraction or entity in the whole oil, leaves room for doubt whether there are in fact such entities, or whether in addition some physical characteristics of these oils as a whole may not be implicated.

It is doubts such as these which decided against the continuation of work on the further fractionation of the unsaponifiable matter of Sunflower seed oil in spite of the fact that some progress had already been made in a number of pilot studies with alumina and reversed phase partition-chromatography. Similarly, a three-stage molecular still for the fractionation of fatty acids threatened to become redundant before its construction had been completed.

An alternative route is the search for mechanisms rather than substances. The study of a lipid inducing hypercholesterolaemia such as egg fat, proved more attractive in this connection. This will be reported in Part IV of this thesis.

P A R T I V

THE HYPERCHOLESTEROLAEMIC PROPERTIES OF EGG LIPIDS
AND THE ABSORPTION OF CHOLESTEROL

SECTION 16INTRODUCTION

It is known that the consumption of hens' eggs by some animals and man results in an extraordinary increase in the serum cholesterol concentration. This has been shown in the case of man by Okey and Stewart (1953), Messinger, Porosowska and Steele (1950), Cook, Edwards and Riddell (1956), Bronte-Stewart et al. (1956), Gordon, Wilkens and Brock (1958) and others. Rabbits also show a most remarkable response to dietary egg treatment. Thus Pollak (1957) fed one egg daily to these animals and observed an increase in serum cholesterol level of three to fourteen times the normal value, depending on the method of preparation of the eggs. No reports of similar experiments conducted with rats have been found.

There are a number of reasons for the choice of hens' eggs as objects for study for the examination of cholesterogenic mechanisms. Neither the "degree of unsaturation" hypothesis nor the "some substance in the unsaponifiable matter" theory appears to be able to account for the behaviour of eggs. If the assumption is made that the serum cholesterol raising effect of eggs resides in the triglycerides contained in the yolks, it would have to be accepted that a fat with an iodine value of between 70 and 80 has exceptional hypercholesterolaemic properties.

The fatty acid composition of hens' egg triglycerides is to some extent dependent on the diet (Cruickshank, 1934). The following are quoted as representative analysis:

fatty acid	Cruickshank (1934)	Fewster and Young (1959) (Gas chromatography)
	%	%
myristic	32	0.3
palmitic		23.1
stearic		3.6
palmitoleic		4.4
oleic	47	53.4
linoleic	20	15.1
linolenic	2	-

The cholesterologenic effect in man of such a fat, according to the "degree of saturation" hypothesis would be expected to be less than is the case with a quantity of whole egg yolks of comparable fat content.

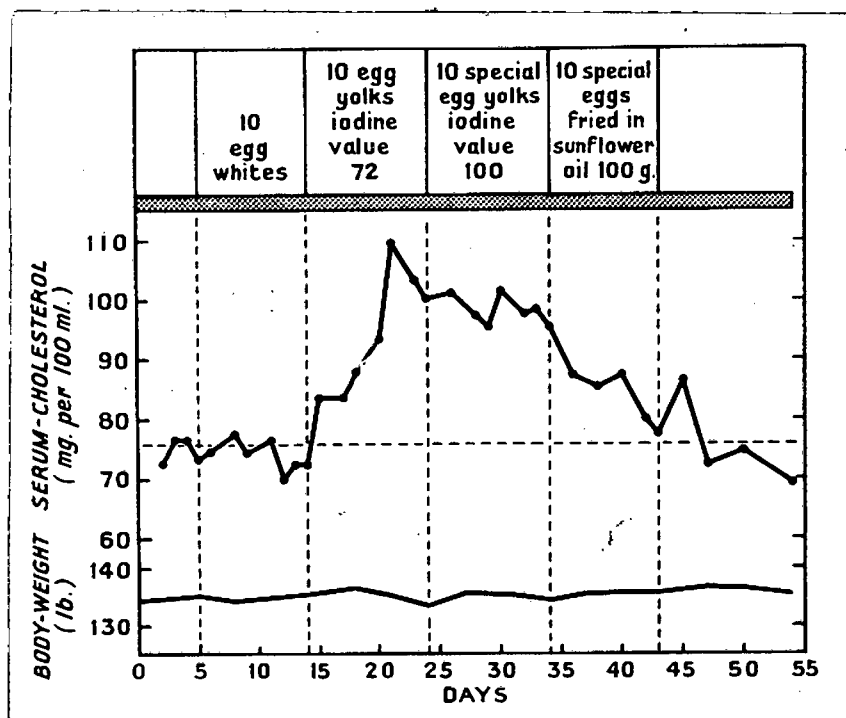


Fig. 25 Serum cholesterol level of male Bantu age 34, during ingestion of egg yolks
(From Gordon, Wilkens and Brock, 1958)

Gordon et al. (1958), furthermore, have shown that the artificial modification and unsaturation of this lipid fraction from iodine value of 72 to an iodine value of 100 does not materially alter the cholesterol raising properties of these "unsaturated eggs" (see Fig. 25). This modification of the fatty acid composition of egg lipids was achieved by manipulation of the birds' diet with high proportions of dehulled sunflower seeds.

In addition to 59% neutral fat, hens' egg lipids contain about 35% of phospholipids and 6% of unsaponifiable matter which is largely composed of cholesterol. Winterstein and Schön (1936) quote 1.342% of this substance in total egg yolk. The latter is thus seen to be the most concentrated source of cholesterol in common foods.

It seems close at hand to implicate the abnormally high cholesterol content of egg yolks for their hypercholesterolaemic properties. This is, however, by no means generally accepted in the interpretation of human experiments. This common sense view has been rejected on account of the belief that dietary pure cholesterol has little or no influence on the serum concentration. Thus Cook (1958) discusses the work of a number of workers in the field and concludes "In man the plasma cholesterol is largely independent of dietary cholesterol." This aspect will be examined in more detail in Section 23 below.

From the above brief discussion it would appear that neither the triglycerides nor the cholesterol content of egg yolks are responsible for their cholesterogenic action in the

case of man. Of the lipids there remains only the phosphatide fraction and the unsaponifiable matter other than cholesterol to be examined. The systematic fractionation of egg lipids with the object of isolating the cholesterol raising factor or discovering its principle is obviously desirable. This will be described in the next section.

SECTION 17THE EFFECT OF TRIGLYCERIDES FROM HENS' EGGSA. Choice of experimental diets

First diet The triglycerides of egg yolks are the obvious choice for a first examination of egg lipids. It is these compounds in other foods that almost invariably exert some sort of effect on the serum cholesterol level. The unsaponifiable matter (in this case largely cholesterol) is here included in this fraction since it accompanies it in nature as well as in most chemical extraction processes. This fraction will be called crude triglycerides.

Second diet For the second diet of the standard four-group experiment the triglycerides were chosen from which the unsaponifiable part had been removed, i.e. the test lipid consisted of pure triglycerides.

Third diet The lipid portion of this diet again consisted of pure triglycerides as in the second diet, but was in this case supplemented by the amount of cholesterol present in the unsaponifiable portion of the crude triglycerides of the first diet. As in all previous experiments the supplement was dissolved in the pure triglycerides.

Fourth diet The rations for this group of animals contained sunflower seed oil as control. The remainder of the diets and

the conduct of the experiment followed the standard pattern described in Section 2.

It must be admitted that the design of these four diets for an experimental test is not strictly systematic. It betrays an a priori conviction that the serum cholesterol raising property of eggs can be attributed to nothing more mysterious than their high cholesterol content. This view was held in spite of the prevailing evidence to the contrary discussed in the previous section. One reason is that Experiment 16 (see Section 23) preceded the present one in time. From the results of this experiment it will be seen that in man dietary cholesterol does in fact raise the serum cholesterol provided it is dissolved in fat. Another strong indication comes from the experiments of Pollak (1957) already mentioned. The remarkable rise in serum cholesterol of rabbits on egg diets is paralleled by the equally remarkable ease with which these animals absorb cholesterol (e.g. Cook and Thomson, 1951). If the same response cannot be observed to the same degree in other species it seems reasonable to postulate at least a vestigial parallel effect in rats and man.

B. Preparation and composition of lipid fractions

The isolation of triglycerides from egg yolk has been described by Rhodes and Lea (1957) who showed that the cold acetone (0°C) extraction of dried egg yolk yields a material consisting of 97% of total neutral lipids which will be called crude triglycerides. No attempts were made to remove the remaining three per cent of phospholipids reported in this

fraction. The author is indebted to Mr. V.M.Wells for preparing a supply of this material. The phospholipids were isolated from the residue by repeated extraction with a mixture of chloroform and methanol.

A third of the acetone soluble fraction was left unchanged. The remainder was saponified and the unsaponifiable matter extracted and purified, using the apparatus and procedure described in Section 12A. The regenerated acids were re-esterified with glycerol according to the method of Hilditch (1956). The reaction mixture was alkali refined in diethyl ether solution to remove excess acids. The re-synthesised triglycerides differ from the natural product in that the acids are now randomly distributed on the glycerol molecule. In view of considerable evidence that triglycerides suffer a certain amount of random interesterification in the intestine before and during absorption, this is no serious disadvantage. The fatty acid composition was determined by Fewster and Young (see Section 16). The remaining results are tabulated in Table 17 (a). On the

TABLE 17a

COMPOSITION OF EGG YOLK LIPIDS

	<u>% of yolk</u>	
Triglycerides (I.V. 69)	19.10	} crude triglycerides
Unsaponifiable matter (U.M.)	1.82	
Digitonin precipitable free * sterols (in U.M.)	1.57	
Phospholipids	10.20	

* only traces of sterol esters were detected

basis of the figure of 1.342 quoted by Winterstein and Schön for the cholesterol content of whole egg yolk the digitonin precipitable sterols were largely composed of cholesterol. In the absence of more precise information a cholesterol content of six per cent in the crude triglycerides was accepted for evaluation in the animal test.

C. Results and Discussion

The results are recorded in Fig. 26 and Table 17b in the usual way and can be summarised as follows. The pure

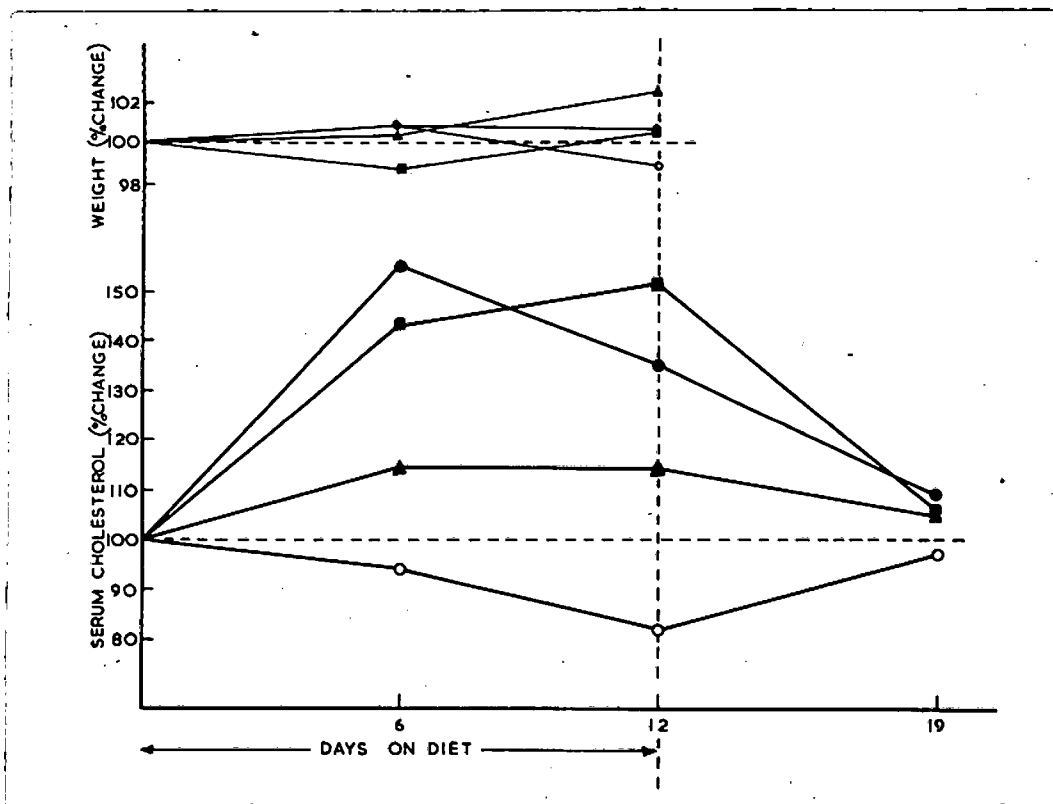


Fig. 26 The effect on the serum cholesterol concentration of hens' egg triglycerides (Experiment 13).

- ▲— pure triglycerides
- crude triglycerides
- pure triglycerides plus 6% cholesterol
- sunflower seed oil control

TABLE 17b - (Experiment 13)

MEAN PERCENTAGE CHANGES IN SERUM CHOLESTEROL CONCENTRATION OF GROUPS OF SIX RATS
AFTER CHANGING FROM LABORATORY CHOW TO EXPERIMENTAL DIETS CONTAINING 20% OF THE
LIPID FRACTIONS LISTED (STARTING VALUE = 100%)

GROUP	DIETS	DAYS ON DIET		(LAB. CHOW)
		6	12 ^{***}	19
I	Crude egg triglycerides	142.4 ± 6.9 (98.7) ^{**}	151.9 ± 10.6 (100.4)	106.2 ± 5.5 (118.8)
II	Pure egg triglycerides	115.1 ± 4.6 (100.4)	114.3 ± 5.3 (102.5)	105.6 ± 3.7 (121.0)
III	Pure triglycerides containing 6% added cholesterol	154.7 ± 8.6 (100.8)	134.3 ± 9.2 (100.4)	108.9 ± 6.0 (121.1)
IV	Sunflower seed oil	94.8 ± 6.7 (100.8)	81.8 ± 5.0 (98.0)	97.2 ± 7.2 (118.6)
Significance of differences (p - values)				
I- II		0.01	0.01	0.9
I-III		0.3	0.2	0.7
I- IV		0.001	0.001	0.3
II-III		0.01	0.1	0.7
II- IV		0.03	0.001	0.3
III- IV		0.001	0.001	0.2

^{*} Standard error of mean ^{**} Mean percentage changes in weight

^{***} Experimental diets discontinued after 12th day.

triglycerides produced a moderate rise in serum cholesterol, relative to the sunflower seed oil control. There is no reason to believe that the extent of this elevation is in any way abnormal if the composition and iodine value of this fat is taken into consideration. The crude cholesterol-rich triglycerides, however, produced the most dramatic elevation so far encountered in this series of rat assays. This elevation was not significantly different from that due to a diet of pure triglycerides to which an equivalent amount of cholesterol had been added. A notable feature of this test is the prompt return to baseline of the cholesterol curves after discontinuation of the experimental diets.

