STUDIES IN CHOLESTEROL METABOLISM

A THESIS

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BY

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CONTENTS.

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CHAPTER I.

INTRODUCTION

"Seek simplicity, and distrust it" - A.N. Whitehead [1].

The biochemistry of the steroids is of unique interest; no other group of relatively simple substances plays so versatile a role in biology. On the one hand are many highly potent steroids occurring in minute quantities e.g. sex hormones, corticosteroids, the vitamins D and the cardiac glycosides. In sharp contrast are certain sterols, exemplified in the animal kingdom by cholesterol - familiar, abundant, easily determined, yet singularly obscure in their physiological significance.

In some restricted fields the functions of cholesterol are known. It is a precursor of the adrenocortical hormones (perhaps not an obligatory one [2]), and is probably important in the biosynthesis of sex hormones too. Present in the skin is 7-dehydrocholesterol, converted to vitamins D_3 by ultraviolet light. Cholesteryl esters are found in large quantities, together with sphingomyelin, in the myelin sheaths of nerve fibres, where these lipids may have an insulating function necessary at least for saltatory conduction.

But what of the cholesterol present in the liver and blood, and to a small extent in all cells? Most tissues, even the arterial wall, can synthesize it [3]. The amount of cholesterol and phospholipid in muscle is increased by prolonged exercise [4], hinting at their importance in cellular function.

Does the plasma cholesterol play a part in the transport of fatty acids? There is no cogent evidence for this. Cholesteryl esters are even less soluble in water than the parent compounds. The roles may even be reversed: studies described in Chapters 4 and 5 support the hypothesis that cholesterol transport is disturbed by certain alterations in dietary fat intake.

Great attention has been paid to cholesterol metabolism in recent years. This is largely due to the assumption that it is of central importance in the pathogenesis of atherosclerosis. Interest has been focussed on the subject by the vast and increasing incidence of coronary heart disease (discussed in Chapter 5). Despite the functional interrelationships of the various lipids in health and disease, cholesterol has received preferential treatment, due to the ease with which it may be measured; the Lieberman-Burchard reaction has cast a blue-green shadow on our understanding of lipid metabolism.

It has been recognised in the past decade that the quantity[5,6] and quality [7,8,9] of dietary fat are important determinants of the serum cholesterol level. Their effects on other classes of circulating lipid have been less well documented. Recent investigations into coronary atherosclerosis have largely centred around the hypothesis that dietary fat is an important aetiological factor, the presumed link in the causal chain being hypercholesterolaemia. Atheromatous plaques were shown by Windaus in 1910 to contain a high proportion of cholesterol [10]; atheroma is predisposed to in many diseases associated with an elevated serum cholesterol, e.g. diabetes, myxoedema and xanthomatosis. The

relationship of atherosclerosis to dietary fat on the one hand and to abnormal blood lipid composition on the other may be of the utmost importance; but a review of the literature (Chapter 5) shows how little unanimity there is. Experimental results have been obscured by ill-conceived speculation, "a world where flesh and blood are less real than paper and ink", as Mumford [11] said in a related context.

There is a lack of fundamental knowledge of the actions of various dietary fats on human lipid metabolism. Consider one well-worn aspect, the serum cholesterol; several mechanisms determine its level (see Figure 1, 1). If the dietary hypothesis of the aetiology of atherosclerosis is to be analysed thoroughly, it is clearly essential to know precisely how dietary fats act.

The present experiments were largely devoted to this problem of how the serum cholesterol level of man is affected by certain dietary fats. Likely possibilities were altered rates of cholesterol synthesis, catabolism or excretion.

Inconsistent animal studies provided little support for the first suggestion. Dietary cottonseed oil and linoleic acid (which reduce the serum cholesterol) increased cholesterol synthesis [12,13] but Alfin-Slater et al [14] could detect no difference in cholesterol synthesis on feeding cottonseed oil. The addition of sodium linoleate to liver slices reduced cholesterol synthesis [15]. It was felt that studies of cholesterol catabolism and excretion would certainly be easier to perform and might provide less equivocal results. Such experiments are described in Chapters 2 and 3. They led to the theory

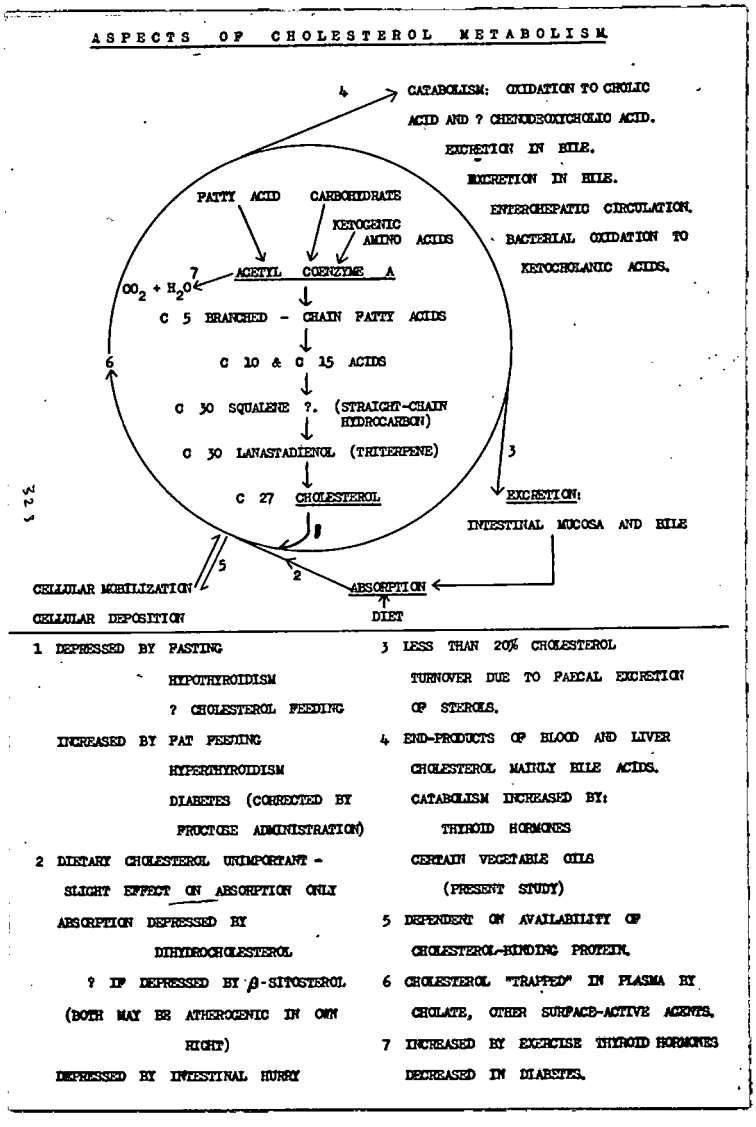

Figure 1. 1. Factors determining the serum cholesterol level.

that certain oils reduce the serum cholesterol level by increasing its rate of catabolism.

In seeking other links in the causal sequence, one was guided by the knowledge that plasma cholesteryl esters contained highly unsaturated fatty acids, particularly linoleic acid. This acid cannot be synthesized in the mammalian body, but is present in large quantities in all oils which have been shown to reduce the serum cholesterol. Was it possible that the feeding of unsaturated oils altered the composition of circulating cholesteryl esters, facilitating their catabolism? Sinclair [16] has suggested a related mechanism. Curran [17] has shown that cholesteryl esters obtained from rabbits are more readily hydrolysed when the animals are fed unsaturated fats than saturated ones. Experiments described in Chapter 4 showed how strikingly the plasma cholesteryl esters change on altering the dietary fat. Thus the quality as well as the quantity of cholesterol compounds in the blood are influenced by diet.

These observations prompted a controlled survey of the composition of circulating cholesteryl esters in coronary heart disease. The type and amount of fat consumed may be important factors in its aetiology. The disease is exceedingly rare in the Bantu, and this has been ascribed to differences in diet; cholesteryl esters were therefore analysed in this group. Esters obtained from atheromatous coronary arteries were also studied, as it was of interest to know their relation to circulating esters.

Analytical techniques had to be developed for some of these investigations, and are described in the Appendices. These

methods were time consuming; for example, two technicians required eight days for a batch of six cholesterol ester analyses. The number of experiments was therefore less than would otherwise have been desirable.

To reduce the number of experimental variables, only two dietary fats were extensively used. Hydrogenated coconut oil and sunflower seed oil were chosen, (the former almost saturated, the latter highly unsaturated). The results do not necessarily hold for saturated and polyunsaturated fats in general, nor for any other classes of fat.

These studies led to the theory that certain dietary unsaturated oils reduce the serum cholesterol level by promoting catabolism of that sterol; elevation of the cholesterol level by hydrogenated coconut oil was not due to diminished catabolism.

Differences were found in the degree of unsaturation of circulating cholesteryl esters between patients with coronary heart disease, normal White and normal Bantu subjects. Dietary factors could explain these differences. The esters were most unsaturated in the Bantu, and least so in patients with coronary artery disease. Those of atheromatous coronary arteries contained far less unsaturated fatty acid than plasma.

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CHAPTER 2.

Dietary Fat and the Faecal Bile Acid Excretion.

There is little doubt that the main end-products of cholesterol catabolism are the bile acids. The advent of isotopic tracer techniques - deuterium and more recently ^{14}C labelling has vastly facilitated study of the metabolism of this sterol. It had formerly been believed that most cholesterol was excreted unchanged in the bile, and that it was eliminated in the faeces largely in the form of coprosterol and coprostanol. Urinary sterol excretion, even including steroid hormone catabolites, is quantitatively unimportant in relation to sterol turnover, and tracer techniques have lately shown that the sterol nucleus is not broken down to expired carbon dioxide [1].