The conclusions to be drawn from this experiment are obvious. A greater part of the very marked hypercholesterolaemic property of egg yolk is probably due to its cholesterol content. The design and outcome of the whole experiment seems in fact trivial, were it not for the persistent light it throws on the question: if dietary cholesterol when dissolved in fat raises the serum concentration, how can fat devoid of this substance have a similar if less marked influence?

It may be criticised that it has not been shown that the feeding of whole egg yolks produces the same effect as that of an equivalent amount of crude triglycerides or pure triglycerides plus cholesterol. The marked differences in effect on rabbits, of eggs prepared in different ways, would make a strictly quantitative comparison difficult. The physical state of the cholesterol in yolk is presumably of

importance.

The investigation of the phospholipids and the unsaponifiable matter other than cholesterol has likewise been neglected in favour of a more detailed study of the conditions under which cholesterol is absorbed.

SECTION 18THE ABSORPTION OF DIETARY CHOLESTEROL

The title of this section introduces a subject on which much has been written. In spite of this, however, no clarity exists about the precise conditions under which cholesterol is absorbed by the rat. In Section 17 it was shown that cholesterol, if dissolved in egg triglycerides, is easily absorbed. The question arises whether the presence of fat is necessary for this to occur. It is the object of the next two experiments to contribute to an answer to this question.

A, The effect of large doses of cholesterol fed with and without sunflower seed oil (Experiment 14a).

Experimental

It will be remembered that 0.2 per cent of cholesterol in the diet either in crystalline form with fat or dissolved in fat could not be shown to influence the serum cholesterol significantly (Experiment 4, Section 9C). The dose was accordingly increased to 2 per cent and tested both in the absence and presence of fat in the diet, the latter consisting of 20 per cent of sunflower seed oil. In order to make this experiment comparable with Experiment 4, a diet with a total cholesterol content of 0.2 per cent (1 per cent in sunflower seed oil) was again included as

was also the sunflower seed oil control diet. In all cases except in the fat-free diet the cholesterol was dissolved at 150°C in the sunflower seed oil before incorporation in the remainder of the rations. A certain portion, no doubt, subsequently crystallized out again before the food was consumed.

Results

See Fig. 27a and Table 18a.

The results appear to give a decisive answer to the problem of the role of fat in the absorption of cholesterol as

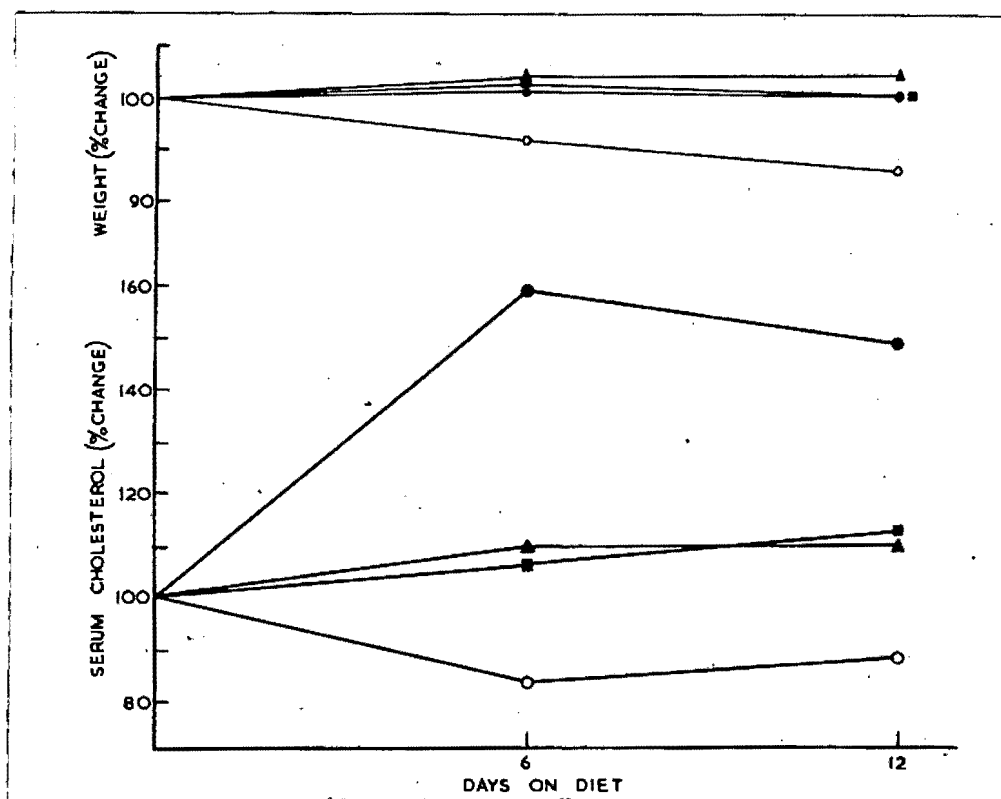


Fig. 27a The effect of large doses of cholesterol fed in absence and presence of oil (Experiment 14a).

- 10% cholesterol in sunflower seed oil (2% of total diet)
- 1% cholesterol in sunflower seed oil (0.2% of total diet)
- ▲— 20% sunflower seed oil control
- fat free

TABLE 18a - (Experiment 14a)

MEAN PERCENTAGE CHANGES IN SERUM CHOLESTEROL CONCENTRATION OF GROUPS OF SIX RATS AFTER CHANGING
FROM LABORATORY CHOW TO EXPERIMENTAL DIETS CONTAINING 20 PER CENT OF THE LIPIDS LISTED
(STARTING VALUES = 100%)

GROUP	DIET	DAYS ON DIET	
		6	12
I	Basic fat-free plus 2% cholesterol	84.0 $\pm$ 3.54* (95.9) **	88.8 $\pm$ 5.41 (92.5)
II	Sunflower seed oil	109.6 $\pm$ 4.70 (101.8)	110.8 $\pm$ 5.37 (101.8)
III	S.S.O. containing 1% cholesterol (0.2% of diet)	107.1 $\pm$ 3.16 (101.4)	112.9 $\pm$ 3.75 (100.0)
IV	S.S.O. containing 10% cholesterol (2% of diet)	159.5 $\pm$ 11.9 (101.0)	149.2 $\pm$ 7.79 (100.0)
Significance of differences (p - values)			
I-II		0.01	0.02
I-III		0.001	0.01
I-IV		0.01	0.001
II-III		0.7	0.7
II-IV		0.01	0.01
III-IV		0.01	0.01

* = standard error of mean

** = mean percentage change in weight

measured by a change in serum concentration. The difference in serum concentration of nearly 100 per cent on feeding the same amount of cholesterol (10% in fat, 2% of diet) with and without fat, is impressive. As in Experiment 4 a comparatively small amount of sterol (1% in the fat, 0.2% of the diet) in the diet is without detectable influence, thus confirming the earlier experiment.

Of considerable interest is the fact that the fat-free but cholesterol-rich concentration curve is significantly lower than that due to a diet containing sunflower seed oil without cholesterol which is considered to be a cholesterol lowering oil in the case of man. The small loss in weight of the animals on the fat free ration, however, indicates some caution in the interpretation of these data.

B. The effect of feeding cholesterol and oil separately
(Experiment 14b)

The last experiment revealed what appears to be a profound interdependence of dietary cholesterol and dietary fat. Neither substance alone will produce maximum effects. The aspect which requires clarification is whether the rise in serum cholesterol is simply due to increased absorption of cholesterol fed in a fatty medium or due to some other mechanism requiring the supply to the body of both cholesterol and fat. The latter alternative is likely to be the more complicated concept and also difficult to visualize. It would be desirable to rule it out if possible.

An elegant way to supply both fractions to the body, out of contact with each other, would be to feed

cholesterol by mouth simultaneously with an oil emulsion by intravenous injection. To do this with rats would probably require a high degree of experimental skill. A compromise on a less ambitious level is the feeding of both cholesterol and oil by mouth but separated in time. This has been done in the present experiment, which differs from the preceding one by the change of two diets only.

Experimental

First diet. Sunflower seed oil containing 10 per cent (2% of diet) of cholesterol as far as possible in dissolved form.

Second diet. As first diet for seven days. Thereafter the cholesterol was added in crystalline form to the fat-free diet and the oil fed separately twice daily (at 8 hours interval). This was done directly into the mouth by means of a pipette.

Third diet. Sunflower seed oil control diet (without cholesterol).

Fourth diet. Fat free diet (without cholesterol).

Results

See Fig. 27b and Table 18b.

The results of Experiment 14b are essentially the same as those of Experiment 14a except for one important point. The separation of cholesterol and oil in the diet of one group of animals after the seventh day was reflected in an immediate and significant drop in serum concentration

relative to a control, where no separation was effected. This lowered concentration was only slightly higher than that due to a cholesterol-free diet with equivalent oil content.

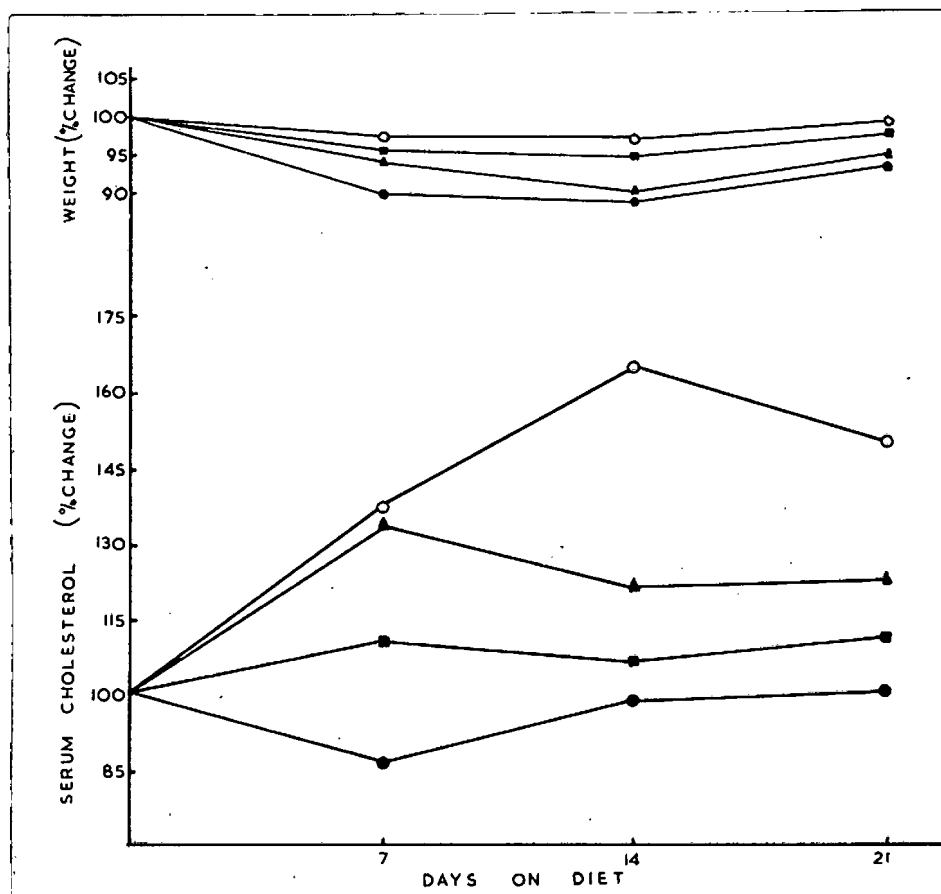


Fig. 27b

The effect of feeding cholesterol and oils separately (Experiment 14b).

- 10% cholesterol in sunflower seed oil (2% of diet)
- ▲— 10% cholesterol in sunflower seed oil fed separately after first seven days (see text)
- 20% sunflower seed oil control diet
- fat free diet

The fat-free and cholesterol-free ration produced serum levels which were not lower than those produced with a similar diet containing 2 per cent of cholesterol in Experiment 14a. Although this comparison is not strictly

TABLE 18b - (Experiment 14b)

MEAN PERCENTAGE CHANGE IN SERUM CHOLESTEROL CONCENTRATION OF GROUPS OF SIX RATS AFTER CHANGING
FROM LABORATORY CHOW TO EXPERIMENTAL DIETS CONTAINING 20 PER CENT OF THE LIPIDS LISTED
(STARTING VALUES = 100%)

GROUP	DIETS	DAYS ON DIET		
		7	14	21
I	Sunflower seed oil plus 10% cholesterol (2% of diet)	134.8 $\pm$ 5.0* (96.8)**	163.8 $\pm$ 8.9 (97.2)	148.1 $\pm$ 8.4 (99.3)
II	Sunflower seed oil plus 10% cholesterol fed separately after 7th day	134.1 $\pm$ 3.9 (98.5)	121.7 $\pm$ 2.3 (91.2)	123.0 $\pm$ 4.1 (95.0)
III	Sunflower seed oil	109.7 $\pm$ 7.7 (95.9)	105.3 $\pm$ 6.8 (95.9)	110.8 $\pm$ 3.9 (98.5)
IV	Fat-free	86.4 $\pm$ 5.9 (89.2)	99.3 $\pm$ 3.6 (88.7)	100.5 $\pm$ 5.2 (93.1)
Significance of differences (p - values)				
I-II		0.9	0.001	0.02
I-III		0.02	0.001	0.01
I-IV		0.001	0.001	0.001
II-III		0.02	0.05	0.05
II-IV		0.001	0.001	0.01
III-IV		0.03	0.4	0.1

* Standard error of mean

** Mean percentage change in weight

legitimate since the data belong to two different experiments, these serum levels occupy the same quantitative position in relation to the sunflower seed oil control curve in both cases. The average weight losses are likewise of the same order in both groups. It is therefore concluded that in the absence of dietary fat, 2 per cent of dietary cholesterol could not be shown to influence the serum level.

C. Discussion and Conclusions

In Section 17 it was shown that dietary cholesterol in the presence of egg triglycerides has a marked cholesterogenic property. The present two experiments have demonstrated that egg triglycerides can be substituted by sunflower seed oil without materially altering the course of the experiment. Furthermore, they have demonstrated that the presence of sunflower seed oil and presumably also other fats is a necessary condition for this phenomena to take place. In other words, it is reasonable to conclude that dietary fat must be present for the absorption of dietary cholesterol by the rat. Thus the reported inert nature of cholesterol in the diet of man (Section 16) has not been confirmed in the case of rats.

Nearly 24 years ago, Cook (1936) fed cholesterol with and without fat to rats, measured the faecal output of this substance and concluded that"cholesterol is absorbed by rats only when fat is present in the diet." No further discussion of the subject is apparently required.

Unfortunately less conclusive results have been reported by some investigators in more recent times.

In close agreement with the present findings are those of Swell, Flick, Field and Treadwell (1955) who fed a combination of cholesterol, sodium taurocholate and olive oil to rats. In the absence of both bile salt and oil, the addition of two per cent of cholesterol to the diet had a negligible effect on the serum cholesterol level. The supplementation of cholesterol with one per cent of sodium taurocholate alone, however, caused an elevation which was comparable to that produced by the simultaneous presence of all three substances in the diet. These authors suggested that dietary fat serves a dual purpose: it stimulates the flow of bile necessary for cholesterol esterase activity and it provides the fatty acids necessary for cholesterol esterification.

In the same year, Pihl (1955) reported his results from balance studies with rats. He found cholesterol to"be readily absorbed on a diet devoid of fats", admitted however, that dietary fat stimulates cholesterol absorption when it is present in large amounts relative to cholesterol. By using mostly the colorimetric Schoenheimer-Sperry method for the "cholesterol" determination in faecal fat the saturated transformation products like dihydrocholesterol and cholestanol (coprosterol) were neglected. Thus Coleman (1956) found that cholesterol represented less than 20 per cent of the total digitomin-precipitable sterols in the faeces of rats on a fat free diet.

In agreement with the present results, Aftergood, Deuel and Alfin-Slater (1957) reported the lowest serum cholesterol of rats on fat free diets. The addition of 12 per cent of cottonseed oil caused a rise in serum level from an average of 42 mg./100 ml to 64 mg./100 ml.

As has been mentioned in Section 16 the rabbit absorbs dietary cholesterol with utmost ease. Even in this animal, however, Steiner and co-workers (1959) observed a three-fold increase in serum concentration when 0.5 per cent of cholesterol in the diet is supplemented by 12 per cent of either safflower or coconut oil.

The conclusions that have been tentatively drawn from Experiments 14a and 14b, can be summarized again as follows:-

1. Comparatively large doses of dietary cholesterol have a marked hypercholesterolaemic effect if given dissolved in fat.
2. In the absence of fat even large doses of dietary cholesterol are without influence on the serum level of rats.
3. Dietary unsaturated fats represented by sunflower seed oil produce higher serum cholesterol levels than a fat free diet.

Substantial, if by no means unanimous, support for these statements have been quoted from the literature. Very little support can be enlisted, however, for the

application of most of these principles to the human sphere.

It may reasonably be asked why so much attention has been paid to the study of the conditions under which dietary cholesterol is absorbed, as this investigation is concerned primarily with dietary triglyceride fats. An answer to this question will be given in Part V of this thesis.

P A R T V

DISCUSSION AND CONCLUSIONS

THE SOLUBILITY HYPOTHESIS

SECTION 19DISCUSSION AND CONCLUSIONS (EXPERIMENTS 1 - 14)

It may be appropriate at this stage to pause for reflection. A considerable body of data has been collected. More than 36 different lipid preparations have been tested in 14 separate experiments. Before attempting to draw any conclusions from these data as a whole, however, the main findings are summarized again as follows:

The Rat as a Suitable Experimental Animal

Contrary to the expectations to be derived from a study of the literature, the rat has shown itself to be, in the hands of the author, an excellent experimental animal for the purpose for which it was used. The earlier preliminary conclusions arrived at in Part II under 'methods' were fully justified. Although the magnitude of the response of rat serum cholesterol concentration to the two representative fats, i.e. coconut and Sunflower seed varied slightly from experiment to experiment and from colony to colony, highly significant differences were found in all cases. Furthermore, when testing certain fat preparations and using only 6 animals per group, finer intermediary gradations between the effects of coconut oil and Sunflower seed oil can be obtained, which can still be shown to be significantly different from either of the high and low control fats or their equivalents. The transient responses obtained in some earlier

experiments were due to inexperienced experimentation. In one case with fish oil and butter (Experiment 3), serum cholesterol differences were kept up for at least 7 months and there is no reason to believe that they cannot be maintained indefinitely. Essentially similar results were obtained with animals of uncertain origin, bred in three different laboratories.

Effect of Unsaponifiable Matter

Passing to actual results obtained from specific tests, it was found in Part III that the unsaponifiable matter of Sunflower seed oil is partly responsible for the hypocholesterolaemic effect of the latter. It follows from this that no particular fatty acid or grouping of different acids can by itself account for all the properties of this fat. It was shown, for example, that three different oils with identical fatty acid compositions had different effects, and that two oils with very different iodine values and linoleic acid contents can have very similar effects. It is concluded at this stage then, that the cholesterologenic property of an oil depends on both the "sap." and "unsap.", i.e. on some property of the oil as a whole.

Effect of Cholesterol

The findings described in Part IV disclose that under certain conditions dietary cholesterol has a profound serum cholesterol elevating effect. The significant fact

emerges, however, that this elevating effect is produced only with comparatively large doses of cholesterol and only when fed simultaneously with, and dissolved in, large amounts of fat. In other words, an interesting and perhaps highly significant connection has been established between cholesterol on the one hand, and fat on the other.

Postulated Conditions of Cholesterol Absorption

The situation then, is as follows. On the one hand there exist fats devoid of cholesterol such as coconut fat which raise the serum cholesterol level. This is, however, not entirely due to any peculiar fatty acid composition. On the other hand there seems to be little doubt that large amounts of cholesterol, when administered in dissolved form in apparently neutral or non-raising fats, produce an increased serum cholesterol level.

Can these two apparently conflicting aspects be reconciled by a common explanation? To put it in other words, are the effects of dietary saponifiable matter, unsaponifiable matter and dietary cholesterol, necessary manifestations of the same biological phenomenon, which incidentally may not even have a chemical basis? If so, it is conceivable that further attempts at concentrating serum cholesterol lowering substances from triglyceride oils may prove to be fruitless. In fact, such attempts have been made by others (see Sections 2 and 3), but generally with little success. Incursions into the field of physiology have so far been deliberately

avoided. They may, however, become necessary.

As discussed in Section 18, it is significant that large doses of dietary cholesterol are apparently not appreciably absorbed in the absence of dietary fats. The presence of fats may be necessary for the absorption of dietary cholesterol and presumably also endogenous or biliary cholesterol from the intestinal tract.* It is also reasonable to assume that this cholesterol, whatever its origin, must be dissolved in fat in order to be effectively absorbed. If the prerequisite to absorption is its state of solution in fat, the solubility of cholesterol in fats would determine the rate of absorption. The solubility of cholesterol may be different in different fats, thus perhaps accounting for different serum cholesterol concentrations produced by the latter. In the next Section the determination of the solubility of cholesterol in a variety of fats at body temperature will be described.

#

That appreciable quantities of endogenous cholesterol are available for re-absorption in the intestine has been demonstrated by Danielsson and Gustaffsson (1959) who found that germ free rats on a diet with completely steroid-free synthetic fat, excrete at least 2.1 mg. of cholesterol daily. Since this does not include the presumed re-absorbed sterol, more endogenous cholesterol may originally have been available in the intestine. Scaled up, weight for weight to the human scale this represents a daily excretion of at least 0.6 gms. per day.

SECTION 20THE SOLUBILITY OF CHOLESTEROL IN TRIGLYCERIDE OILS
(IN VITRO experiment)Experimental

The determination of the solubility of cholesterol in triglyceride oils is essentially a simple matter. In practice, however, a certain amount of experimentation is required to obtain reliable results. The apparatus and procedure eventually used after a series of modifications is briefly described, as follows.

An aquarium tank formed the basis of a constant temperature bath. A toluene - mercury contact thermometer was constructed to act as thermo-regulator. This operated an electrical relay which in turn regulated a conventional immersion heater. The wattage of the latter was made variable by an external series resistance. A "quick heating up" circuit was also provided. By these means and an electrical water stirrer the temperature was kept constant at $37 \pm 0.05^{\circ}\text{C}$.

Twenty gm. of oil and 1.5 gm. of reagent grade cholesterol were placed in 6" x 1" open test tubes and suspended in the constant water bath. Agitation of the mixture was effected by bubbling a steady stream of nitrogen through each tube. To avoid cooling effects due to contact with the gas, the latter was preheated to the required temperature by preliminary passage through glass coils in the bulk of the tank.

After two hours^{*} the excess cholesterol was allowed to settle and the supernatant oil sucked off by means of vacuum in sintered glass Knich filter sticks of porosity 3. Fig. 29 illustrates the whole apparatus during the filtering operation.

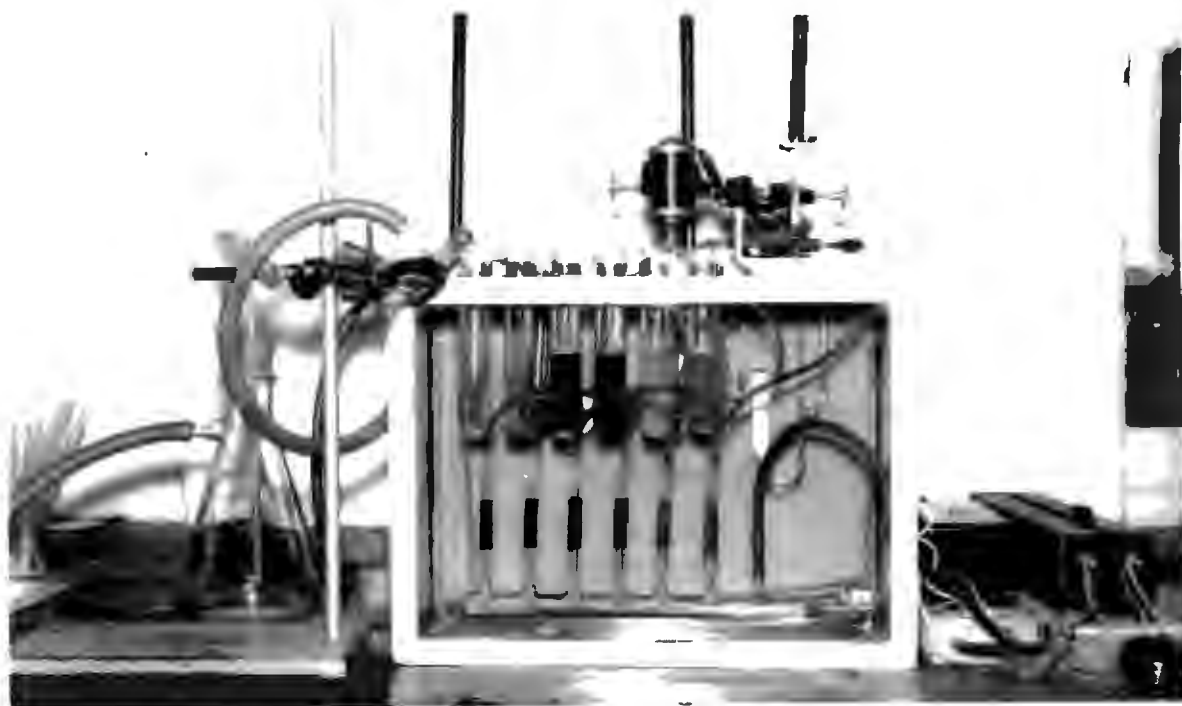


Fig. 29. Apparatus for the determination of the solubility of cholesterol in triglyceride oils.

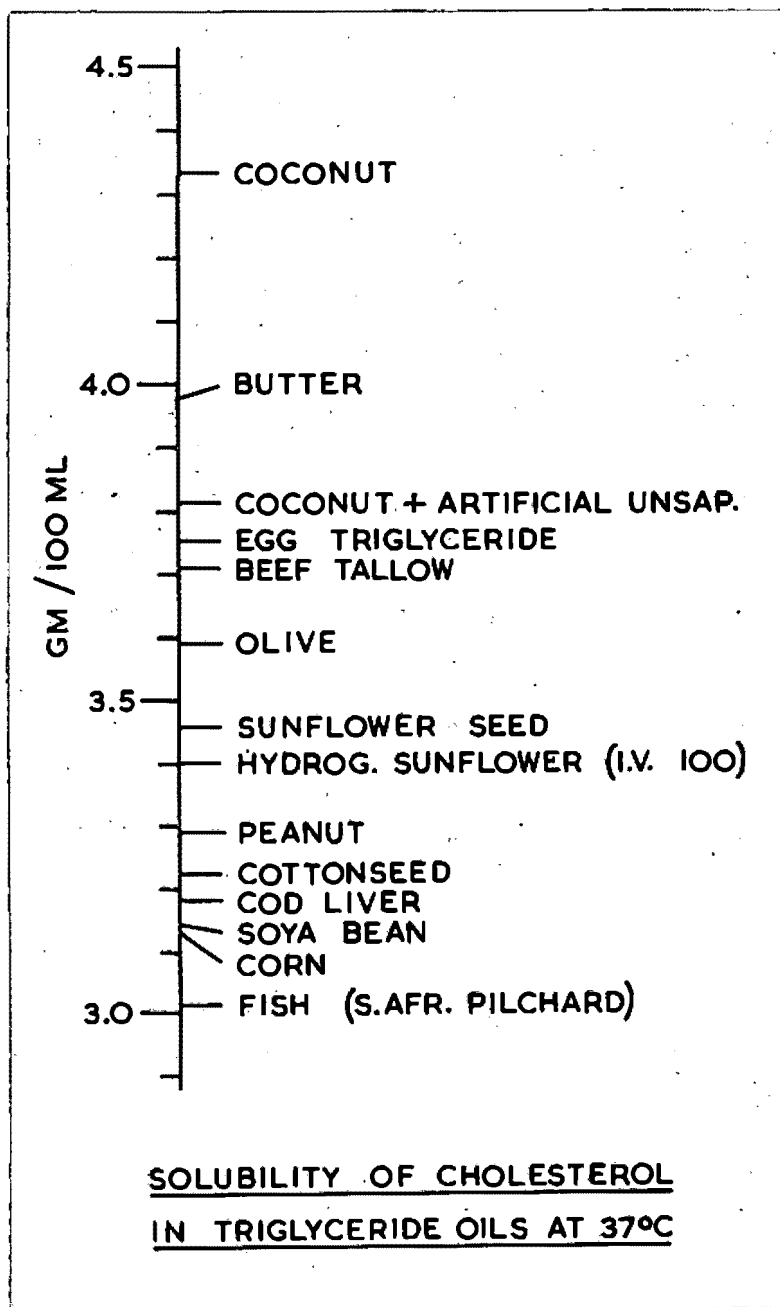
The dissolved cholesterol in the oil saturated at 37°C was estimated according to the method of Abell et al.(1952). Since most oils contain appreciable quantities of Liebermann-Burchard colour-producing substances (e.g. unsaturated hydrocarbons, sitosterols, carotenes, vitamin A etc.), a cholesterol determination in the oil before saturation with the sterol served

^{*} Experience has shown that saturation levels are reached in less than 15 minutes' agitation time. For safety, a time of two hours was allowed in routine tests.