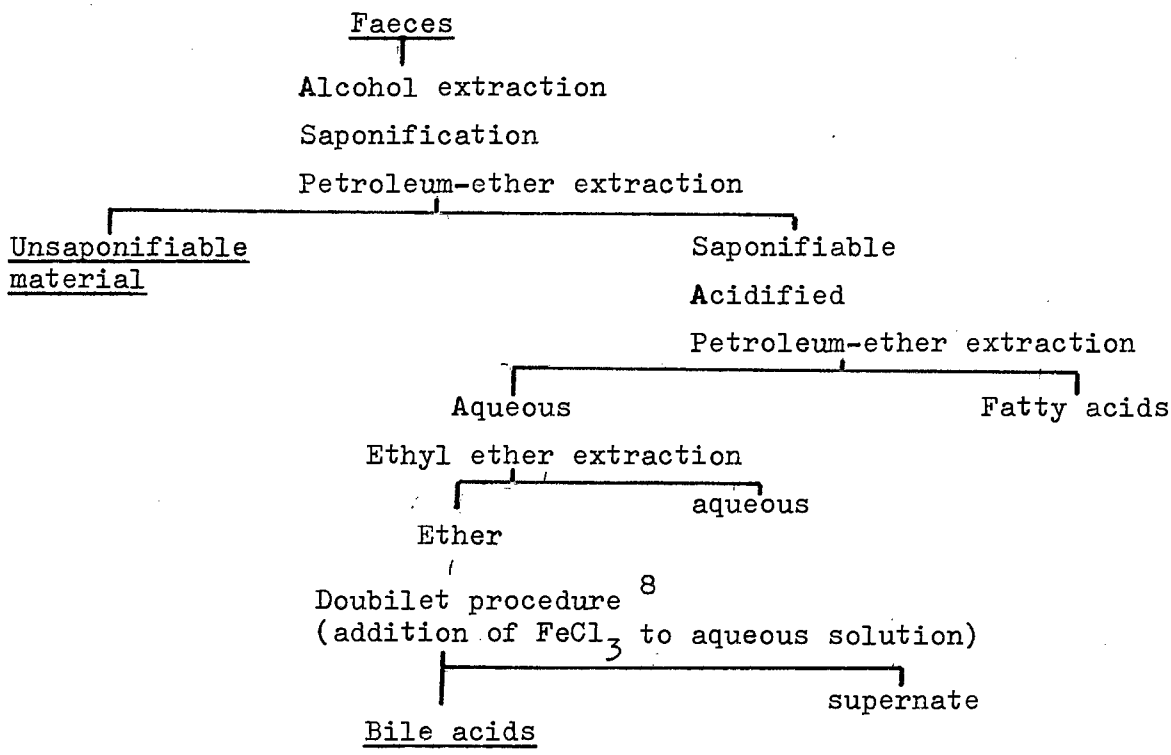
Bloch et al [2] were the first to suggest that cholesterol is catabolized to bile acid; Siperstein and his associates [3,4,5] and Bergström [6] have demonstrated that the radioactivity of injected cholesterol-4-^{14}C appears almost exclusively in the bile acid fraction of bile (both in rats and in a human subject with a bile fistula); This is also true, though not quite to the same extent, of the bile acid fraction of faeces [5]. Similar findings have been reported by other workers [1,22,23,24].

In the study on a human subject, Siperstein [4] administered labelled cholesterol intravenously (dissolved in Tween 20). It is instructive to recall some of his results in detail: 40% of the ^{14}C

was excreted within 50 hours; of this, 98% appeared in the bile; only 7% of the ^{14}C was found in the unsaponifiable matter i.e. cholesterol, and 90% in the bile acids, principally glycocholic acid. Similar experiments on animals have recently shown that some radioactivity also appears in chenodeoxycholic acid [7].

On administration of cholesterol-4-^{14}C to normal rats, 95% of the radioactivity ultimately appeared in the faeces [5]. Of this 81 ± 7% was on the bile acid fraction, the remainder in neutral material. For later comparison, the flowsheet in these studies is as follows:

Table 2, 1. Fractionation of Faecal Lipid.



The possible mechanisms by which certain unsaturated oils lower the serum cholesterol have been reviewed in Chapter I; one likely suggestion was that such oils promote the degradation or possibly the excretion of cholesterol. These hypotheses had also the value of appearing reasonably simple to prove or disprove, thus conforming to the aphorism that "a good hypothesis dies young."

Experimental design did not provide much difficulty but it was immediately apparent that the necessary analytical methods were not available, at least in a form suited for use on the large numbers of samples anticipated. In the stable conditions of the metabolic ward, the subjects were fed a diet varying only in the quality and/or quantity of fat. In this internally controlled experiment, observations were made on the faecal bile acid and sterol output, during periods of low fat intake, high intake of saturated fat, and high intake of saturated oil. The serum cholesterol level was followed, and fat absorption and the dry weight of faeces observed.

It was evident from a survey of the literature that no straightforward technique was available for the determination of total faecal sterols; procedures involving digitonin precipitation do not determine the 3α- sterols (sterols possessing an - OH group orientated in the trans position at carbon 3; they are probably present to a moderate extent in faeces [9]).

Chromatography on alumina of the unsaponifiable fraction of the faecal lipid [9,10] showed that in addition to coprostanol, coprosterol and cholesterol, several other sterols were present in smaller quantities.

Detailed fractionation by Riddell and Cook [9] showed that the unsaponifiable matter in rat faeces contained cholesterol (if present in the diet), coprostanol, and unknown substances not precipitated by digitonin. Choice of an analytical method in the projected experiment was guided in part by the fact that two procedures in frequent use in this laboratory were (i) the van de Kamer determination of faecal fats [11], in the course of which the unsaponifiable fraction of faecal lipid could readily be isolated, and (ii) the Abell procedure [12] for serum cholesterol determination, employing the Lieberman-Burchard reaction. By the latter method colour development occurs with the greater part of the faecal sterol, including coprostanol which gives less colour than cholesterol on a molar basis; the saturated derivative of cholesterol, cholestanol, gives no colour, and certain other quantitatively minor sterols in faeces, e.g. 7 - dehydrocholesterol, produce a maximum intensity of colour at velocities greater than that of cholesterol. Carroll and Noble [10] found that in human faeces, figures obtained by gravimetric determination of faecal sterol after digitonin precipitation corresponded reasonably with those obtained when the Lieberman-Burchard was used.

It was therefore decided to apply this colour reaction to the unsaponifiable material obtained in the course of the van de Kamer procedure, but it was recognised that qualitative variations in the faecal sterol might modify the results somewhat.

Further practical and theoretical difficulties were encountered in the determination of faecal bile acid. A fairly exhaustive search of the literature revealed only two analytical procedures using modern chemical techniques, while earlier methods (on which, incidentally, many of our present concepts of bile acid physiology are based), appeared disconcertingly unlikely to possess any useful degree of selectivity. Chromatographic isolation of certain of the faecal bile acids is common to the recently published methods of Carey and Watson [13] and of Abell, Mosbach and Kendall [14]. The former workers indicated their procedure in a paper describing essentially qualitative studies on faecal bile acids; in the course of these they isolated deoxycholic acid which they found to be the predominant faecal bile acid. A somewhat similar technique was employed by the latter group, in demonstrating reciprocal changes in serum cholesterol and faecal bile acid during thiouracil administration to dogs.

Nature of the faecal bile acids.

In 1911 Fischer [15] isolated deoxycholic acid from faeces. This was confirmed by Carey and Watson, chromatographically and by infra-red spectroscopy. In their passage along the intestine, the bile acid conjugates secreted by the liver are largely hydrolysed. Cholic acid, though the predominant bile acid in the bile of most human subjects, is conspicuously absent from faeces, and its fate is uncertain. Linstedt and Norman [16] administered cholic acid-4-^{14}C to rats and showed its conversion to a complicated mixture of acids;

this was largely prevented by prior sterilization of the alimentary tract with antibiotics. Other workers have demonstrated the ability of Escherichia coli to convert cholic acid to a mixture of mono- and diketocholanic acids [17,18], while Alkaligenes faecalis is able to oxidise it to 3, 7, 12 triketo-cholanic acid [19]. It seems likely that cholic acid is oxidised in the bowel to a mixture of ketonic acids, which substances achieve considerable importance when it is recalled that cholic acid itself is in the main biliary end-product of cholesterol metabolism.

Some degree of uncertainty therefore surrounds the nature of the faecal end-products of cholesterol catabolism; they occurred (in Siperstein's studies) in the acidic, ether-soluble, petroleum ether-insoluble fraction of the faecal lipid, (see Table 2,1) and were precipitated by ferric ions. These properties strongly suggest that the substances are bile acids. The tracer studies of Linstedt and Norman, and the in-vitro properties of certain intestinal bacteria support the contention that the faecal ketocholanic acids are the main catabolic products of cholesterol. Further evidence for this has been adduced by Cook et al [20], who found that cholesterol-fed rats excrete an increased amount of petroleum-insoluble acids, which they thought likely to be ketocholanic acids. Such conclusions being presumptive, it was felt that the projected study of cholesterol catabolism during fat and oil feeding experiments would require a technique for the determination of total faecal bile acids. Gross qualitative changes

were detected by paper chromatography; selective quantitative procedures [13,14] would have led to considerable difficulties of interpretation pending a more exact knowledge of the nature of the faecal derivatives of cholesterol.

The development of a technique for the estimation of total bile acids is described in Appendix I. The profusion of colorimetric and spectrophotometric methods for bile acids in bile was not of great help; the concentration of bile acids in faeces is far lower than that in bile, and it was suspected that the degree of contamination of such extracts might invalidate optical methods.

Normal faecal bile acid excretion.

The faeces of ten normal adult males receiving a low-fat diet (5-10 g/day) were collected in 5-day batches, the samples being stored at -15°C. They were homogenized thoroughly, and duplicate determinations made. The daily total acid excretion was 53-301 mg. mean 149, S.D. 50.5. These figures are of the same order of amount as the mean of 80 mg. deoxycholic acid/24 hours obtained by Carey (personal communication) in a single subject.

The wide normal range of bile acid excretion (at least by the present method) must be borne in mind when interpreting experimental data. The day-to-day variation in a single subject may be minimized by ensuring constancy of diet and regularity of bowel habits (Gordon & Lewis [25]).

Discussion.

The procedure is relatively simple to perform on a fairly large scale, obviating as it does such time-consuming operations as column chromatography, Soxhlet extraction and saponification. However, its selectivity is low, and its validity rests on the assumption that only the bile acids are present in significant quantity in the solution titrated. In support of this are the observations that (i) the titrimetric figure is in general agreement with the Doubilet and Mosbach procedures, and comparable to Carey's figure for excretion of deoxycholic acid obtained by column chromatography; (ii) all the material titrated forms insoluble ferric salts, a property common to the bile acids; (iii) paper chromatography shows deoxycholic and probably certain ketocholanic acids in the appropriate order of amount.

Quantitative resolution of the bile acid mixture would have provided more detailed information, but, as has been stated, uncertainty regarding the precise nature of the faecal bile-acid end-products of cholesterol catabolism would have created difficulties in interpretation. It maybe recalled that the isolation procedure used in the routine method is closely similar to that employed by Siperstein and his group in isolating the radioactive fraction from the faeces of rats given cholesterol-4-^{14}C.

Experimental:

Subjects. Seven non-white male subjects were investigated in the metabolic ward at Groote Schuur Hospital. Their clinical status was assessed by Professor J.F. Brock and Dr. H. Gordon, the latter having the task of arranging their diets and supervising the ward. A specially trained staff was responsible for the collection of specimens. The age of the subjects, their ethnic group, diagnosis and course appear in Table 2, 2.

Prior to admission they had lived in relative poverty due to their disabilities or to prevalent social conditions; they had received satisfactory general diets for not less than one month, and usually far longer, while in hospital. Their individual maladies had been managed as indicated in the table. At the time of the experiments, none had active disease as assessed clinically and by analysis of the urine, chest X-ray, erythrocyte sedimentation rate, routine blood counts, electro-cardiography, determination of serum proteins, urea, and thymol and zinc turbidity and Wasserman reaction; their stools were free from pathogens. During the experiments their physical activity was minimal, but reasonably constant.

Diets. The basal 2000 Caloric diet consisted of 60 g. casein, and maize meal, corn grits, white bread, black tea, sugar, syrup, 200 g. each of pumpkin and squash to provide roughage, and a vitamin preparation. This was administered for not less than one week before commencing basal observations, and continued throughout the experiment, as far as possible using foodstuffs from a single batch stocked by the diet kitchen.

Subject	Age	Ethnic Group	Clinical Summary
A.M.	25	Bantu	Flexion contractures of arms following burns 18 months previously.
G.J.	25	Bantu	Acute encephalitis 2 months previously. Residual aphasia and intellectual deterioration.
M.P.	28	Bantu	Cerebrovascular accident 3 months previously. Residual hemiparesis.
W.K.	34	Bantu	Plantar warts.
V.M.	21	Coloured	Vascular malformation in midbrain. Haemorrhage 3 months previously.
J.G.	57	Coloured	Syphilitic aortic aneurysm, treated one year previously. Asymptomatic at present.
A.S.	19	Bantu	Cerebral bilharzia. Treatment with antimony completed one week previously.

Table 2, 2.

To this diet was added one or both of the following :

(1) Coconut oil, hydrogenated to iodine value 6, and containing 303 mg/100 g. of Lieberman-Burchard chromogen in the unsaponifiable fraction. Much of the fatty acid was of short chain length, resulting in a consistency acceptable to the subjects despite the very low iodine value.

(2) Sunflower seed oil, deodorized and of refined grade, iodine value 135, containing 751 mg./100 g. Lieberman-Burchard chromogen. The predominant fatty acid was linoleic acid [26].

Collection of Specimens.

Blood was drawn before breakfast three times weekly, for cholesterol determination by the method of Abell et al [12] as modified by Anderson & Keys [27]. In this laboratory a variation of 12 mg.% was significant at the 5% level.

Faeces were collected in 24-hour batches, weighed, then stored at -15°C. until analysis. They were homogenised in a Waring blendor, with the addition of water if necessary. Samples were weighed into flasks for the van de Kamer procedure [11]. for faecal fats; the unsaponifiable material was extracted during this procedure with petroleum ether (B.P.68-70°). Faecal fats and neutral sterols were determined daily. Before taking aliquots for measurement of sterol and fat content, proportional aliquots of stools were obtained; these were pooled in 3 or 5-day batches and again homogenised; further aliquots were then weighed on to aluminium foil for bile acid determination.

Experiment 1.

Four subjects were studied, (A.M., G.J., M.P., and W.K.). After a week of stabilization on the basal diet supplemented by 500 Calories of cane syrup or sugar, observations were commenced; the Caloric intake was 2500. Twelve days later, 100 g. of fat was substituted isocalorically for carbohydrate; this was administered for 12-15 days. Subjects W.K. and M.P. received hydrogenated coconut oil (HCO) while G.J. and A.M. were fed sunflower seed oil (SSO). At the end of this period, the HCO was replaced by 100 g. SSO in the diets of the first two subjects, while G.J. and A.M. received HCO instead of SSO.

Results.

The results appear in Table 2, 3; figures 2, 1 and 2, 2 show the results of the experiments on W.K. and A.M. In three of the four subjects the bile acid output fell slightly below the basal figure during HCO administration, synchronising with a distinct rise in the serum cholesterol. During SSO feeding, the reduction in serum cholesterol was accompanied by a pronounced elevation of faecal bile acid excretion in three subjects. There was thus a negative correlation between serum cholesterol and faecal bile acid excretion. On the other hand the faecal bile acid output did not bear any relationship to the dry weight of faeces or the faecal fat, suggesting that malabsorption of bile acids due to intestinal hurry would not account for the changes. A total of eighteen observations were made (in duplicate) during each of the three feeding periods. Use of the t test indicated that the difference between bile acid output in the basal and SSO

Subject	Diet	Serum cholesterol mg.%	Faecal bile acid mg./24 hrs.	Faecal sterol mg./24 hrs.	Dry weight of faeces g./24 hrs.
W.K.	Basal	86	147	520	16.7
	Basal + HCO	105	122	790	24.3
	Basal + SSO	56	407	1080	25.4
M.P.	Basal	156	220	380	19.1
	Basal + HCO	259	188	650	20.9
	Basal + SSO	139	314	750	16.5
G.J.	Basal	124	110	370	12.8
	Basal + SSO	93	113	840	22.7
	Basal + HCO	163	74	570	17.5
A.M.	Basal	113	92	210	15.3
	Basal + SSO	86	205	850	28.4
	Basal + HCO	147	125	710	22.5

Table 2, 3. Faecal excretion of bile acid and sterol during manipulation of serum cholesterol by administration of hydrogenated coconut oil and sunflower seed oil.

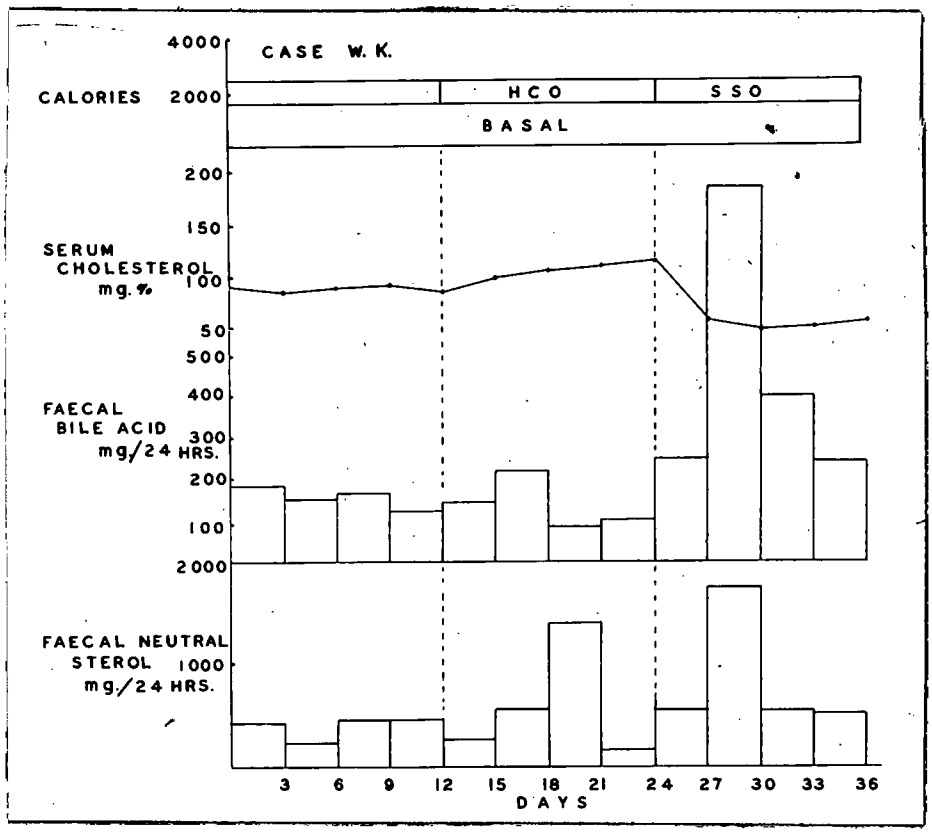


Figure 2, 1. Case W.K. Effect of dietary fat on the serum cholesterol, faecal bile acid excretion and faecal neutral sterol excretion.

periods was significant at the 1% level, while that between basal and HCO periods failed to achieve significance at the 5% level.

The faecal neutral sterol excretion in all subjects was considerably higher during HCO administration than during the basal period, and higher still when SSO was fed. Definite conclusions could not be drawn from these observations; faecal sterol output bore no obvious relationship to the serum cholesterol. It was noted however that the faecal sterol excretion correlated quite closely with the dry weight of faeces and to some extent with the faecal fat. The fact that the dietary sterol was not constant during the experiment (during HCO feeding it was 227 mg./24 hours higher than in the basal diet, and during the SSO period 560 mg. above basal) renders this experiment somewhat inconclusive.

Subject G.J. gained 3 pounds during the experiment. The weight of the other subjects was unchanged.

Formal balance studies carried out by Gordon et al [28] showed that fat absorption exceeded 93% throughout these studies.

Experiment 2.

When SSO replaced HCO in the diet in Experiment 1, a reduction of serum cholesterol below the basal figure was achieved in every subject. Bronte-Stewart et al [29] had noted also that supplementation of a diet containing HCO with SSO reversed the rising trend in the serum cholesterol, though the fall was seldom as dramatic as when SSO was substituted for HCO. In the present experiment, the resultant effect of these oils on the bile acid and neutral sterol excretion was studied.

Three subjects were investigated (V.M., A.S. and J.G.). They received the basal diet (2000 Cals.) for one week, after which observations were commenced, and continued for 10 days. HCO 75 g. was then added to the diet (now 2675 Cals.), and after a further 10 days SSO 75 g. was introduced as well. After 20 days on this regime (3350 Cals.), SSO was withdrawn, a basal-plus-HCO period completing the experiment. Oils were from the same batches as used in Experiment 1.

Owing to the exigencies of the Metabolism Ward timetable a further variable was introduced into the experiment: for the last 5 days of SSO administration the oil was previously heated to 210°C for 2 hours in an open aluminium saucepan to simulate domestic usage and so determine whether the possible isomerization or oxidation of fatty acids during cooking would modify the effect of SSO on the serum cholesterol level.

Results.

These are recorded in Table 2, 4 and Figure 2, 3.

Subject	Diet	Serum Cholesterol mg.%	Faecal Bile Acid mg./24 hrs.	Faec.sterol mg./24 hrs.	Dry weight of Faeces g./24 hrs.
A.S.	Basal	78	112	840	22.4
	Basal + HCO	106	134	880	20.0
	Basal + HCO + SSO	96	218	1270	32.5
	Basal + HCO	120	132	750	24.0
J.G.	Basal	131	129	400	16.0
	Basal + HCO	178	152	650	22.5
	Basal + HCO + SSO	164	428	810	30.2
	Basal + HCO	196	124	750	26.0
V.M.	Basal	129	170	750	14.5
	Basal + HCO	173	188	1000	23.0
	Basal + HCO + SSO	150	353	900	21.0
	Basal + HCO	167	153	800	24.0

Table 2, 4.

Serum cholesterol and faecal excretion of bile acid and sterol during administration of hydrogenated coconut oil alone and with sunflower seed oil.