TABLE 19

THE SOLUBILITY OF CHOLESTEROL IN TRIGLYCERIDE FATS AND OILS AT 37°C IN GM/100 ML

	Mean				Remarks
Coconut fat				4.33	Expts. 1 - 14
Butter fat (originally contains 0.18 gm./100 ml)	3.94	4.00		3.97	Expt. 3 (incl. 0.18 gm./100 ml)
Coconut + "Artificial Unsap."				3.81	Expt. 12, Section 14
Egg triglycerides				3.75	Expt. 13, Section 17
Beef tallow				3.71	Expt. 3, Section 9
Olive oil				3.59	
Sunflower seed oil, Sample I				3.48	Expts. 1 - 14
Sunflower seed oil, Sample II	3.48	3.40	3.50	3.46	
Sunflower seed oil, hydrogenated	3.39	3.44	3.36	3.40	I.V. 100, Expt. 16, Section 23
Arachis oil	3.28	3.32	3.26	3.29	Expt. 3, Section 9
Cottonseed oil				3.22	
Cod liver oil				3.12	
Soya bean oil				3.14	
Corn oil				3.13	
Fish body oil (sardina ocellata)	2.85	3.20	2.98	3.01	Expt. 3, Section 9
Coconut fat + sitosterol Sample I				0.93	Coconut fat saturated with β -sitosterol at 37°C
Coconut fat + sitosterol Sample II				0.68	
Sunflower seed oil propane "overhead" fraction				3.52	Expts. 8 and 9, Section 13
Sunflower seed oil propane "bottom" fraction				3.31	

Fig. 30.

as a blank. In a few cases the results were checked by the micro-ferric chloride procedure described earlier, as well as by the more fundamental method of precipitation with digitonin. Up to six solubility determinations were made simultaneously in one batch.

The relative values of the solubility in different oils were more reproducible from batch to batch than their absolute values. For this reason, a representative of both hyper- and hypocholesterolaemic oils, i.e. coconut oil and sunflower seed oil were carried through each batch as controls. This enabled the solubility determined in an unknown oil to be corrected relative to coconut oil for which a mean value of 4.32 gm./100 ml was accepted from a large number of determinations.

Results

The solubility of cholesterol in a variety of oils including some special oils used in previous rat experiments are recorded in Table 19. The reproducibility of the determinations can be estimated from the duplicate values obtained with the same oil in different batches. For visual ease of comparison, Table 19 has been reproduced in the form of a graphical scale in Fig. 30.

The discussion of the significance of these results as a whole will be found in the next Section.

SECTION 21THE SOLUBILITY HYPOTHESIS

"Firm progress in biological research is dependent on the proper use of hypothesis."

A.C. Frazer, 1955

A. Statement of Hypothesis

Even a superficial inspection of Fig. 30 in the previous Section reveals a striking correlation between the solubility of cholesterol in different fats and oils on the one hand and the serum cholesterol concentration produced by the same fats and oils on the other. This correlation would seem to be in conformity with the mechanism of cholesterol absorption as postulated in Section 19. Whether this correlation is, in fact, a consequence of the above postulate, if true, or due to some other related mechanism cannot be settled at present, but the following hypothesis can nevertheless be stated tentatively at this stage -

The property of different dietary fats which determines their effect on the serum cholesterol may be their different capacity to dissolve cholesterol.

If this statement can be demonstrated to be true, not only would the problem set in Section 1 be solved, but at the same time this solution would suggest a mechanism of action which is both simple and reasonable. In the

remainder of this thesis all efforts will therefore be directed towards a critical examination of the validity of the above hypothesis. Is the solubility concept a reasonable one, or do its implications clash with accepted facts of cholesterol metabolism?

B. Consequences of the Solubility Hypothesis

Any hypothesis or theory, if valid, should not only explain known facts but predict new ones. A number of both categories are listed below.

- I. All dietary fats and oils have a hyper-cholesterolaemic effect relative to a completely fat-free diet. It follows that the apparent lowering effect of some oils is of a relative nature only. This has been found to be the case in Experiment 14, Section 18, where a typical cholesterol "lowering" fat like Sunflower seed oil produced higher serum cholesterol levels than a fat-free diet. The same has yet to be shown for fish oil.
- II. The lowest and the highest levels producible by fats can be defined. The lowest level is that due to a fat-free diet. On the other hand, there is an amount of dietary cholesterol above which no further absorption should take place when administered with a given quantity of fat. This amount is reached when the dietary fat in its passage through the intestine is saturated by cholesterol. That this prediction is true will be shown in Experiment 15 described in the next Section.

The above argument can also be reversed as follows: there is an amount of dietary fat above which no further absorption of cholesterol takes place when administered with a given quantity of (endogenous) cholesterol. This amount is reached when the supply of the latter is exhausted. Whether this situation is likely to arise in the rat is unknown, since the total endogenous cholesterol supply is unknown.

- III. Dietary cholesterol is not easily absorbed when given in crystalline form, i.e. out of its natural habitat, even when fat is fed at the same time, provided the latter is kept out of contact with the cholesterol as far as possible. This is illustrated by Experiment 14 (a), Section 18.
- IV. Relatively small amounts of dietary cholesterol even if given in dissolved form in fats exert a perceptible influence on the serum cholesterol level only when these amounts form an appreciable proportion of the total cholesterol pool (i.e. dietary and endogenous). This is illustrated by Experiment 4, Section 9; Experiment 13, Section 17; Experiment 14, Section 18; and Experiment 15, Section 22. The negligible effect of 1% of cholesterol in dietary fat as compared with the considerable effect of larger quantities is consistent throughout these experiments. The controversy whether or not dietary cholesterol influences the serum cholesterol is apparently resolved.
- V. The rate of absorption of cholesterol is also a function of the solubility in the accompanying fat. No data are available on rats but a human experiment (Experiment 16, Section 23) indicates that this may be so in the case of man.

- VI. The controversy of " 'sap.' versus 'unsap.' " is resolved, both chemical fractions of the fats being necessarily implicated in the regulation of the serum cholesterol level. It is seen for example that the reduced serum cholesterol level produced by dissolving constituents of Sunflower seed oil "unsap." in coconut fat (Experiment 12, Section 14C) is reflected by a reduced solubility of cholesterol in this preparation (cf. Fig. 30, Section 20). These dissolved substances apparently have the property of partially blocking the solubility of cholesterol. In other words, they may compete with cholesterol for the solvent power of fats. It is for example known to students of the Phase Rule that the solubility of one substance in another can be materially altered by the presence of comparatively small amounts of a third compound. This phenomenon has in fact been used for the detection of impurities (Bowden, 1945).
- VII. Although related to the above, the question of the serum cholesterol reducing property of β -sitosterol merits separate treatment. Pollak (1953) suggested as a mechanism of action of β -sitosterol the formation of "mixed crystals" with cholesterol in the gastro-intestinal tract. This relatively insoluble association complex is thought to be responsible for the impaired absorption of exogenous and endogenous cholesterol. This "mixed crystal" hypothesis is not incompatible with the solubility concept although the latter can dispense with it.
- The negligible solubility of cholesterol at 37°C in coconut oil which had previously been saturated with sitosterol at that temperature (see Section 20) would lead one to expect this modified oil to have

very low hypercholesterolaemic properties. Whether this is actually the case with the natural experimental conditions used in the present rat tests is unknown but is being studied.

VIII. The extraordinary cholesterogenic properties of dietary egg fats are explained in both their qualitative and quantitative aspects. In Experiment 13 it was shown that the cholesterol content of crude egg triglycerides is partly responsible for high serum cholesterols. Since the crude egg triglycerides contained 8.6% of cholesterol, i.e. more than required to saturate the pure triglycerides (Fig. 30 Section 20), the actual serum level observed is also the maximum producible under the conditions of the experiment; i.e. even higher quantities of cholesterol in egg lipids would not result in yet higher serum cholesterols. Provided then, that there is cholesterol present in the diet in excess of that soluble in accompanying fat, the precise theoretical maximum level attainable is determined by the solubility as discussed under II above.

It is thus conceivable, or indeed a necessary conclusion that eggs of other animals containing more cholesterol than is present in hens' eggs but at the same time also fats of lower solvent power would have lower cholesterogenic properties than hens' eggs. This has, in fact, been shown in the case of penguin eggs in human experiments. (Bronte-Stewart, Wells and Wilkens, 1960). The triglycerides from these eggs contain up to 18% of cholesterol, yet produce only a moderate rise in human serum cholesterol. The correctness of the present explanation has yet to be tested by

a determination of the solubility of cholesterol in penguin egg triglycerides which have been freed from all unsaponifiable matter.

- IX. The faecal excretion of cholesterol is likely to be higher on diets of low solubility oils than on high solubility oils, that is in inverse ratio to the ease of absorption of cholesterol. This could conceivably be tested by experimentation with germ free rats. The faecal excretion of cholesterol will be more fully discussed in Section 26.

C. Conclusion

Up to the present the solubility hypothesis has proved to be extremely fertile in that it is capable of producing reasonable explanations of many facts which hitherto appeared entirely unconnected. It is also capable of predicting new facts. Some of these predictions have been tested and will be described below. The relevant literature on the cholesterol metabolism of rats will be discussed together with that of man in Section 26 when these new concepts will be discussed in relation to the cholesterol metabolism as a whole. Although offending against the general plan of exposition, this joint discussion of rat and man has been done to avoid tiresome and unnecessary repetition.

SECTION 22THE SOLUBILITY OF CHOLESTEROL IN SUNFLOWER SEED OILIN VIVO Experiment - (Experiment 15)

In Section 21B it was predicted that there is a quantity of dietary cholesterol above which no further absorption should take place, when administered with a given quantity of fat. This maximum amount should be related to its solubility in the accompanying fat. In other words it should be possible to determine the approximate solubility of cholesterol in fat in an in vivo experiment. Is this so? There is here an excellent opportunity to test the validity or otherwise of the solubility hypothesis.

Experimental

A standard colony of 24 rats was divided into six dietary groups of four rats each. All groups were offered ad. lib. the standard experimental diet containing 20 per cent of sunflower seed oil. In addition to the above the different groups received supplements of cholesterol of 0, 2, 4, 6 and 15 per cent of the fat fed, i.e. from 0 to 3 per cent in the total diet. As far as this was possible, the sterol was dissolved at 150°C in the sunflower seed oil before the latter was incorporated in the remainder of the fat-free rations. A single set (in duplicate) of serum cholesterol determinations were made at the end of 12 days on the special diets.

Results and Discussion

Due to the small number of animals within a group (four) the results are presented without statistical treatment. All individual serum cholesterol values are therefore recorded both at the beginning and at the end of the experiment. As on previous occasions, the details given in the table (Table 20) are summarised in graphical form (Fig. 31).

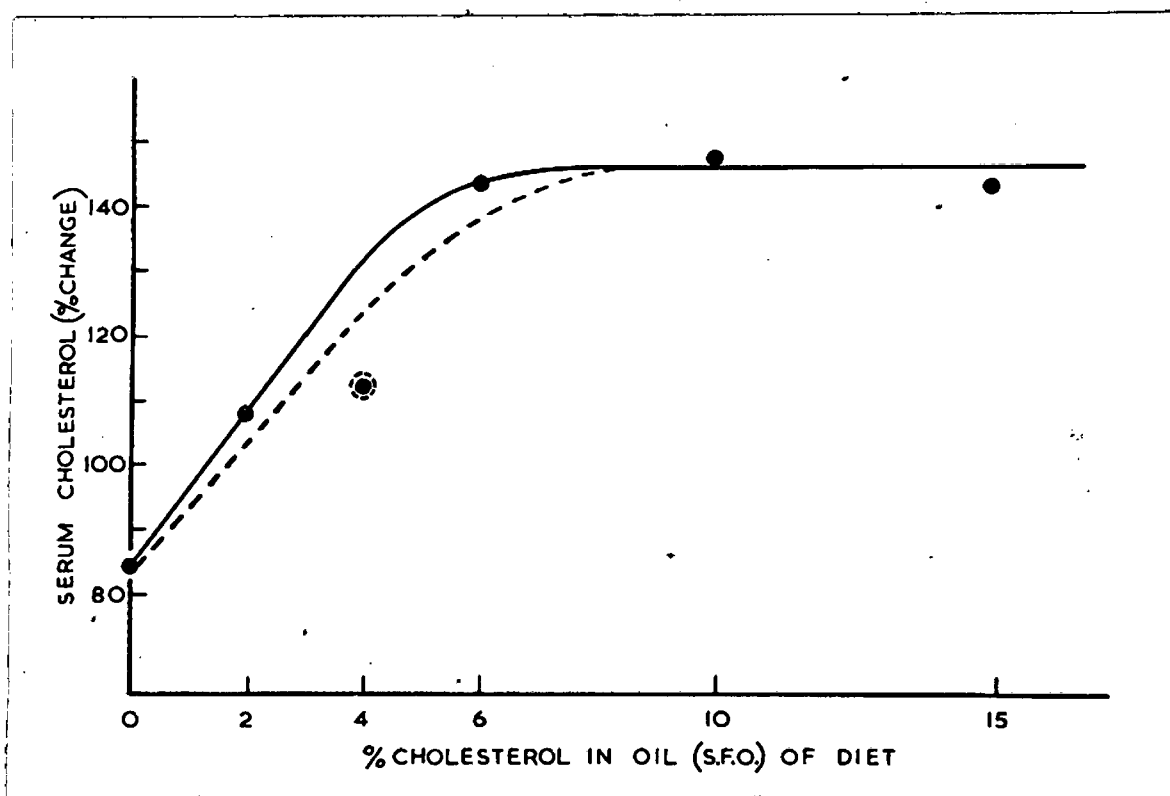


Fig. 31 The effect on the serum cholesterol of increasing quantities of dietary cholesterol, fed dissolved in 20% of sunflower seed oil.
 abscissa = per cent cholesterol in oil
 (Experiment 15)

With a single exception (4% group) the means of all serum cholesterol values lie remarkably close to a smooth curve

TABLE 20 - (Experiment 15)

SERUM CHOLESTEROL CONCENTRATIONS IN MG./100 ML OF RATS ON LABORATORY CHOW (A) AND AFTER 12 DAYS ON EXPERIMENTAL S.S.O. DIETS (B) CONTAINING INCREASING AMOUNTS OF DIETARY CHOLESTEROL IN 20 PERCENT SUNFLOWER SEED OIL

RAT NO.	PER CENT CHOLESTEROL IN S.S.O. OF DIET	SERUM CHOLESTEROL			
		A	B	PERCENTAGE CHANGE ($\frac{B}{A} \times 100$)	
				MEAN	
1	0	47	37	78.7	81.3
2		67	54	80.6	
3		54	49	90.7	
4		61	46	75.4	
5	2	49	54	110.2	107.0
6		45	48	108.9	
7		66	72	109.1	
8		50	50	100.0	
9	4	56	64	114.3	112.6
10		62	67	108.0	
11		50	56	112.0	
12		46	53	115.2	
13	6	51	63	123.5	143.1
14		69	117	169.5	
15		59	58	98.3	
16		48	87	181.2	
17	10	38	54	142.1	147.0
18		44	65	147.7	
19		46	76	165.2	
20		66	88	133.3	
21	15	52	—*	—*	143.0
22		53	73	137.7	
23		49	73	149.0	
24		59	84	142.3	

* Sample spoilt

which at first rises steeply but begins to "plateau" at between four and six per cent of cholesterol in the sunflower seed oil of the diets.

This simple experiment has confirmed three predictions of both a qualitative and a quantitative nature.