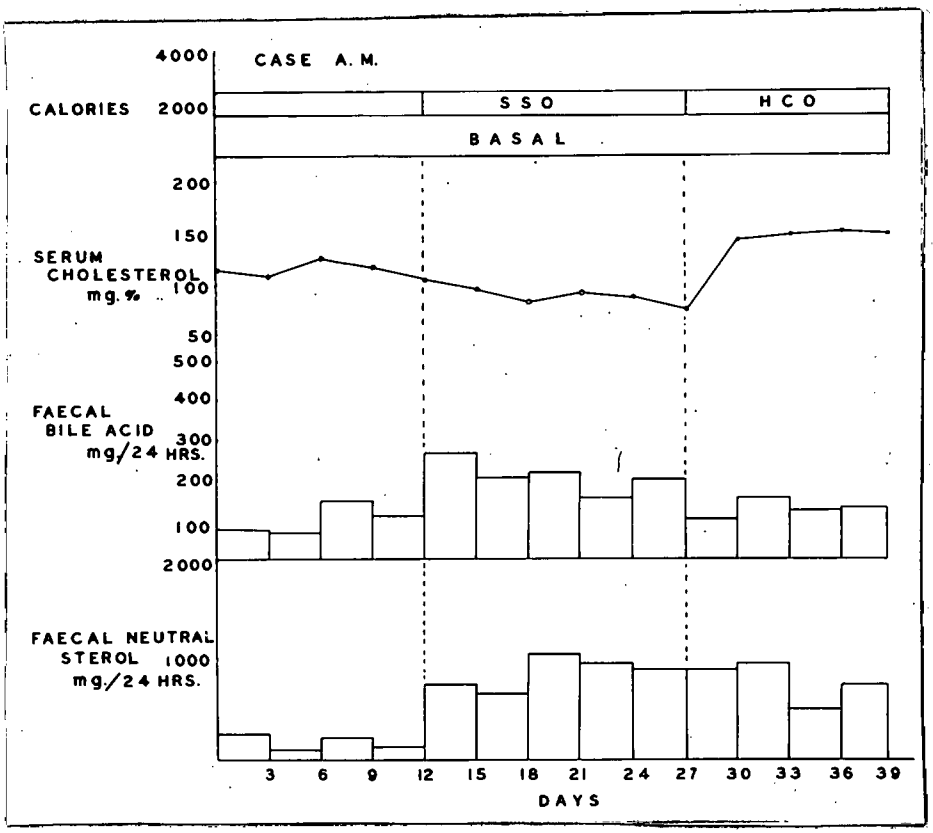


Figure 2, 2. Case A.M. Effect of dietary fat on the serum cholesterol, faecal bile acid excretion and faecal neutral sterol excretion.

In each subject, the addition of SSO to the diet led to an abrupt increase in faecal bile acid excretion, which fell to basal or near-basal levels when the oil was withdrawn.

Sterol output did not conform to a single pattern. In A.S. and J.G. there was a stepwise increase as first HCO and then SSO were added to the diet, but V.M. excreted no more sterol when receiving both oils than on HCO alone. Once again a relation to the dry weight of stools and the faecal fat was apparent. Fat balances during these studies showed that absorption consistently exceeded 95% [28].

On HCO the serum cholesterol rose steeply; the addition of SSO led to moderate reduction, the level failing to reach the baseline in 20 days. There was a second steep rise when SSO was withdrawn. Contrasting with the small extent of the serum cholesterol reduction, bile acid excretion during SSO administration increased markedly, the proportionate rise being approximately the same on the average as in Experiment 1 (in which the fall in serum cholesterol was so striking). Bile acid excretion did not materially vary from the basal on addition of HCO to the diet.

In the 20 days during which the diet provided 3350 calories, all three subjects gained weight.

There was no apparent difference in the serum cholesterol-reducing properties of heated and unheated SSO.

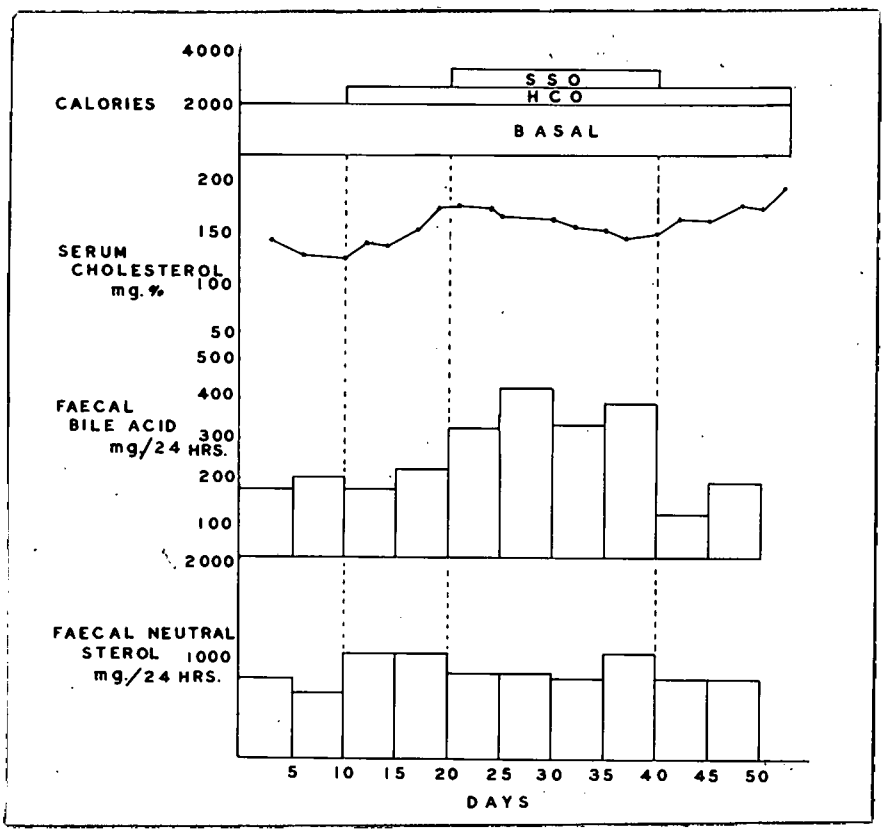


Figure 2, 3. Case V.M. Effect of dietary fat on the serum cholesterol, faecal bile acid excretion and faecal neutral sterol excretion.

Experiment 3 : Qualitative studies

To supplement these determinations, further analyses were performed on the petroleum ether-insoluble organic acids of faeces and also of duodenal contents (aspirated by Miller-Abbott tube passed 60 cm. from the mouth). The latter samples were obtained 5 - 6 hours after the midday meal. The methods were:

1. Paper chromatography using one of the solvent systems described by Sjövall [30], and detecting spots by their fluorescence on heating with antimony trichloride [31]. Sjövall's method had superior resolving power in our hands to other systems [32,33,34]. Isopropyl ether was distilled over hydroquinone on the day it was to be used.
2. Addition of Brady's reagent, ethanolic 2:4 dinitrophenylhydrazine (DPNH) to a solution of the bile acids in 70% ethanol. Formation of a precipitate (of the hydrazone derivative) is a specific test for carbonyl compounds, e.g. ketonic bile acids.
3. Determination of the ultraviolet absorption spectra in 65% sulphuric acid.

These tests were applied to samples obtained in the metabolism ward from a subject who had received HCO for 8 days, and after he had had SSO for a similar period.

Samples were extracted and decolourized as in the quantitative procedure, using 40 ml. duodenal content and 10 g. faeces. Extracts were saponified in sealed pyrex ampoules at 120°C. for 4 hours, using 2N sodium hydroxide. Part of the bile acid fraction was titrated to

determine the aliquot necessary to provide 100-200 µg. of bile acid, which was then chromatographed. Spots were detected by the fluorescence reaction. Some strips were not treated with antimony trichloride; the regions corresponding to the fluorescent spots were eluted with methanol by the procedure of Lewis [41], and their UV spectra in 65% sulphuric acid determined [42].

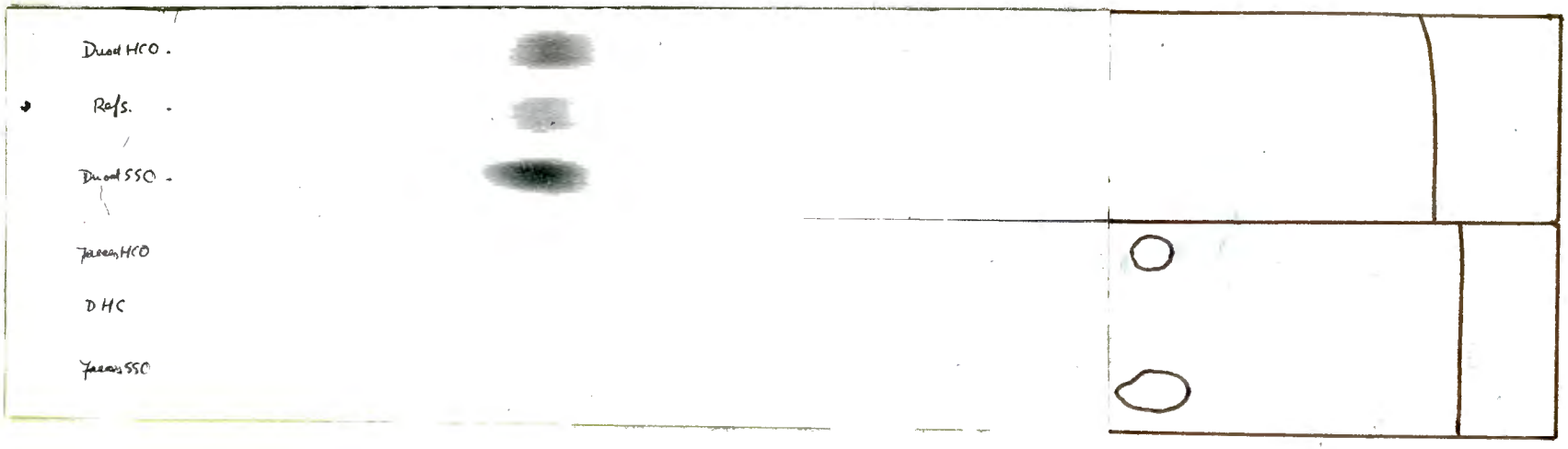
Results

The chromatograms are shown in Figure 2, 3; the following observations were made:-

1) Cholic and deoxycholic acids were present in duodenal contents in roughly equal quantities during the HCO period, but the former predominated when SSO was fed.

2) No keto-acids were detected in duodenal contents in either feeding period using the DPNH reagent. The chromatograms showed no spots with the red-purple or violet fluorescence of ketocholanic acids.

3) The faeces obtained during both periods contained deoxycholic acid but no cholic acid. There was also material running at Rf 0.75, the fluorescent colour of which suggested the presence of a ketocholanic acid. Sjövall [40] has stated that replacement of a hydroxyl by a carbonyl group in a bile acid molecule has little effect on its chromatographic behaviour; one would infer from the low polarity of this substance that the number of oxygen functions is small: it may have been a mono- or diketocholanic acid. During the HCO period this acid was of the order of 1/5 as plentiful as deoxycholic acid; but, significantly the amounts were roughly equal during SSO feeding.



Origin. | Cholic acid. Dehydrocholic acid, (fluorescence only) | Deoxycholic acid. | ? keto-acid, (fluorescence only) | Solvent front

Figure 2.4. Bile acids of duodenal contents and faeces. Photographed in visible light, these chromatograms show cholic and deoxycholic acid spots. Attempts at photographing the strips under ultraviolet light, (to demonstrate fluorescence), were unsuccessful. The ketocholanic acid spots, such as that of the reference compound dehydrocholic acid (DHC) were not coloured. The position is indicated diagrammatically of a spot with violet fluorescence suggestive of a keto-acid; its low polarity suggests the presence of only one oxygen function on the nucleus. The quantity of each reference acid was 50 µg.

4) The absorption spectra in 65% sulphuric acid confirmed the nature of the cholic and deoxycholic acid spots, the former showing peaks at 320 and 391 m μ, the latter at 384 and 272 m μ. The possible keto-acid had a single relatively small peak at 300 m μ; absorption maxima in this region were reported by Eriksson and Sjövall [43] for 3α-hydroxy, 12-ketocholanic acid (302 m μ) and for Δ^{9-11}, 12-ketocholenic acid (297 m μ). Extinction coefficients were not determined in the present study.

5) The DPNH reagent produced a yellow precipitate from the faecal bile acid fractions during both feeding periods, confirming the presence of a ketone. The 2:4 dinitrophenylhydrazone melted at 222 - 228° and decomposed at 236°.

Discussion

These findings were consistent with the view that cholic acid is converted in the lower alimentary tract to one or more keto-acids.

Sunflower seed oil feeding (in comparison to HCO), increases the proportion of cholic acid in the bile acid fraction of duodenal contents, and that of ketocholanic acids in faeces.

DISCUSSION.

The data obtained in the feeding experiments show that reduction of serum cholesterol levels by SSO was accompanied in six out of seven detailed studies by a rise in the faecal excretion of chloroform-soluble, petroleum-insoluble organic acids, and reasons have been adduced for considering this material to consist, at least to a large extent, of bile acids. Elevation of the serum cholesterol by HCO was not associated with a significant change in bile acid excretion.

Qualitative studies on the bile acids of duodenal contents and of faeces supported the view that ketocholanic acids were the main faecal end-products of catabolism of cholesterol. It may be speculated that SSO increases the faecal excretion mainly of ketocholanic acids of low polarity.

On the other hand, the excretion of Lieberman-Burchard-reacting neutral sterols in faeces bore no clear-cut relationship to the serum cholesterol level in the conditions of these experiments.

Reference has been made to the evidence suggesting that faecal excretion of bile acid, in particular of ketocholanic acids, reflects the rate of cholesterol catabolism. The findings of Cook [20] lend support to this concept: cholesterol feeding increased the faecal excretion of petroleum-insoluble acids which had in common with ketocholanic acids that they formed 2:4 dinitrophenylhydrazones and that the infrared spectrum was typical of bile acid and showed a marked carbonyl inflexion. Their data, and most of Siperstein's

and Bergström's, were obtained in experiments on rats, but Siperstein & Murray [4] have shown in man that at least part of the catabolic pathway of cholesterol is similar; their bile fistula subject, after intravenous administration of cholesterol-4-^{14}C, secreted ^{14}C mainly in the form of glycocholic acid.

The present studies may therefore be interpreted as suggesting that increased cholesterol catabolism takes place when SSO is administered. This was not observed in one of the subjects however (G.J.). The oil lowered the serum cholesterol in all subjects: in Experiment I, (isocaloric substitution) the level at the end of the SSO period averaged 56% of that after HCO was given; in Experiment 2 (supplementation of HCO with SSO), the reduction was less striking averaging 19%.

It was of interest to consider the quantitative relationship between the increase in faecal bile acid output during SSO administration and the amount of cholesterol leaving the circulation; the limitations of experimental design, and of the techniques employed, were such as to render such a calculation highly approximate. Plasma volume was calculated from Wilson's formula V (in ml.) = 20W + 73H - 3250, where W is body weight in Kg. and H is height in inches; and the molecular weight of deoxycholic acid was employed, to conform with the calculation in the determination of faecal bile acids. The increase of faecal bile acid excretion was not computed from the mean figures for the SSO feeding period, but specifically for the number of days during which the serum cholesterol was <u>falling</u>; as a base-line the

mean bile acid excretion during the preceding period was taken. The calculation was carried out on data obtained from the four subjects in Experiment I.

It is interesting that the mean ratio for the four subjects is close to unity, the bile acid increase being equivalent to 115% of the cholesterol leaving the circulation.

Yet it is not justifiable to assume that the cholesterol catabolized is drawn entirely from the plasma. It is known that the serum and tissue cholesterol are in dynamic equilibrium, that of the liver being especially readily exchanged. Nor is it legitimate to assume that the rate of cholesterol synthesis remains constant during the experiment; evidence will be presented in Chapter 3 to suggest that this is not the case.

In Experiment 2, where HCO and SSO simultaneously exerted their antagonistic effects on the serum cholesterol, reduction in the latter was relatively small; a similar calculation showed that the faecal bile acid increment was 2 - 3 fold greater than was necessary to account for the cholesterol lost from the circulation; Cholesterol was conceivably being mobilized from the tissues, but it was also possible that its synthesis was increased.

It is generally held, though without direct experimental support, that dietary saturated fats elevate the serum cholesterol by increasing synthesis of this sterol. The biosynthesis of cholesterol has been the subject of intensive research in recent years, and has been reviewed by Bloch [36] and by Friedman and Byers [37].

Acetyl coenzyme A is assumed to be the link between fat assimilation and cholesterol synthesis; it is produced in the course of β-oxidation of fatty acids, and incorporated into cholesterol *in vitro* by liver slices and by other tissues [36].

Evidence was not obtained for reduced catabolism of cholesterol during HCO feeding; the mode of action of HCO on the serum cholesterol would appear to be one of increased cholesterol synthesis. This would account for the findings in Experiment 2. The excessive rise in faecal bile acid output during SSO feeding, when compared with the extent of the fall in serum cholesterol, would be ascribed to increased cholesterol synthesis from HCO.

The possibility has not been excluded that changes in faecal bile acid excretion represent altered rates of intestinal reabsorption rather than of hepatic secretion.

> The observation that faecal bile acid excretion bears no relationship to the dry weight of faeces, or to the faecal fat, offers little support to the concept of variable reabsorption; but this possibility merited a direct study of bile acid secretion in bile fistula subjects. Such experiments would be favoured by more precise analytical methods, and by a clear knowledge of the nature of the biliary end-products of cholesterol metabolism.

Reference must be made to a recent communication in abstract from Ahrens' group [38]. These investigators studied a single hypercholesterolaemic subject for several months, and noted the faecal excretion of ^{14}C after intravenous administration of cholesterol-4-^{14}C.

They observed an increase in faecal excretion of "cholesterol and its end-products" when the serum cholesterol was reduced by corn oil, and a decrease during butter oil feeding, which elevated the cholesterol level; they concluded that an intestinal excretory mechanism was responsible for regulation of the serum cholesterol. According to Ahrens (personal communication) the variation in radioactivity took place in the neutral (as opposed to the bile acid) fraction. These intriguing findings cannot be commented on extensively until a detailed account is published, but some discrepancy between their and our results is evident; it is important to note that their studies were over a far longer period than our own, and that our transient state observations more faithfully reflect the variations in faecal lipid composition associated with a changing serum cholesterol level. It is quite conceivable, too, that cholesterol handling was abnormal in their hypercholesterolaemic patient, or that the cholesterol injected (presumably as a solution in a surface-active agent such as Tween 20), was in an unphysiological form. Kinsell [44] has recently published experiments in which ethyl linoleate, trilinolein, a preparation of arachido-phosphate and safflower oil were used to reduce the serum cholesterol. His figures for faecal sterol excretion do not show reciprocal changes with the serum level.

While tracer studies of cholesterol catabolism have proved that bile acids are the main end-products, it is facile to ignore the fact that roughly 20% of the radioactivity in the faecal lipid of rats fed cholesterol-4-^{14}C was in the unsaponifiable fraction [5]. It should also be recalled that these and other tracer studies extended over a few days at most.

Little is known of the nature or metabolism of the natural ketocholanic acids. Shimuzu and Shimuzu [39] have perfused the keto-acid

dehydrocholic acid through the liver (in dogs), and obtained evidence for its conversion to neutral C19 and C21 substances; these remain to be characterized. They are excreted in the bile and urine. Whether this pathway is of importance in the metabolism of the intact animal is at present an open question. Information is lacking about the extent to which ketocholanic acids formed in the intestinal tract are absorbed. Nevertheless, the perfusion studies indicate a possible pathway by which cholesterol could be broken down to neutral substances by hepatic conversion to bile acids, oxidation of these to keto-acids by the intestinal flora, reabsorption and further degradation by the liver to neutral substances.

SUMMARY.

The catabolism of cholesterol to bile acid is discussed; the development of a method for the determination of bile acids in faeces is described in Appendix I. In six out of seven internally controlled studies, faecal bile acid excretion increased when the serum cholesterol was reduced by feeding sunflower seed oil. The increase was due mainly to raised excretion of ketonic bile acid; support was obtained for the view that faecal ketocholanic acids are derived from cholic acid.

One acceptable interpretation of these data is that the fall in serum cholesterol, when sunflower seed oil is introduced into the diet, is due to accelerated catabolism of this sterol. The action of hydrogenated coconut oil in elevating the serum cholesterol is not explained by decreased cholesterol breakdown.

ADDENDUM.

Antonis [45], using a different analytical procedure, has confirmed the observation of a pronounced rise in faecal bile acid excretion on feeding a vegetable oil (in three out of four subjects).

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CHAPTER 3.

The Effect of Certain Dietary Oils on Bile Acid Secretion in Subjects with Bile Fistulae.

Investigations on bile fistula subjects during dietary manipulation of the serum cholesterol were prompted in part by the results of the experiments described in Chapter 2; these were interpreted as showing that increased catabolism of cholesterol probably takes place when the serum cholesterol is reduced by administration of sunflower seed oil. The possibility was not excluded, however, that variations in faecal bile acid output resulted from alterations in the extent of reabsorption of bile acid. Interpretation was further complicated by the degree of uncertainty surrounding the precise composition of the faecal bile acid end-products of cholesterol breakdown, which necessitated a technique of relatively low selectivity for determining these acids.