1. The phenomenon of a plateau.
2. The height of the plateau. This is at essentially the same level as that obtained with 10% of cholesterol in sunflower seed oil in Experiments 14a and 14b after 12 days' feeding.
3. The approximate level of dietary cholesterol at which the plateau forms. The slight discrepancy (3.5% predicted, 4 - 6% found) need cause no concern. This may have been due to incomplete saturation at marginal concentrations, to additional solubilising powers of bile acids or to experimental error. Whatever other absorption mechanisms are at play appear to play a comparatively minor role only.

Conclusions from Part V

The ability to plan and predict the outcome of an experiment under conditions as unfamiliar as those obtaining at the present one, has served to give considerable strength to the views expressed in Part V of this thesis. The outlines of a true understanding of the central problem of this investigation are beginning to appear at last. For this reason the final task of the application of the experience gained from animals to man can be attacked with confidence. This will be attempted in the last part (Part VI) of this thesis. Without the establishment of this final link, the ultimate value of the present labours would be severely limited indeed.

PART VI

APPLICATION TO MAN

SECTION 23THE ABSORPTION OF DIETARY CHOLESTEROL BY MAN(Experiment 16)

"No lipid parameter is measured more frequently or discussed as endlessly as is serum cholesterol."

David Kritchevsky,
1959

Introduction

The greatest obstacle to the application of the solubility hypothesis to man is the widely held belief that dietary cholesterol has little or no influence on the human cholesterol level. If this is so, it would be difficult to explain why endogenous cholesterol should form an exception to this rule. Beveridge and his associates (1959) have recently summed up the history of the subject by the remark that "...a review of the literature would lead one to the conclusion that this sterol in amounts up to several times that of the usual daily intake has no demonstrable effect on the plasma lipid levels in man."

If the above conclusion is true, it would also have to be accepted that man differs from all hitherto tested mammals in his reaction to dietary cholesterol. In the absence of any special reasons for occupying this unique position, man's implied immunity to dietary cholesterol must

be regarded with some scepticism.

With the experience gained and the views expressed in these pages as background, it is not difficult to venture a guess as to a possible reason for the failure to detect a positive relationship between dietary and serum cholesterol in man.

The reason may be that in most reported experiments the sterol was fed under conditions which made it difficult or impossible for this substance to dissolve in dietary fat. In the following simple human experiment cholesterol was fed in its natural habitat, i.e. dissolved in fat.

Experimental

The author was fortunate in having been able to make use of an existing well organised experimental machinery for human nutritional studies in the Department of Medicine. This has been established for lipid studies by Dr. B. Bronte-Stewart and his associates in the course of their original studies (1956) on the effect of different dietary fats on the serum cholesterol level (see Sections 1 and 2).

The subject for the present experiment was a healthy male Bantu 35 years of age. During the experiment he lived in a metabolism ward and received a basal diet low in fat content (8 - 12 gm. daily). With minor variations, the basal diet consisted of maize meal, pumpkin, Casilan, bread and sugar and except as indicated below was kept constant with respect to calories (2500 daily) and protein (88 gm. daily).

During the experiment the volunteer slowly gained weight (113 lbs to 119 lbs) and suffered no digestive disorders.

Lipid supplements

The lipid supplements fed in nine successive dietary periods were as follows:

1. No supplements
2. Lipids present in 10 egg yolks fed as whole yolks.
3. Equivalent amount of lipids in the form of 3 gm. cholesterol dissolved at 150°C in 60 gm. hydrogenated sunflower seed oil (I.V. 100).
4. 60 gms. sunflower seed oil (100 I.V.), no cholesterol.
5. As for 3 above
6. 3 gm. crystalline cholesterol without fat, sprinkled over food.
7. No supplement.
8. As for 3 above.
9. No supplement.

Serum cholesterol concentrations were estimated on alternate days by the method of Abell et al. (1952).

Results and Discussion

The results reported in Table 20a and illustrated in Fig. 32 show that the high serum levels created by the ingestion of egg yolk were maintained by an equivalent amount of cholesterol given dissolved in fat. Withdrawal of the cholesterol while continuing the fat supplement resulted in a marked drop which was reversed after restoration of the sterol to the diet. The reverse procedure, i.e. the withdrawal

of the sunflower seed oil, while continuing the cholesterol supplement was accompanied by a most dramatic drop of the serum concentration to base level.

If the reaction of this single human subject to dietary cholesterol is a reflection of what happens in the population at large there seems to be no basis for the statement referred to in Section 16, namely that "In man the plasma cholesterol is largely independent of dietary cholesterol."

The present results are thus at variance with the views advanced by most workers in the field. Keys and associates (1956) for example have stated that "... in adult man the serum cholesterol level is essentially independent of the cholesterol intake over the whole range of human diets." Similar opinions have been expressed, among others, by Bronte-Stewart et al. (1956), Ahrens et al. (1957) and Gordon (1959).

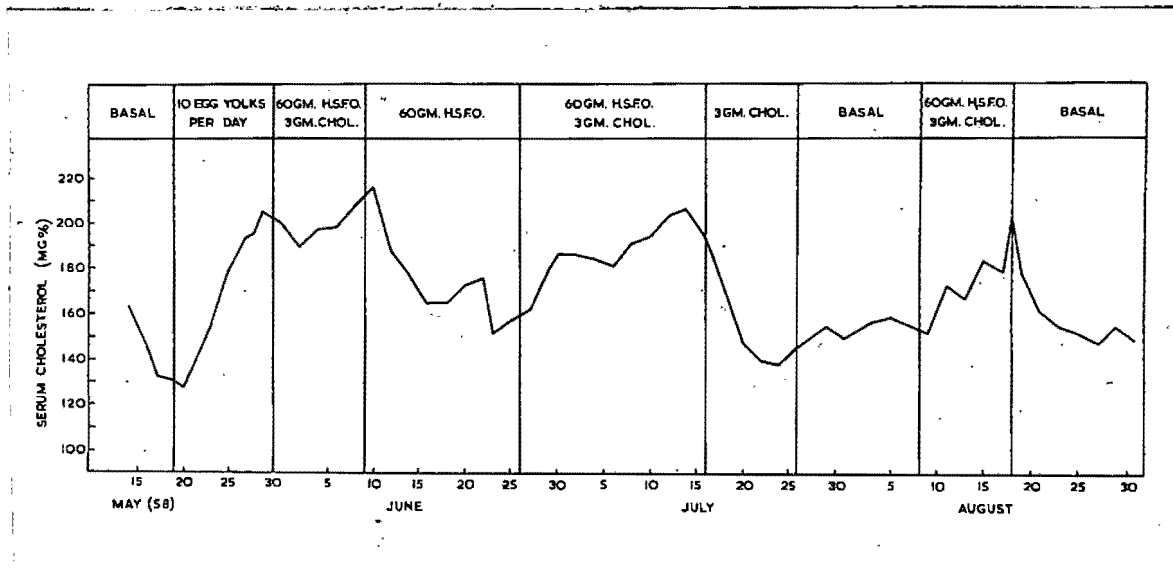
Table 20a/

TABLE 20a

THE EFFECT OF FEEDING CHOLESTEROL IN THE ABSENCE
AND PRESENCE OF HYDROGENATED SUNFLOWER SEED OIL
TO MALE BANTU AGED 35 (Experiment 16).

Dietary period	Duration of period (days)	Supplement (see text)	Serum cholesterol (mg./100 ml)	
			I*	II**
1	8	nil	136	130
2	12	Egg yolk	198	205
3	9	Chol.+fat	201	208
4	17	fat	161	157
5	20	Chol.+fat	201	194
6	10	Chol.	140	145
7	13	nil	156	154
8	10	Chol.+fat	188	202
9	12	nil	150	148

* Mean of last three readings of period
** Last reading of period.

Fig. 32

While the present studies were in progress a new approach to the problem was likewise pioneered by Beveridge and his group (1959a). They found that the hypercholesterolaemic properties of a volatile molecularly distilled fraction from butter was largely due to the cholesterol (3.83%) which had concentrated in this fraction. The cholesterol-poor fractions led to relatively small increases in serum cholesterol levels, but when supplemented with an amount of cholesterol equivalent to that provided by the cholesterol-rich fraction, increases of comparable magnitude were obtained. Their explanation for this effect is, however, less clear, for they suggest that the substance with which cholesterol reacts (why?) is a specific type of triglyceride having certain fatty acids attached at the alpha, beta and alpha₁ positions on the glycerol moiety.

In an even more recent abstract (1959b) these same workers reported the results of feeding increasing quantities of cholesterol (up to 1200 mg. per day), together with a cholesterol-poor butter fraction, to man. At dietary cholesterol levels of above 400 gm. per 950 calories, the serum concentration of the sterols plateaued. No explanation is offered by these authors for this phenomenon. Its significance is at once apparent if the results with

rats of Experiment 15, Section 22, are recalled.

It is concluded from this section that the response of man to dietary cholesterol does not differ materially from that of rats. When fed in its natural habitat this sterol is equally well absorbed by both species. The way has been cleared for an attempt to apply the solubility hypothesis to man.

SECTION 24APPLICATION OF THE SOLUBILITY HYPOTHESIS TO MANA. Effect of Natural Fats and Oils

In Section 9E it was claimed that under the conditions created and used in this work, the serum cholesterol of rats reacts similarly to that of humans to dietary fat treatment. In the previous section it was shown that dietary cholesterol, if dissolved in dietary fat, elevates human serum cholesterol. There is, therefore, no reason why the solubility concept should not apply to man. Is this so?

Fortunately, several groups of workers have produced lists of a variety of oils together with their quantitative effects on the serum cholesterol concentration of man. One of the most carefully conducted investigations of this nature is that due to Ahrens and associates who summarised their results in a diagram reproduced in Fig. 3 Section 2.

Using only oils for which cholesterol solubility figures have been determined to date and including Ahrens' data on menhaden oil (1959a), this diagram has here been re-arranged into a conventional graph. For this purpose groups of individual values for each oil were condensed to means, and a linear relationship was assumed. The

result is shown in Fig. 33, and illustrates the opinion expressed by Ahrens as recently as 1959 (b) that the degree of unsaturation is the property of oils which best correlates with serum cholesterol levels.

In a comparable graph, iodine values have been replaced by the solubilities of cholesterol in oils at 37°C (see Fig. 34). It is evident both by visual inspection and by comparison of the correlation coefficients, that the correlation of solubilities with serum cholesterol is superior when compared with that of iodine values.

In constructing the second graph it was assumed that the same oils of different origins have identical compositions. This, of course, is not true especially in the case of beef tallow and fish oil. In the latter case, menhaden oil has actually been replaced by the body oil of the related South African pilchard (*sardina ocellata*). If, in addition, the unavoidable lack of precision of the serum cholesterol data is taken into account, an actual perfect correlation, if it exists, is not incompatible with these experimental data.

The "abnormally" low serum cholesterol levels obtained with corn oil (see Armstrong et al., 1957; also prediction equation of Keys, Anderson and Grande, 1958) deserve special mention. This oil, according to the data supplied by Ahrens, actually produces slightly higher values than here predicted from solubility values. These and

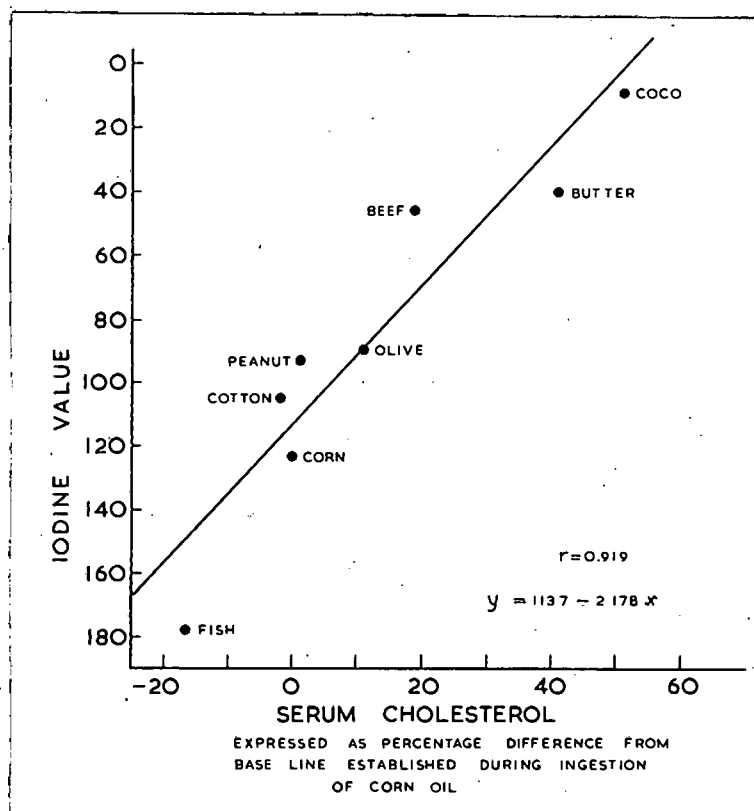


Fig. 33. Relationship between iodine value and serum cholesterol concentration (adapted from Ahrens et al., 1957).

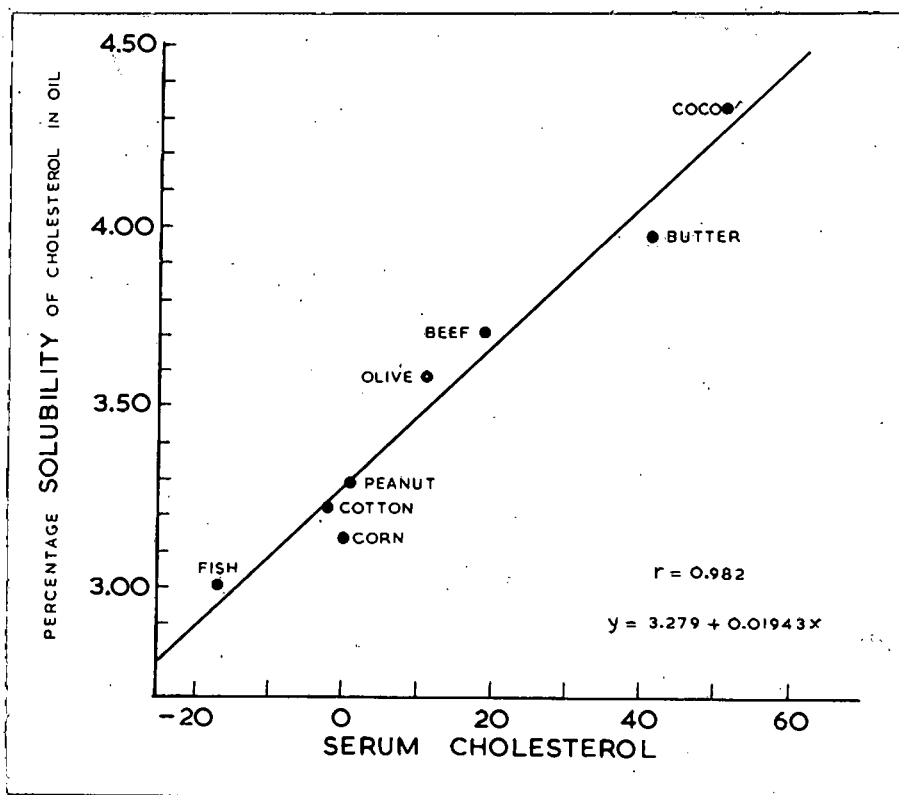


Fig. 34. Relationship between solubility of cholesterol in oil at 37°C and serum cholesterol concentration.

other minor variations are, however, easily within range of experimental error. Whatever the shortcomings or the underlying mechanisms of this correlation are, the degree of correlation ($r = 0.982$) is too high for this relationship to be due to chance.