Such considerations indicated a more direct study of bile acid metabolism. It was evident that by analysis of bile obtained during feeding experiments from subjects with fistulae, both problems were eliminated. A third advantage was the opportunity to observe more precisely the time relations of changes in serum cholesterol level and bile acid secretion.

Certain questions arose, however, as to the validity of such studies on patients with bile fistulae, which may drain the body of 5 g./24 hours of bile acid and cholesterol :

1) Kier [1] has reported "very low" cholic acid secretion for 3 - 4 days after cholecystectomy, and related "rapid recovery" to normal liver function. Deoxycholic acid secretion, on the other hand, remained constant during the postoperative period. Bergström [2], reviewing this and related papers, preferred to regard the immediately postoperative figures as normal; and he attributed the increase to an attempt by the liver to compensate for the loss of bile acid normally reabsorbed by the intestine. It has been recently shown that establishment of bile fistulae in rats markedly increased the rate of bile acid synthesis, a plateau being reached in 3 - 4 days [3].

Yet it appeared from these and related studies [1,3] that after initial variability the secretion of bile acids in any individual remained reasonably constant. In the present experiments this pattern of bile acid output had first to be confirmed, while ensuring that the composition of the diet remained as constant as possible. Such confirmation was readily obtained; it was considered that patients with bile fistulae were suitable experimental subjects provided that at least five days elapsed postoperatively before starting observations.

2) Another apparent obstacle was the possible impairment of fat absorption in subjects deprived of the digestive actions of the bile salts; it may be noted in anticipation that faecal fat determinations were performed in all but one experiment, absorption amounting to 80 - 90% during fat feeding. Pessoa et al [4] found 65 - 75% absorption in animals with complete bile fistulae. Though abnormal, the absorption of fat was clearly not so low, in the present investigations, as to vitiate the effects of fat feeding on the serum cholesterol.

Fats in general are cholagogues, i.e. their ingestion increases the flow of bile into the duodenum a) by causing the gall bladder to contract through the mediation of the hormone cholecystokinin, and b) by relaxation of the sphincter of Oddi [5]. Magnesium sulphate is also a cholagogue. Substances which increase hepatic secretion of bile, i.e. choleretics, include the bile salts themselves, and possibly the duodenal hormone secretin. In the earlier literature, two papers have described the choleretic effect of certain dietary fats:-

1. McMaster and Elman [5] demonstrated a doubling of hepatic bile secretion, commencing about 20 minutes after ingestion of a fatty meal. This choleretic effect may have been due to reabsorption of bile salts discharged into the intestine by the contracting gall bladder, and perhaps also to release of secretin.

2. McClure et al [6], also carrying out observations over a period of up to 4 hours, introduced various substances through indwelling duodenal tubes in normal subjects and patients who had undergone cholecystectomy; subsequently they aspirated and analysed duodenal contents. Both in normal and in cholecystectomy subjects they found cottonseed oil more effective than hydrochloric acid, magnesium sulphate, peptone and dextrose (in decreasing order) in stimulating "hepatic secretion" of furfural chromogens (presumably cholic acid), cholesterol and bile pigments. Over a 4-hour period the increase in bile acid flow was similar in both groups of subjects.

In the present studies on bile acid secretion, diets containing saturated and unsaturated fats were fed to patients with bile fistulae; in one experiment cottonseed oil was administered parenterally, as an emulsion for intravenous use.

Analytical procedures.

The concentration of bile acid in human fistula bile is about 0.5 - 1%, and its determination is comparatively simple. Several methods have become available during the past 25 years [7,8,9,10,11,12]; others have been listed in the course of a most valuable review on the bile salts by Haslewood [13]. Many are colorimetric, depending for example on the Petterkofer reaction for cholic acid determination [7,12] and on colour development with salicylaldehyde for deoxycholic acid. Absorption spectrophotometry of a solution of bile acid in 65% sulphuric acid is the basis of the Kier [1] and Mosbach [11] procedures, and has been modified by Erikson and Sjövall [14]. Estimations of sulphur and amino-nitrogen have been used as indices of bile acid conjugates (with taurine and glycine). Fluorescence methods are available, and also procedures depending on the lowering of surface tension, as indicated by haemolysis.

Sjövall [15] has contributed a quantitative paper-chromatographic technique for determination of mixtures of bile acids, used in conjunction with his spectrophotometric method. He and Bergström have developed a reversed-phase column-chromatographic procedure [16,17] for semiquantitative resolution of bile acids; Mirvish [18] has lately modified this, obtaining quantitative recoveries. Chromatographic methods have the considerable advantage over purely optical methods that the constituents are isolated before determination. To this end, for example, Wooton [19] resolved bile acid mixtures by adsorption chromatography before estimation by infra-red spectrophotometry. As a corollary to the separation of the bile acids, there is the further

consideration that qualitative information is obtained; there are not two but several bile acids, and significant increase in any one of those normally present in minor quantities would probably be detected chromatographically.

On practical as well as theoretical grounds, Mirvish's procedure appeared to offer particular promise. The alternative, of quantitative paper chromatography, had the advantage that several samples could simultaneously be chromatographed, but one's experience in the past [20,21] had been that quantitative paper methods, while exceedingly useful in the determination of minute quantities of, for example, steroid hormones in body fluids, required the most meticulous attention to detail, and especially to the preparation of paper to produce consistent and low blank values. Mirvish's modification of Sjövall's column method, in combination with a conventional procedure for saponification and extraction, was chosen for the present experiments, (see Appendix 2). Serum cholesterol and faecal fat were determined by methods referred to in Chapter 2.

Subjects, diets and collection of samples.

It was intended originally to use subjects having chronic bile fistulae, those for example who were undergoing treatment for postoperative strictures of the common duct and required indwelling T-tubes for several months. Unfortunately, seven months of "lobbying" in the surgical wards of Groote Schuur and associated hospitals produced only one patient with a long term fistula, in whom some bile continued to reach the duodenum. Attention had therefore to be

diverted to patients in whom fistulae had only recently been established. In view of considerations listed earlier in this chapter, experiments on such subjects appeared entirely acceptable.

The features of the patients are summarized in Table 3, 1.

	Age	Sex	Race	Operation	Interval between operation and first basal observation.
R.D.	50	F	Coloured	Cholecystectomy and choledochostomy following exploration of common bile duct. Carcinoma of gall bladder.	Nil
P.M.	30	F	Bantu	Cholecystectomy and choledochotomy for stone in common duct.	5 days
J.S.	36	M	White	Sphincterotomy for relapsing pancreatitis.	8 days
P.N.	68	M	Coloured	Cholecystectomy and choledochotomy for stone in common duct.	5 days

Table 3, 1.

A T-tube was inserted into the common bile duct; this was indicated in cases R.D., P.M. and P.M., following exploration of the common duct, and served the function in case J.S. of splinting the dilated sphincter of Oddi.

None of the patients had overt infection of the biliary tract. Nor was there clinical evidence of disturbed liver function, and at the time of the experiments the serum bilirubin, proteins, cholesterol, thymol and zinc turbidities were normal. Routine blood

counts and chest X-ray showed no abnormality; electrocardiography was carried out only on case P.N. who had no detectable lesion.

The patients remained under the care of their surgeons during the experiments; the postoperative course of R.D., P.M. and P.N. was entirely satisfactory.

J.S. had had a history of recurrent abdominal pain and backache, and noticed jaundice a week before admission. Preoperatively his serum bilirubin was 2.8 mg.%. A history of alcoholism was not obtained. A diagnosis of chronic relapsing pancreatitis was borne out at operation. Sphincterotomy was not initially successful in re-establishing normal bile excretion, but after a month the T-tube gradually ceased to drain. Pancreatic function, at least at the time of the experiment, was apparently normal as judged by serum amylase, fasting blood sugar, and faecal trypsin (rapid digestion of Congo red-fibrin).

In contrast to the faecal bile acid experiments (Chapter 2), in which all the subjects were male, two females and two males with bile fistulae were studied. The duration of the experiments was necessarily somewhat shorter than in those on normal individuals.

In patients R.D., P.M. and J.S. Wangensteen drainage was used to ensure complete recovery of the bile. This was probably unnecessary, as collection by syphonage was quite satisfactory in P.N. On the basis of stool colour the fistulae were considered to be complete; faecal bile acid excretion was undetectable or minimal in two or more determinations on each patient.

The patients received a basal diet of 2000 Calories containing 5 - 8 g. fat and 75 g. protein. This was commenced as soon as possible postoperatively, but food intake was irregular for up to 60 hours after operation.

Of the oils administered, two were similar to those employed in studies of faecal bile acid excretion : sunflower seed oil (SSO) of refined grade was used from a single batch, and had an iodine value of 135. Coconut oil (HCO) was hydrogenated to an iodine value of 6. The oils were given in divided doses, with meals, the intake being 75 g. per day; they were well tolerated. The third oil, cottonseed oil (CSO), iodine value 115, was obtained in a preparation for intravenous use ("Lipumol"), by courtesy of Upjohn Laboratories. It was emulsified with Pluronic F68 and a small amount of soybean phosphatide, and also contained 2½% dextrose. This emulsion, on intravenous administration, has been shown to lower the serum cholesterol significantly in four subjects, who showed no untoward reaction to the infusion (Gordon and Lewis [22]). A comparable preparation, containing the emulsifying agents and dextrose but no CSO, failed to affect the serum cholesterol in the same subjects. In the present study an infusion containing 100 g. of CSO was administered daily over 4 hours commencing at the beginning of each bile collection period.

Blood was drawn at the same time each morning for cholesterol determination. Bile was collected in 24 hour batches using thymol as preservative. Faeces were collected over 24-hour periods, for fat determination in subjects 2 and 3, and for bile acids in all four.

Results.

1) Case R.D. Figure 3, 1. Table 3, 2.

A constant diet of 2000 Calories, and 5 - 8 g. fat, was taken except on the first postoperative day. The bile acid and cholesterol output were computed for the first day from the 19 hour collection following operation; 24-hour collections were subsequently made. Cholic acid secretion doubled during the first 24 hours, and showed little variation after the third day. Secretion of "deoxycholic acid" was low on the first day after operation, and remained relatively inconstant. The deoxycholic acid peak presumably contained also chenodeoxycholic acid, these acids together amounting to about two-thirds the output of cholic acid once a steady state had been reached. No other substances were detected in the chromatograms.