B. In vivo solubility determination in Man

An experiment resembling that described in Section 22 has been reported by Karvinen and co-workers on man. In balance experiments on 16 subjects, the apparent absorption of cholesterol was studied when 1, 3, 6 and 9 gm. of cholesterol was added in the diet mixed with 37 and 50 gm. of a mixture of animal and vegetable fats. These workers summarized their results in a diagram which is reproduced unchanged in Fig. 35. They interpret these data as "demonstrating that the human intestine has a limited capacity for absorption of dietary cholesterol". The apparent fall in absorption on ingestion of 9 gm. of cholesterol daily they attribute to an "artefact due to experimental error".

Fig. 35/

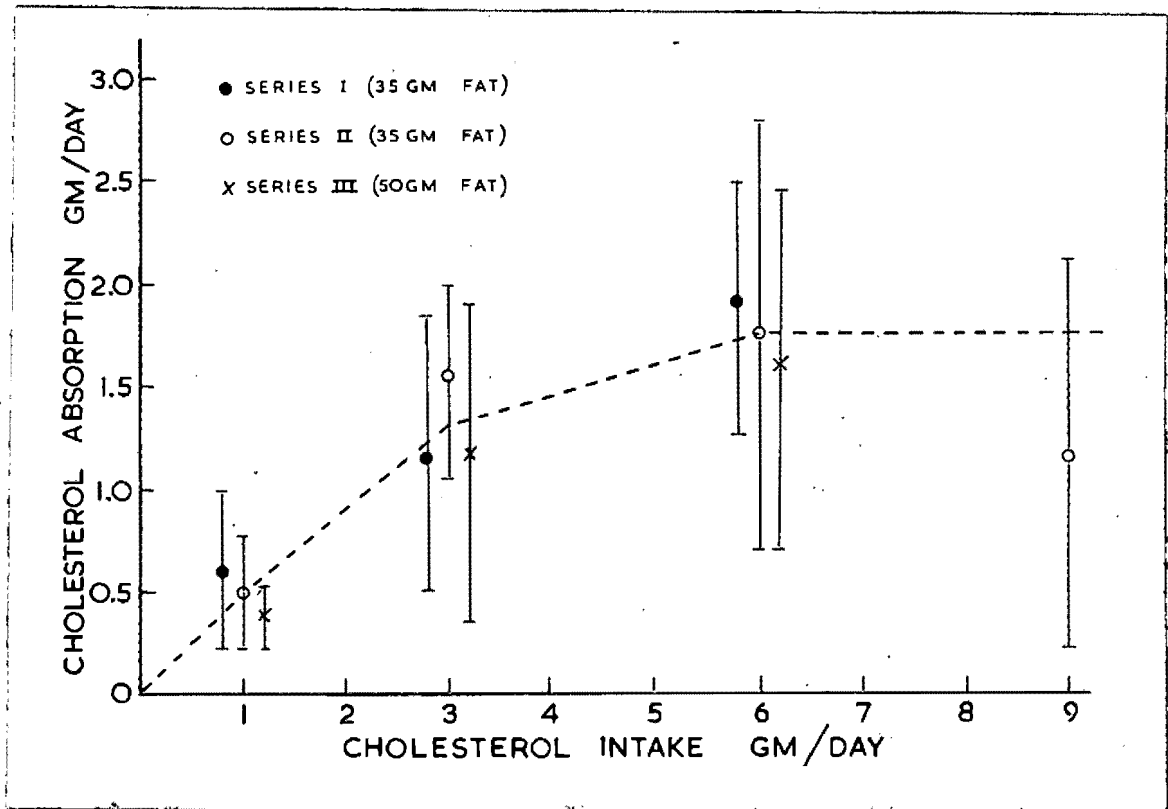


Fig. 35: Capacity of the human intestine for the absorption of dietary cholesterol.

(Reproduced from Karvinen et al., 1957)

These same results are here interpreted as demonstrating that amounts of dietary cholesterol, in excess of that soluble in accompanying fat, are not absorbed. On the assumption that the absorbed cholesterol was previously present in saturated solution in fat, the data supplied by Karvinen can be used to calculate the mean solubility of cholesterol in fat to be close to 4 gm./100 gm. This is in close agreement with the probable

solubility value of the animal - vegetable oil mixture used by Karvinen, somewhere between 3.5 and 4 percent.

C. The serum cholesterol raising properties of egg lipids

The results of Experiment 16 described in the previous Section are now interpreted as follows. The steep rise in serum cholesterol on feeding egg yolk lipids is due to the excessive amount of cholesterol dissolved in these lipids. The same amount of cholesterol dissolved in hydrogenated Sunflower seed oil maintains this rise. On subsequent withdrawal of the dietary dose of cholesterol one observes a fall to a level which is, however, still above the basal line owing to the comparatively smaller amount of endogenous cholesterol, which is now absorbed along with the fat. The amount of endogenous sterol from both bile and intestinal juice available for re-absorption has been estimated by Karvinen et al. (1957) to be about 1 gm. daily. Resuming the cholesterol diet with the Sunflower seed oil again results in an elevation of cholesterol which on this occasion however, rises but slowly due to the lower solubility of cholesterol in Sunflower seed oil as compared with that in egg triglycerides. When next the fat is withdrawn but the cholesterol continued, neither dietary nor endogenous cholesterol can any longer be absorbed and the concentration promptly falls to basal level. The "enigma" (Gordon et al., 1958) of the cholesterologenic property of eggs is apparently solved.

D. The effect of β -sitosterol

It is significant that appreciable cholesterol depressing effects with sitosterol have been obtained in man only with very large doses of this substance. Thus Farquhar and Sokolow (1957 and 1958) fed as much as 18 gm. per day to human subjects to produce only moderate falls in serum cholesterol levels. Up to 45 gm. daily was fed by Best and co-workers (1954) to humans to produce maximum effects, while Beveridge et al., (1957a) fed similarly high dosages (7 gm.) of β -sitosterol in their earlier experiments. In later studies the same workers (1957b and 1958) recorded some success with more natural doses when the sterol was given dissolved in oil.

One reason for this may be that this sterol was fed in crystalline form, i.e. out of its 'natural habitat'. This reason, in other words, may be analogous to that advanced in Section 23 for the alleged lack of effect of dietary cholesterol. The solubility hypothesis predicts minimal serum cholesterol with oils saturated with sitosterol. Experimental verification would be complicated by the fact that sitosterol is absorbed to the extent of about 10% by rats, as has been demonstrated by Gould (1955) with tritium labelled β -sitosterol. A figure of 4% or more has been quoted for man by the same author one year later. Conventional methods for the determination of serum cholesterol can not be applied since these methods are not specific for cholesterol,

but in addition measure most unsaturated sterols including sitosterol. This fact has been overlooked by many workers, including the writer.

E. The effect of hydrogenated oils

The equal solubilities of cholesterol in natural and hydrogenated (I.V. = 100) Sunflower seed oil (see Fig. 30, Section 20) is difficult to explain on the basis of the reports by Bronte-Stewart et al. (1956) and Ahrens et al. (1957). These workers found that the hydrogenation of unsaturated oils leads to higher serum cholesterol levels than those noted with the natural oils. It has been suggested that this effect may in part be due to the production of isomeric trans-acids during the hydrogenation process.

There is no unanimity of opinion on this point. Thus Beveridge et al. (1958a and 1958b) report equal effects with natural and hydrogenated (I.V. 69) corn oils, while Gordon et al. (1958) states that they have "frequently observed that certain.....saturated fats, such as hydrogenated Sunflower seed oil (I.V. 74) do not raise the serum cholesterol". Ahrens et al. (1957) also report a case in which a hydrogenated corn oil preparation had just as great a hypocholesterolaemic effect as the natural oil. The problem of the effect of the hydrogenation of oils can thus be regarded as still unsolved. The determination of solubility values of oils actually used in successful human experiments has yet to be done.

F. Effect of oils relative to a fat free diet

If the differential absorption of cholesterol from the intestine with different oils is the mechanism responsible for the solubility - serum cholesterol relationship it would appear that the lowest possible serum cholesterol obtainable by manipulation with dietary fats is to feed no fats at all. This seems to be the case with rats (Experiment 14, Section 18). An increase in serum cholesterol of man relative to a fat free diet with so-called serum cholesterol "depressing" oils has been reported by a number of investigators notably Keys (1950 and 1952), Hildreth et al. (1951) and Meyer and associates (1954). As has been suggested by Sinclair (1958) these works belong to the

".....Proto-Keysian period
When all fats in equal measure
Raised cholesterol in serum
Butter, sardines, walrus liver,..
....."

Some recent evidence from human experience points in the opposite direction, i.e. a hypocholesterolaemia is observed following the addition of some oils to a diet low in fat (Bronte-Stewart et al., 1956). This is in apparent contradiction to the solubility concept in its present form. It will be remembered, however, that the absorption of sterols from the intestine is obviously not the only mechanism whereby cholesterol gets into the blood. That fluctuations in hepatic synthesis can, for example, be induced by changes

in the rate of absorption of cholesterol from the intestine will be discussed in the next sections. A further possible explanation of the depression-below-fat-free phenomenon will also be offered which invokes the solubility concept.

G. The rate of change in serum concentration

The difference in solubility of cholesterol in various oils seems at first sight to be too small to account for the very different effects that these oils can have on the serum cholesterol level. The following simple calculation will show, however, that this may not be so.

Supposing a human subject consuming 50 gm. Sunflower seed oil daily has a steady serum cholesterol concentration of 200 mg./100 ml. At what rate will his serum cholesterol rise on changing from Sunflower seed oil to the same quantity of coconut oil in the diet?

The following additional data are given:

Total serum volume	3,000 ml
∴ Total circulating cholesterol	6 gm.
Endogenous cholesterol available for re-absorption (Karvinen et al., 1957)	1 gm./day
Solubility of cholesterol:	
in Sunflower seed oil (actual 3.46)	3%
in Coconut oil (actual 4.32)	4%

Assuming arbitrarily that the endogenous cholesterol is re-absorbed by coming in contact with, and saturating half the dietary fat (i.e. 25 gm. fat) in the intestine, one

can say:

Rate of absorption:

during the ingestion of S.S.O. oil $\frac{1}{4}$ gm./day

during the ingestion of coconut oil $\frac{4}{4}$ gm./day

Therefore, extra absorption on changing
from sunflower seed oil to coconut oil $\frac{1}{4}$ gm./day

Other factors remaining constant, this is equivalent to a daily increase in total circulating cholesterol of $\frac{0.25}{6.0} \times 100 = 4.2\%$ which, in the present example, corresponds to an approximate rise of 60 mg./100 ml per week. This is well within the range of observed fact. (see e.g. Gordon et al., 1957). This serum cholesterol enriching process will continue until the regulating mechanism of the liver presumably stops it by decreased synthesis.

Needless to say, the above very approximate calculation is rather arbitrary. It is merely intended to illustrate that small differences in solubility could conceivably be responsible for quantitative effects of the same order of magnitude as found by practical experience.

Similar calculations could be invented to account for the different rates of increase of serum concentration when cholesterol is fed together with egg fat (comparatively high solubility) or sunflower seed oil (comparatively low solubility) as was found in Experiment 16.

H. Discussion

The concept of the re-absorption of endogenous cholesterol from the intestinal tract is by no means a new one. The related entero-hepatic circulation of bile acids is indeed described in most textbooks on physiology. As far as is known, Bloor (1943) was the first to apply this principle to cholesterol. He pointed out that the part played by the bile in supplying cholesterol to the blood has generally been overlooked, and the cholesterol of the bile, re-absorbed during fat absorption, may be sufficient to explain the increase in blood cholesterol after feeding fat. The essential role of fat in the absorption of cholesterol has already been discussed in Section 23.

In 1949 Gubner and Ungerleider refer to biliary cholesterol as but "one phase in the physiologic circulation of cholesterol (intestine — blood — liver — intestine) in its role as an adjunct in fat transport..." Two years later Hildreth, Mellinkoff, Blair and Hildreth (1951) observed an increase in serum cholesterol in three human subjects after vegetable fat feeding and suggested the same mechanism as an explanation for these findings. "Its (biliary cholesterol) re-absorption, together with the absorption of ingested cholesterol, appears to depend upon the simultaneous absorption of fatty acids. It may well be that this fatty acid — cholesterol absorption relationship... shares in the regulation of serum cholesterol concentration".

A case has been made for intestinal absorption processes being the mechanism underlying the solubility - serum cholesterol level correlation. There is, however, no reason to believe why similar principles should not be operative in the body wherever fat and cholesterol is transported across a membrane from one tissue to another, such as from the blood stream to the liver cells. The alteration of the partition coefficient of cholesterol between two lipid phases or lipid and aqueous phases due to a change in the lipid composition at either side of an interface, is indeed a phenomenon which might explain the movement, deposition or removal of this sterol at different sites of the body by simple physical concepts. This view is strengthened by recent work of Gould, Jones and Wisaler (1959). They employed the injection of titium-labelled cholesterol to demonstrate the equilibration of blood cholesterol with various tissues in the human body, including atherosclerotic lesions.

SECTION 25THE RATE OF DIFFUSION OF CHOLESTEROL
FROM SERUM TO DIFFERENT FATS.A. The intravenous injection of fat

There is an interesting experimental fact which requires elucidation and that is the successful depression of the serum cholesterol level in man by the intravenous injection of an emulsion of cottonseed oil by Gordon (1957). An example of such an experiment is illustrated in Fig. 36.

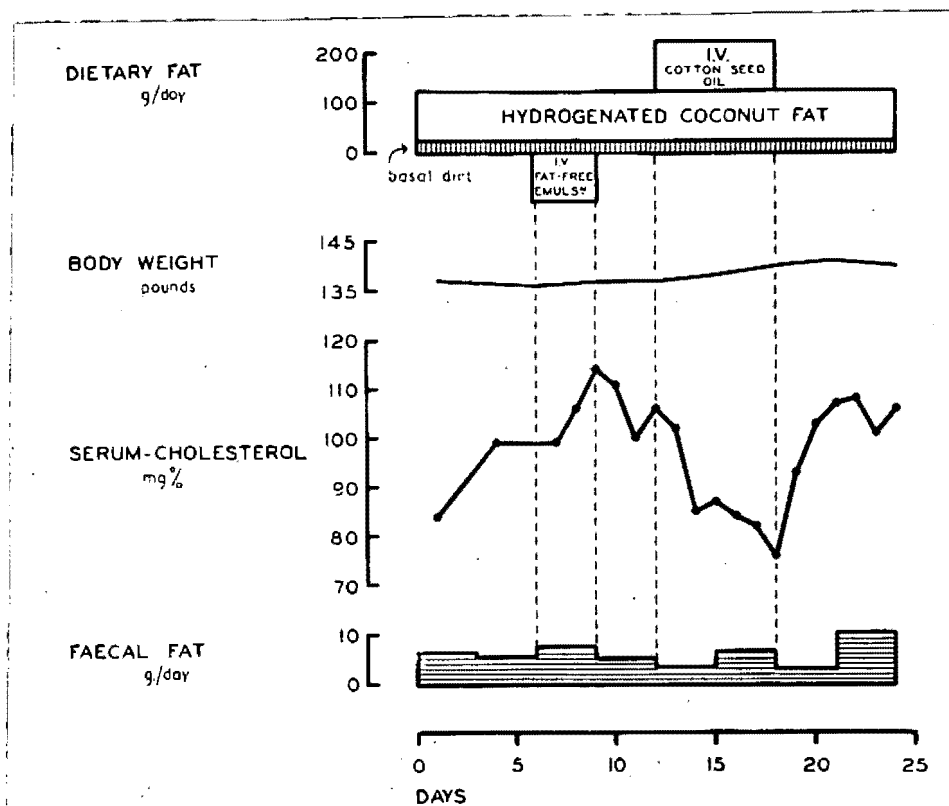


Fig. 36: Effect on the serum cholesterol of the intravenous injection of an emulsion of cottonseed oil (Gordon, 1957).

Since the injection of fat directly into the bloodstream obviously by-passes the normal intestinal absorption process the resulting depression of the serum cholesterol appears to be in direct contradiction to the views advanced in these pages. To find a loophole for escape from this disturbing situation is the object of the last experiment.

B. The diffusion of cholesterol from serum to different fats. (Experiment 17)

The experimental procedure was as follows:

To 10 ml of human serum contained in a 6" x 1" boiling tube is added 10 ml of the triglyceride oil. The securely stoppered tube is shaken vigorously. The resulting fine emulsion is then incubated at 37°C in a constant temperature bath for two hours. During this time the tube is shaken periodically by hand whenever the emulsion threatens to break up. Immediately after removal from the bath the emulsion is transferred to a centrifuge tube and spun at high speed (7,000 r.p.m.) for 30 minutes. Samples are then carefully withdrawn from the upper oily layer and the serum layer below for cholesterol determinations.

The three oils used here, namely coconut oil, olive oil and fish oil have been chosen from the centre and both extreme ends of the solubility scale obtained in Section 20.

The results of this pilot experiment are summarised in Table 21 and show clearly that some cholesterol has been

TABLE 21
(Experiment 17)

Diffusion of cholesterol from serum to triglyceride oils

<u>Coconut oil</u>	Before contact	After contact	Difference	Average Transfer
tube 1 (O)	84	98	+14	17.7
(S)	256	237	-19	
tube 2 (O)	84	100	+16	
(S)	256	234	-22	
<u>Olive oil</u>				12.5
tube 1 (O)	181	193	+12	
(S)	256	241	-15	
tube 2 (O)	181	193	+12	
(S)	256	245	-11	
<u>Fish oil</u>				10.5
tube 1 (O)	533	544	+11	
(S)	256	244	-12	
tube 2 (O)	533	547	+14	
(S)	256	251	- 4	

Cholesterol concentration in mg./100 ml (FeCl_3 - colour) in oil (O) and serum (S) before and after intimate contact of equal volumes for 2 hours at 37°C .

transferred from the serum phase to the oil phase, presumably by diffusion. They also show that a high solubility oil (coconut oil) absorbs more cholesterol than a low solubility oil under the same conditions, with the olive oil somewhere in between. Under the conditions of this experiment the amount of transfer of cholesterol is admittedly small, but can be more than doubled by extending the time of contact from two to four hours as has been shown in another pilot experiment with Sunflower seed oil. The optimum conditions for the rate of diffusion are at present under study. Efforts are also made to improve the precision of the method.

C. Discussion

The above very simple experiment shows that triglyceride oils can be used to "extract" cholesterol from serum in much the same way as conventional fat solvents like diethyl ether. Moreover, it shows that coconut oil is a better solvent than, for example, fish oil. The rate and extent of transfer or diffusion of cholesterol from the serum to the triglyceride droplets, can be visualised as depending on the time of contact, the surface area of contact, the concentration in serum and oil, and the partition coefficient of cholesterol between these two phases; in other words, on the relative solubility of this sterol in the two phases. The precise state in which cholesterol is present in the serum is relevant but will not be discussed further than remembering that it is slightly soluble in the aqueous layer.

It is conceivable that the mechanism by which intravenously injected cottonseed oil lowers the serum cholesterol concentration is based on a similar principle. The injected oil merely dissolves and extracts the sterol from the serum and possibly other sites and transports it to the liver or some other part of the body. It may behave like chylomicrons in this respect. Thus, the successful in vivo removal of aortic atheroma of rabbits by repeated infusions of phospholipid by Beyers (1958) is here interpreted as a simple "extraction" process.

Frazer (1955) is of the opinion that the "....eventual partition of the lipid molecules between the oil and water phase may affect their distribution in the body after absorption." On this view, intravenously injected coconut oil with its higher solvent capacity for cholesterol, would be expected to be an even more efficient hypocholesterolaemic agent than cottonseed oil. These phenomena could be pictured as resulting from another manifestation of the solubility theory "in reverse" in the blood stream.

The mechanism discussed above might be capable of throwing light on yet another hitherto unexplained experimental fact. This is the lowering of the serum cholesterol of human subjects already consuming 100 gm. of a high solubility fat such as dripping or hydrogenated coconut fat by the further addition of 100 gm. of sunflower seed oil daily to their diet (Bronte-Stewart et al., 1956; Gordon et al., 1957).

What happens in this case may be as follows: -

The first 100 gm. of coconut fat in the diet is sufficient to cause the re-absorption of all available endogenous cholesterol from the intestine. Extra dietary fat, in excess of this amount will then be absorbed into the blood stream free from cholesterol and therefore with unimpaired solvent capacity. These triglycerides therefore behave like intravenously injected oil, thus reversing the process and lowering the serum cholesterol again. It would be interesting to repeat the experiments referred to above using coconut fat instead of Sunflower seed as the second additional dietary supplement. An even greater reversing effect might thus be obtained. No records of carefully conducted studies could, in fact, be found of the relationship between varying amounts of the same fat in the diet and the serum cholesterol level.

SECTION 26CHOLESTEROL METABOLISM AS AFFECTED BY
DIFFERENT DIETARY FATSA. Circulation of cholesterol

The concept of the differential absorption of endogenous cholesterol due to different dietary fats must remain idle speculation if it cannot be shown to dovetail into wider established aspects of cholesterol metabolism. This will be attempted by describing in more detail the circulation of cholesterol (intestine - blood - liver - bile - intestine) as outlined in Section 24. In many instances, already trodden ground is thus re-covered. This is worthwhile if it reveals new and wider relationships.

Two diagrams are shown, which depict the state of affairs when either high or low solubility oils are fed. These are represented respectively by coconut and corn oil. Absorption through the capillaries into the portal circulation has been omitted from the diagram. Neither neutral fats (Frazer, 1955) nor cholesterol (Biggs, Friedman and Beyers, 1951) follow this route.

There is good evidence to assume that dietary cholesterol-free fats are able to influence the serum cholesterol for an indefinite period of time in rats (Experiment 3, Section 9) and man (Gordon and Brock, 1958). The metabolism of the fraction of cholesterol responsible for these effects, therefore, reaches a state of dynamic

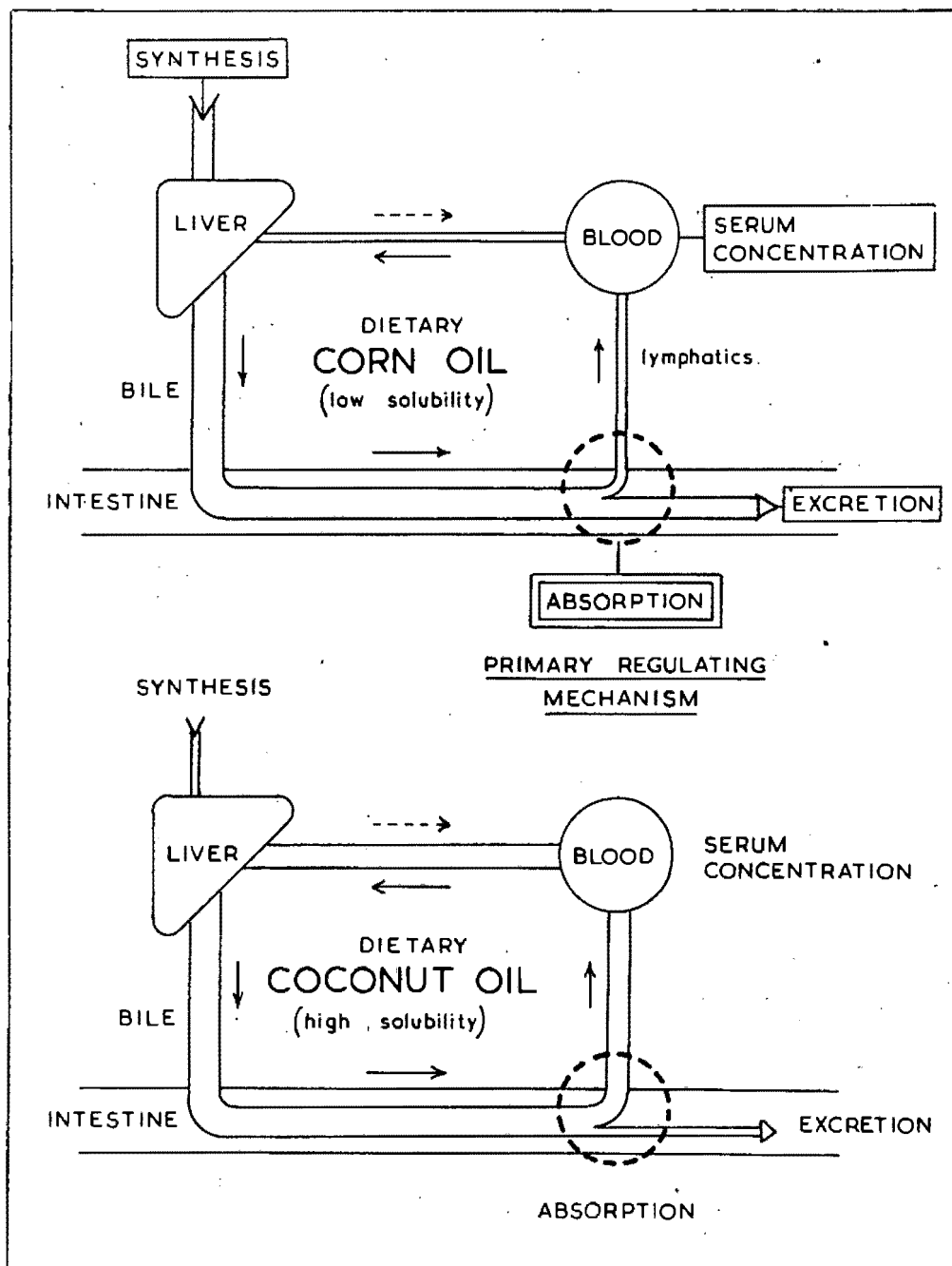


Fig. 37 The circulation of cholesterol.

equilibrium and the rate of synthesis must equal the rate of excretion of cholesterol and its end-products. The higher absorption rate of cholesterol due to coconut fat as compared with that due to corn oil implies a lower rate of excretion. This, in turn, is necessarily linked with an equally lower rate of synthesis. If this were not so, the body would in time be either completely drained of cholesterol or progressively enriched with this substance. Different dietary fats will therefore influence not only absorption and excretion but will also affect the rate of hepatic synthesis in a characteristic way. These three aspects will now be treated in more detail in relation to existing experimental evidence.

B. Intestinal absorption

Direct proof of differences in the rates of resorption of endogenous cholesterol due to saturated or unsaturated dietary fats would be difficult to obtain experimentally. "Absorption" as measured by a change in serum concentration may be misleading, as this change may be due to other causes. The determination of cholesterol in chylomicrons in chyle of rats fed with and without olive oil and cholesterol by Bragdon (1959) described in Section 18 might profitably be extended to other oils. A tracer method might conceivably be devised for this purpose. What amounts to indirect proof of differential absorption will be furnished under D. below.

Lin, Karvinen and Ivy (1955), working with rats, are responsible for the only report that could be found in the records of an attempt to ascribe differences of absorption of dietary cholesterol to differences of solubility of this sterol in dietary lipids. The following solubilities are recorded.

	% solubility of cholesterol	Temperature °C	% absorption
Oleic acid	22	38	74
Tallow	4.6	39	59
Corn oil	2.7	38	64
Triolein	2.7	39	49

Although oleic acid caused significantly higher absorption as compared with corn oil, it was not as different as the solubilities. For this reason the above investigators did not consider the solubility of cholesterol in fat an important factor.

According to Frazer (1955) fatty acids, as distinct from triglycerides, are absorbed in the aqueous phase, through the portal route. This mechanism is likely to be radically different. If this is so, the in vitro solubility of cholesterol in pure oleic acid has little bearing on the processes at play in capillary absorption, and the concentration in the systemic circulation is unlikely to be directly affected.

The similarity in behaviour of cholesterol and neutral fat during intestinal absorption impressed Frazer (1955) who suggested that cholesterol might be transported across

the intestinal wall in solution in fat. This view arises out of his partition hypothesis, according to which "complete hydrolysis of glycerides to fatty acids and glycerol is not an obligatory step in the process of fat absorption. Lipids do not have to be brought into water-soluble form before they can be removed from the small intestinal lumen".

Glover and Morton (1958) criticised Frazer's concept of transport by simple solution as being too uncomplicated since it does not account for the marked selectivity of the intestine in absorbing some sterols and rejecting others. These authors do not suggest, however, how such a selection mechanism could be visualised more easily as taking place in the aqueous phase.

The relative ease of esterification of cholesterol with different fatty acids or the relative absorbability of the esters has been suggested as being responsible for the different rates of absorption, e.g. Lin et al. (1955). This view cannot easily be dismissed, but it is not supported by recent work by Swell and associates (1959) who believe that cholesterol is absorbed in the free state. This agrees with the observations made by a group of investigators led by Ganguly (1959) who fed free cholesterol, its acetate and palmitate in a minimum quantity of oil to rats. From 60 to 90 minutes later, the same amount of cholesterol, predominantly in the free state, was found in all cases in the mucosal cells.

C. Excretion

The elimination of cholesterol from the body takes place as unchanged cholesterol, its bacterial transformation

products in the intestine such as coprosterol (coprostanol) and finally as bile acids.