Post-operative day.	Diet	Cholic Acid g./24 hrs.	Deoxycholic Acid g./24 hrs.	Biliary Cholesterol mg./24 hrs.	Serum Cholesterol mg./100 ml.
1	2000 Calorie	1.46	1.40	522	172
2	(Basal)	2.80	2.62	408	
3		3.03	2.10	430	
4		4.04	2.94	390	158
5		3.77	2.35	305	
6		5.16	3.81	426	
7		4.86	3.76	406	
8		4.21	2.82	445	150
9		4.77	3.99	409	
10		3.89	3.64	363	
11		3.43	1.90	431	
12		4.23	2.46	308	155
13		4.48	2.65	396	

Table 3, 2. Case R.D.

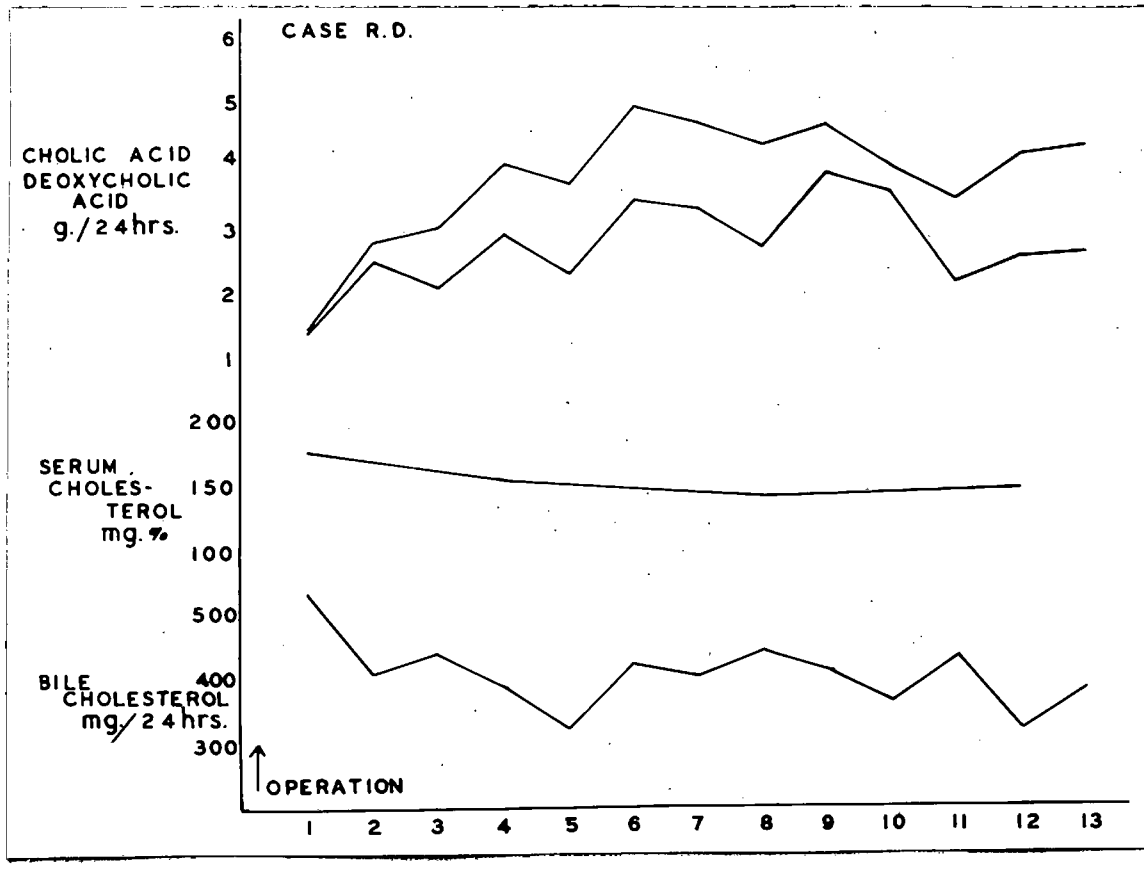

Figure 3, 1. The pattern of bile acid secretion after establishment of a bile fistula.

2) Case P.M. Figure 3, 2. Table 3. 3.

After two observations on bile composition had been made with the patient on basal diet, HCO 75 g./24 hours was administered for 4 days; it had no effect on bile acid or cholesterol output, but increased the serum cholesterol. When SSO was substituted for HCO at the same dosage, a four-fold increase in cholic acid secretion commenced within 24 hours. During the basal period, "deoxycholic acid" secretion was roughly one-third that of cholic acid; it showed no significant change throughout the experiment. Bile cholesterol output showed no recognisable pattern. Faecal fat determinations showed the absence of a gross degree of steatorrhoea. The serum cholesterol rose moderately during the HCO period, and feel steeply when SSO was fed.

		Cholic acid g./24 hrs.	Deoxycholic acid g./24 hrs.	Biliary cholesterol mg./24 hrs.	Serum cholesterol mg./100 ml.	Faecal Fat g./24 hrs.
Sept.						
9	Basal	6.63	2.42	363		
10	Basal	8.65	2.52		156	6.2
11	Basal + hydrogenated coconut fat.	6.20	2.66	310	155	
12		5.31	1.58	315	161	7.5
13		6.22	1.65	289	169	7.4
14		7.26	2.58	348	164	7.7
15	Basal + 100 g. sunflower seed oil.	20.68	2.87	360		
16		19.78	1.81	333	137	12.0
17		26.50	1.71		134	
18		23.14	1.68	301		11.1

Table 3, 3. Case P.M.

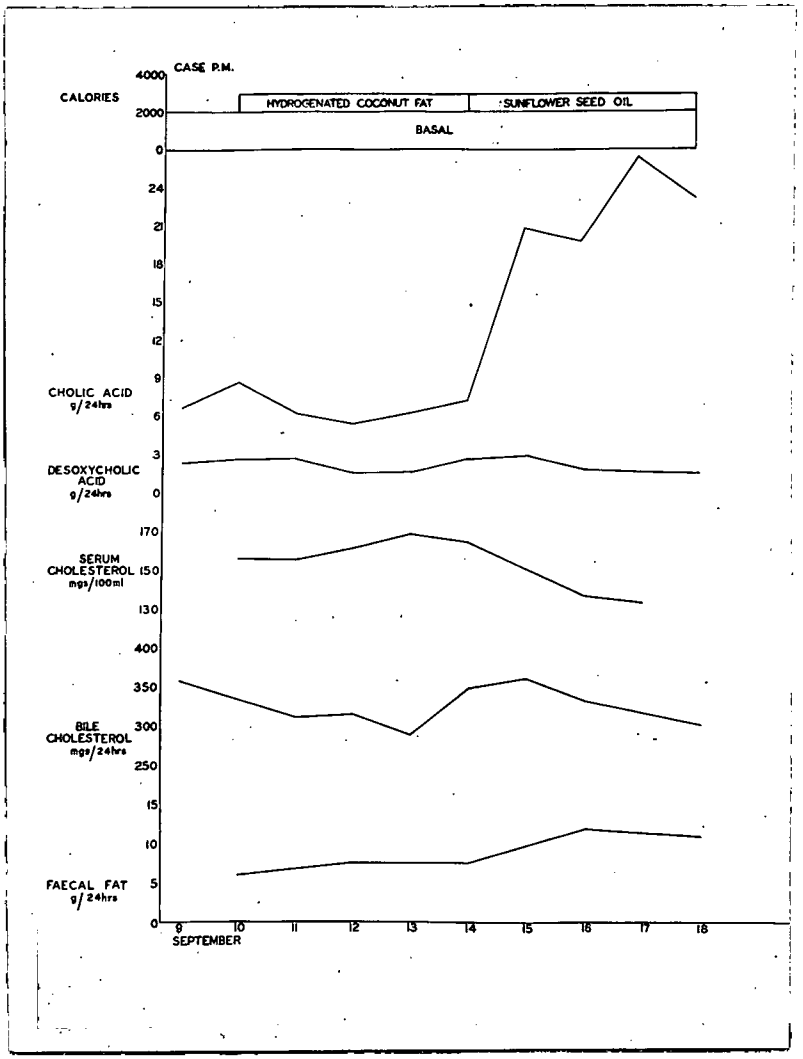

Figure 3, 2. Case P.M. Changes in bile acid and cholesterol secretion during administration of hydrogenated coconut oil and sunflower seed oil.

3) Case J.S. Figure 3, 3. Table 3, 4.

This experiment was similar to the previous one except that the order of administration of SSO and HCO was reversed. SSO produced a rapid increase in cholic acid output, accompanied in this case by a slower and relatively minor rise in deoxycholic or chenodeoxycholic acid; much smaller amounts of these dihydroxy-acids were secreted by this patient. Reduction in the serum cholesterol began 24 hours after the change in cholic acid secretion. Replacement of SSO by HCO reversed the downward trend in the serum cholesterol; cholic acid output fell rapidly, but did not reach the basal level in three days. Biliary cholesterol excretion showed no clear-cut trend beyond a possible tendency to parallel the serum cholesterol. Faecal fat excretion only slightly exceeded the normal.

	Diet	Cholic acid g./24 hrs.	Deoxycholic acid g./24 hrs.	Biliary cholesterol mg./24 hrs.	Serum cholesterol mg./100 ml.	Faecal Fat g./24 hrs.
Sept.						
9)	Basal	6.7	0.6		208	
10)	Basal	7.8	0.4	450	215	2.9
11)	Basal +	21.8	0.5	505	218	3.0
12)	100 g.	21.1	1.6	396	190	6.2
13)	sunflower	18.6	2.3	304	180	8.4
14)	seed oil	22.0	2.5	309	186	7.9
15)	Basal +	14.9	1.9	302	170	
16)	100 g. hydro-	11.1	0.7	492	177	7.1
17)	genated coconut fat.	9.2	0.8	306	187	

Table 3, 4. Case J.S.