Various authorities have assigned different importance to one or the other groups of compounds as catabolic end-products. These differences of opinion need be of no concern here, since emphasis is placed on variations in total end-products only. According to the mechanism outlined at the beginning of this Section, decreased absorption results directly in increased excretion of cholesterol (and probably its hydrogenation products). This is more or less obvious. It also leads to increased liver synthesis, however, If it is further assumed that the same ratio of the total cholesterol synthesized in the liver is at all times converted to bile acids, increased excretion of the latter substances results.

To summarize; dietary change from coconut to corn oil may be accompanied by increased excretion of cholesterol and its end-products and bile acids, and vice versa. Extrapolating from this argument, the highest excretion would be expected with a fat free diet.

Experimental evidence from rats is scanty and inconclusive. Lin, Karvinen and Ivy (1956) found that ".....tallow slightly decreased the endogenous elimination of cholesterol, whereas corn oil and triolein definitely increased it." In a later study (1957), these investigators unfortunately worked with free acids which probably follow a different absorption path. Wilson and Siperstein (1959a) injected rats consuming either corn oil or lard with cholesterol

-4-C¹⁴ and found no difference in excretion products. Nor did these workers (Wilson and Siperstein, 1959b) detect any influence on the concentration of these substances in the bile. Since intestinal absorption - the primary regulating mechanism - was by-passed, these results are not surprising.

Fortunately more data are available from human experiments : Gordon, Lewis, Eales and Brock (1957a,b) were the first to show that the substitution of hydrogenated coconut oil by Sunflower seed oil in the diet is accompanied by an increase in the faecal excretion of both bile acids and neutral sterols. The average increased excretion in four such experiments with 100 gm. of fat in the diet was 350 mg. daily. Although the Liebermann-Burchard-positive sterols measured were not an accurate reflection of the sterol excretion, this figure is of the same order as that calculated from the change in absorption of endogenous cholesterol in similar circumstances (see Section 24 G).

In a modification of the experimental conditions the extra addition of 75 gm. of sunflower seed oil to the diet of subjects already consuming the same amount of coconut fat caused an increased excretion in both bile acids and neutral sterols. This was accompanied by a fall in the serum cholesterol level. These experiments have been interpreted as demonstrating the special properties of sunflower seed oil as a promoter of cholesterol catabolism. No suggestion was made, however, as to how this might happen. A different interpretation is possible. An explanation has been suggested in Section 25 for

the decrease in serum cholesterol level following the extra addition of large quantities of any fat. The extra fat, comparatively free from endogenous cholesterol, once in the blood stream, dissolves out the cholesterol from serum and transports it to the liver, until the serum cholesterol drops to a new equilibrium value. During this transitional period then, extra cholesterol is transported to the liver without a change in the rate of absorption. Extra excretion might reasonably be supposed to follow. The important point made here, however, is that extra coconut oil in the diet might have had an even greater effect. Unfortunately, no control experiment was conducted. The comparatively low excretion of sterols and bile acids on a low-fat diet remains unexplained.

Conditions resembling more closely to the steady equilibrium state under consideration here obtained when Haust and Beveridge (1958) recorded the following bile acid excretion in man during the last four days of an eight day experiment:

Butter	(60% of cal.)	0.72 ± 0.36	gm./4 days
Corn	"	1.00 ± 0.47	"
Fat free	"	1.11 ± 0.52	"

Although confirming a comparably higher excretion with a low solubility oil, the highest excretion was observed on a fat-free diet !

Increased faecal sterols with corn oil has likewise been reported by Hellman et al. (1957) and more recently by Rosenfeld and Hellman (1959), whereas Antonis (1959) found more faecal sterols and bile acids with Sunflower seed oil than

with butter in the diet.

It is concluded that there is good evidence for increased excretion of total catabolic end-products with low, as compared with high, solubility oils in the diet. In addition, one report exists which records maximum excretion on a fat-free diet.

D. Liver Synthesis

The link between the hepatic synthesis of cholesterol and its solubility in dietary fats is remote, and perhaps for this reason a most fascinating one. Established more firmly, this link would furnish another indirect proof of the differential resorption of endogenous cholesterol due to different fats.

Effect of dietary cholesterol

There is a good deal of evidence that dietary cholesterol suppresses the synthesis of this substance in the liver. The classical experiment of Gould and Taylor (1950) with dogs and rabbits demonstrated that dietary cholesterol suppressed hepatic synthesis to a few per cent of the control rate. These workers used the conversion of C^{14} labelled acetate into cholesterol as an index of synthesis in both liver slices and in vivo experiments. Similar conclusions were arrived at by Tomkins and Chaikoff (1952) who worked on rat liver slices. By feeding a diet containing 5% cholesterol, hepatic synthesis was almost completely suppressed.

Both Alfin-Slater et al. (1952) and Franz et al. (1954) reported a higher liver cholesterol concentration on cholesterol fed rats than in controls. This higher liver

cholesterol was associated with a lower synthesis of this sterol. Morris et al. (1957) working with rats found that increasing the dietary intake of cholesterol containing cholesterol -4-C¹⁴ from 0.05% to 2% of the diet, decreased the synthetic component of serum cholesterol from 67 - 80 per cent to 10 - 26 per cent.

The suppression of hepatic synthesis by dietary cholesterol can thus be taken as an established fact in some animals but has yet to be shown in man. Hepatic cholesterol synthesis then, is regulated at least in part by the rate of absorption of this sterol from the intestine. According to the solubility hypothesis, high and low solubility oils (e.g. coconut and corn) cause different rates of absorption of dietary or endogenous cholesterol. Other things being equal, it follows that dietary high solubility oils suppress liver synthesis and vice versa. Support for this view comes from a number of sources.

Effect of high and low solubility oils

Over 20 years ago, Eckstein (1938) reported a larger sterol synthesis in rats consuming corn oil than in those on a comparable diet of coconut fat. This sterol balance study, however, gives no account of a possible interference by excreted bile acids and its interpretation is therefore open to criticism.

More recently Avigan and Steinberg (1958) using modern tracer techniques studied the incorporation of 1-C¹⁴ acetate into the unsaponifiable matter of the liver of rats

consuming either coconut or corn oil and found a higher incorporation with the latter oil. Similar conclusions were arrived at by Merrill (1958) who reported increased incorporation of sodium acetate $1-C^{14}$ into cholesterol with dietary linoleic acid, in spite of its lowering action on the serum cholesterol.

Further supporting evidence was presented by Wood and Migicovsky in 1958. These authors fed unsaturated oils and fatty acids to rats and report "a stimulating effect" on the incorporation of C^{14} acetate into cholesterol, both in vivo and in liver homogenates. Equal amounts of saturated material such as coconut oil and lauric acid had the opposite effect. Only three days' feeding was required to demonstrate these phenomena. Finally, Wilson and Siperstein (1959b) failed to distinguish corn from lard as affecting the incorporation of acetate into cholesterol in liver slices. The unhappy choice of lard as an experimental fat, however, becomes apparent on inspection of Fig. 3, Section 2. Its serum regulating property is not conspicuously different from that of corn oil.

Summarizing this brief review, one can say that there is substantial experimental evidence that unsaturated oils have a greater stimulating effect on hepatic cholesterol synthesis than saturated fats. The parallelism between the depressing effect on liver synthesis of dietary cholesterol on the one hand and the relatively greater depressing effect of high as compared with low solubility oils on the other, is impressive.

It is suggested that these two phenomena are based

on the same primary mechanism, namely the rate of intestinal absorption of cholesterol, both dietary and endogenous. Another independent link has thus been established between liver synthesis and absorption. While not furnishing a formal proof of differential absorption as influenced by different dietary fats, this link provides strong supporting evidence.

E. Conclusion

There can be little doubt that the picture drawn here of the metabolic inter-relationships of cholesterol as affected by different dietary fats and the solubility principle, is both fragmentary and grossly over-simplified. Much of the picture is based on controversial evidence. Even the broad outlines, if true at all, require further confirmation. If accepted, however, the following conclusions can be drawn:

The solubility hypothesis does not obviously contradict any established principles of physiology or cholesterol metabolism. It may, in fact, be used to unify knowledge on the subject and explain apparently disconnected phenomena on a common basis.

A brief review of the recent literature has shown that cholesterol-free dietary fats of different composition have characteristic effects on the excretion, serum concentration and liver synthesis of cholesterol. An attempt has been made to demonstrate that these effects are inter-related and

ultimately caused by one primary regulating mechanism - the rate of intestinal absorption of cholesterol. This in turn is believed to depend on the presence in the intestine of endogenous cholesterol and its solubility in accompanying fat.

SUMMARY

The effect of different dietary fats on the serum cholesterol concentration has been discussed. In a series of experiments, conditions have been created under which rats can be induced to imitate humans in this respect.

Using the rat as an experimental animal, the hypocholesterolaemic property of dietary sunflower seed oil has been investigated by the chemical and physical fractionation of this oil. By feeding the saponifiable and unsaponifiable matter of the sunflower seed oil, it was found that the latter fraction was partly responsible for the serum regulating property of this oil.

This finding was confirmed by altering the composition of the unsaponifiable matter by the physical fractionation process of liquid propane segregation. Several fractions of oils with similar fatty acid compositions but differing in both quality and quantity of the unsaponifiable matter were found to have different effects on the serum cholesterol level.

In a further series of experiments, the various known components of the unsaponifiable matter were next fed as pure compounds, dissolved in coconut fat. Sitosterol had little effect, while squalene had a slight cholesterol lowering effect. Combinations of these with β -carotene and α -tocopherol produced a larger and more significant effect.

Although the unsaponifiable matter and some of its components were shown to have serum lowering properties, it was not possible to demonstrate that this fraction was responsible for the action of the oil as a whole. From this it was concluded that it is probably impossible to isolate highly potent serum cholesterol lowering substances from this oil.

In further experiments to elucidate the mechanisms at play, the hypercholesterolaemic properties of hen's egg lipids were studied and traced to the cholesterol content of the yolk. When this sterol was fed alone, however, no increase in serum cholesterol level occurred unless administered in solution in fat.

Attempts to explain the above phenomena led to the postulate that cholesterol is absorbed only when in solution in fat. A hypothesis was next proposed according to which the amount of cholesterol absorbed is a function of its solubility in dietary fat. In the absence of cholesterol, fats would exert their effect on the serum cholesterol by causing the re-absorption of endogenous or biliary cholesterol.

To test this hypothesis the solubility of cholesterol in a variety of fats and oils at 37°C was determined in vitro, and found to correlate with the serum cholesterol level produced when these oils were fed to rats. Thus the high serum cholesterol level produced by coconut fat, for example, was reflected in a comparative high solubility in this fat. The different cholesterol-raising properties of fats originally

free from cholesterol were thus explained. The hypocholesterolaemic effect of the unsaponifiable matter of sunflower seed oil was likewise accounted for.

To test a prediction from the solubility hypothesis it was demonstrated experimentally that there is a certain amount of cholesterol, when fed with a given quantity of fat, above which no further rise in the serum cholesterol is observed. This amount of cholesterol was shown to be related to its solubility in accompanying fat.

The above principles were next applied to the human sphere. From a single experiment it was observed that dietary cholesterol markedly raises the serum cholesterol of man provided it is administered in solution in fat. A good correlation was also obtained between the solubility of cholesterol in different fats and their published quantitative serum cholesterol elevating effects on humans.

In a final chapter it was proposed that the influence of different dietary fats and oils on the excretion and hepatic synthesis of cholesterol can likewise be related to their solvent power for dietary or endogenous cholesterol.

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oooOooo

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With Mr. H. de Wit:

A micro-method for the determination of total serum cholesterol concentration in 0.2 ml of blood.

S. Afr. J. Lab. Clin. Med. (submitted)

Other papers in preparation

NOTE: Half-tone photographs in this thesis were taken by the author.

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SUMMARY OF THESIS

entitled

THE EFFECT OF DIETARY LIPIDS

ON THE

SERUM CHOLESTEROL CONCENTRATION OF THE RAT

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SUMMARY

The effect of different dietary fats on the serum cholesterol concentration has been discussed. In a series of experiments, conditions have been created under which rats can be induced to imitate humans in this respect.

Using the rat as an experimental animal, the hypocholesterolaemic property of dietary sunflower seed oil has been investigated by the chemical and physical fractionation of this oil. By feeding the saponifiable and unsaponifiable matter of the sunflower seed oil, it was found that the latter fraction was partly responsible for the serum regulating property of this oil.

This finding was confirmed by altering the composition of the unsaponifiable matter by the physical fractionation process of liquid propane segregation. Several fractions of oils with similar fatty acid compositions but differing in both quality and quantity of the unsaponifiable matter were found to have different effects on the serum cholesterol level.

In a further series of experiments, the various known components of the unsaponifiable matter were next fed as pure compounds, dissolved in coconut fat. Sitosterol had little effect, while squalene had a slight cholesterol lowering effect. Combinations of these with β -carotene and α -tocopherol

produced a larger and more significant effect.

Although the unsaponifiable matter and some of its components were shown to have serum lowering properties, it was not possible to demonstrate that this fraction was responsible for the action of the oil as a whole. From this it was concluded that it is probably impossible to isolate highly potent serum cholesterol lowering substances from this oil.

In further experiments to elucidate the mechanisms at play, the hypercholesterolaemic properties of hen's egg lipids were studied and traced to the cholesterol content of the yolk. When this sterol was fed alone, however, no increase in serum cholesterol level occurred unless administered in solution in fat.

Attempts to explain the above phenomena led to the postulate that cholesterol is absorbed only when in solution in fat. A hypothesis was next proposed according to which the amount of cholesterol absorbed is a function of its solubility in dietary fat. In the absence of cholesterol, fats would exert their effect on the serum cholesterol by causing the re-absorption of endogenous or biliary cholesterol.

To test this hypothesis the solubility of cholesterol in a variety of fats and oils at 37°C was determined in vitro, and found to correlate with the serum cholesterol level produced when these oils were fed to rats. Thus the high serum cholesterol level produced by coconut fat, for example, was reflected in a

comparative high solubility in this fat. The different cholesterol-raising properties of fats originally free from cholesterol were thus explained. The hypocholesterolaemic effect of the unsaponifiable matter of sunflower seed oil was likewise accounted for.

To test a prediction from the solubility hypothesis it was demonstrated experimentally that there is a certain amount of cholesterol, when fed with a given quantity of fat, above which no further rise in the serum cholesterol is observed. This amount of cholesterol was shown to be related to its solubility in accompanying fat.

The above principles were next applied to the human sphere. From a single experiment it was observed that dietary cholesterol markedly raises the serum cholesterol of man provided it is administered in solution in fat. A good correlation was also obtained between the solubility of cholesterol in different fats and their published quantitative serum cholesterol elevating effects on humans.

In a final chapter it was proposed that the influence of different dietary fats and oils on the excretion and hepatic synthesis of cholesterol can likewise be related to their solvent power for dietary or endogenous cholesterol.