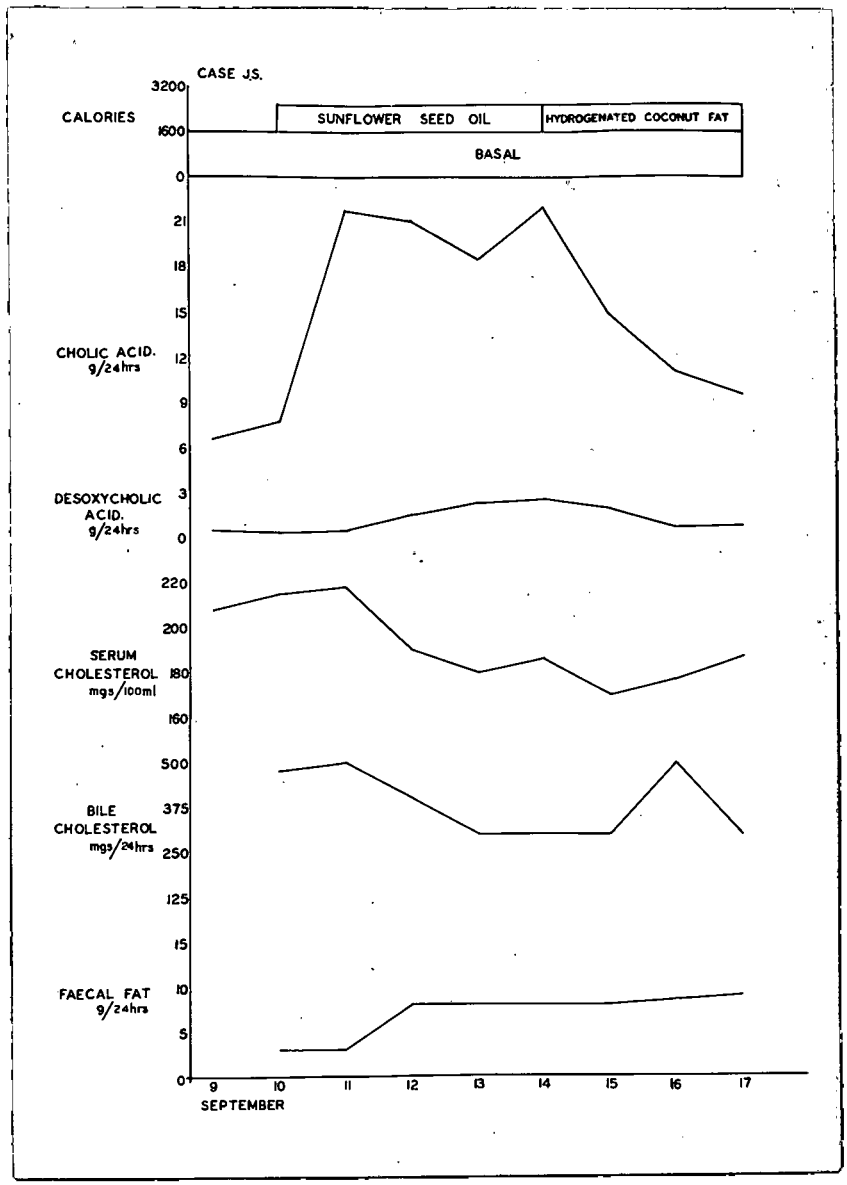

Figure 3, 3. Case J.S. Changes in bile acid and cholesterol secretion during administration of sunflower seed oil and hydrogenated coconut oil.

4) Case P.N. Figure 3, 4. Table 3, 5

A somewhat longer basal period showed remarkably constant bile acid secretion, the ratio of cholic to dihydroxy-acids approximating to unity. Intravenous infusion of CSO daily for 3 days produced a distinct fall in the serum cholesterol. Cholic acid output increased considerably, while "deoxycholic acid" secretion remained unchanged. The increase in cholic acid production was less steep than in the SSO experiments, yet the output fell abruptly to the basal level on the day following the last CSO infusion.

	Diet	Cholic acid g./24 hours	Deoxycholic acid g./24 hours	Serum cholesterol mg./100 ml.
Oct 20		2.04	1.44	
21				141
22	Basal	1.88	1.51	
23		1.80	1.45	146
24		2.10	2.20	142
25	Intravenous cotton-seed oil 100 g.	4.01	1.68	
26		4.80	1.86	101
27		7.40	1.27	100
28	Basal	2.25	1.56	

Table 3, 5. Case P.N.

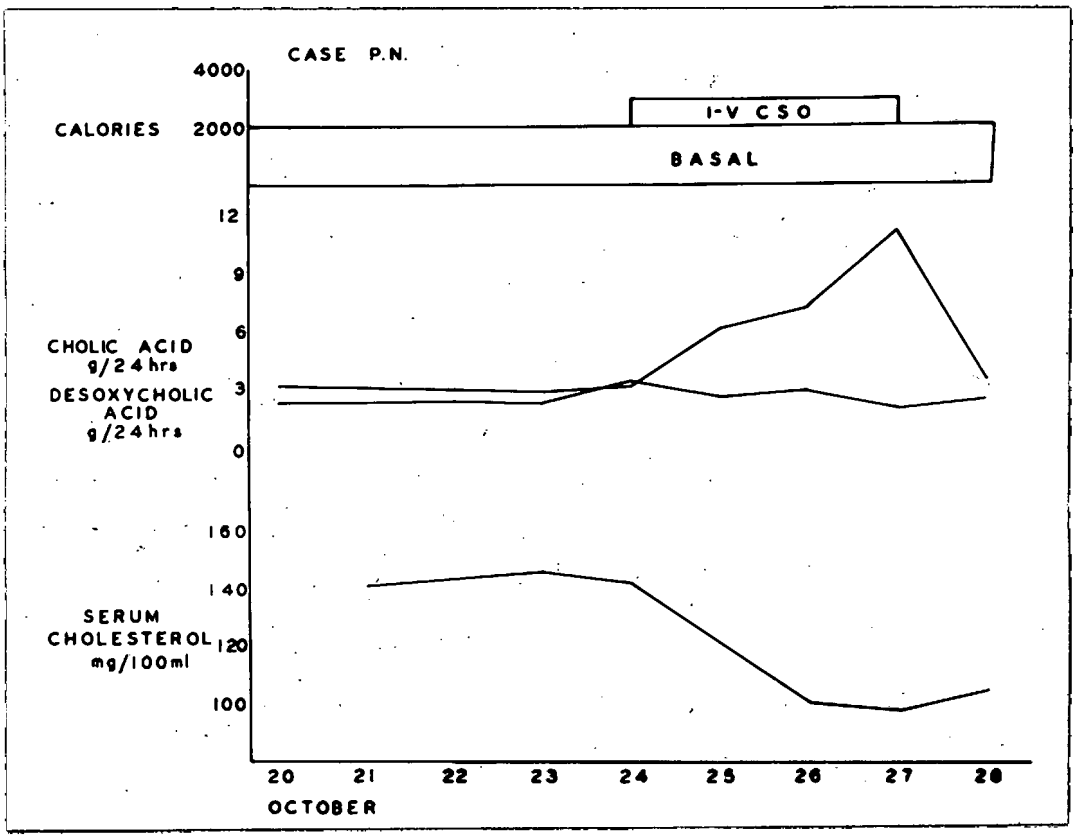

Figure 3, 4. Case P.N. Changes in bile acid secretion during intravenous infusion of cottonseed oil.

DISCUSSION

In these studies on bile fistula subjects, administration of SSO by mouth, or of intravenous CSO, produced within 24 hours an increase in cholic acid output which reached 3 - 4 times the basal level and preceded or accompanied a significant reduction in the serum cholesterol. A small associated increase in output of "deoxycholic acid" took place in one of three experiments.

When HCO feeding followed the basal diet, no change was noted in bile acid production, and when this oil followed SSO, the elevated cholic acid output fell rapidly.

Essential similarity was apparent between these observations and those on the faecal bile acid excretion of normal subjects on similar diets. The analyses of bile was carried out by a highly selective and accurate procedure; and the results obtained in studies on subjects with fistulae indicated

1) that the material determined in faeces was in fact bile acid, despite the relative lack of selectivity of the method used.

2) Secondly, the present experiments were carried out on subjects whose fistulae were completely effective; phenomena associated with reabsorption of bile acids were virtually ruled out. The changes in bile acid output, both in bile and in faeces, therefore represent variations in the rate of hepatic secretion rather than in the degree of reabsorption.

3) The time relationships of the rise in cholic acid secretion and the fall in serum cholesterol are clear only in the

experiment on J.S.; while the former reached its peak within 24 hours of introducing SSO, the latter began to fall 24 hours after altering the diet. In the studies on P.M. both changes commenced during the first day of SSO feeding, while the critical serum cholesterol determination in P.N., 24 hours after the first infusion was not made owing to loss of the sample. In all three cases, an increased cholic acid secretion commenced within 24 hours of introducing the oil, while reduction of serum cholesterol by oral and intravenous oils generally begins after 2 - 3 days. If the sequence observed in J.S. is typical, it supports the notion of a causal relation between increased bile acid synthesis (i.e. cholesterol catabolism) and reduction of serum cholesterol.

What is the relation of dietary oils to the mechanisms regulating the serum cholesterol? It must be emphasized that the present investigations were based on transient-state observations, being concerned with the phenomena associated with rapidly changing cholesterol levels. Diet-induced variations in the concentration of this sterol could in theory be due to changes in the rate of its synthesis, absorption, excretion, catabolism, deposition in the tissues, in the process of cholesterol "trapping" (Friedman and Byers [23]) or in a combination of such mechanisms. The following points are relevant to this problem:

1) Karvinen [24] has shown that cholesterol absorption was not depressed by ingestion of unsaturated oils.

2) Significant changes in faecal sterol excretion could not be demonstrated during dietary manipulation of the serum cholesterol in seven subjects (Chapter 2); changes in bile cholesterol excretion, as determined in the present investigation, could not explain changes

in the serum level. On the other hand, Hellman [29], in a paper analysed in Chapter 2, has ascribed serum cholesterol reduction by corn oil to its increased faecal excretion. Kinsell et al [32] was unable to detect reciprocal changes in the serum cholesterol and the faecal sterol excretion on feeding a variety of preparations containing unsaturated fatty acids.

3) The evidence regarding the effect of unsaturated oils on cholesterol synthesis is contradictory. Wood and Migicofsky [30] have shown that rat liver slices synthesize cholesterol from ^{14}C-labelled acetate at a reduced rate in the presence of certain concentrations of sodium linoleate. Whitney and Roberts [31] administered CSO to rats for several weeks, then sacrificed the animals and measured the incorporation by liver slices, of labelled acetate into cholesterol and glycogen. In comparison with liver slices from a control group, the former showed accelerated cholesterol synthesis at the expense of glycogen formation. This experiment, in which the oil was presented as a dietary constituent to intact animals, appears less artificial than a study based on incubation of liver slices with sodium linoleate. Merrill [33] has referred recently to increased synthesis of hepatic cholesterol on feeding 10% linoleic acid in the diet of rats. Kinsell et al [32] have used the findings of Wood & Migicofsky to explain the effect of dietary oils on a basis of decreased cholesterol synthesis, and dismissed theories based on cholesterol excretion as being inspired by "an archaic belief that cholesterol was bad and must be exorcised". Alfin-Slater et al [34] could not demonstrate any change in cholesterol synthesis on feeding CSO.

Later in this chapter it will be shown that the increase in bile acid secretion on feeding SSO is far greater than is necessary to account for the fall in serum cholesterol level. If, as seems probable, bile acids are derived mainly from cholesterol it would appear likely that SSO increases the synthesis of this sterol.

From the foregoing discussion it may be inferred that no existing theory accounts adequately for the action of certain vegetable oils in the serum cholesterol.

> In the light of the strikingly increased secretion of bile acids during SSO and CSO administration, I would submit that these oils reduce the serum cholesterol by a mechanism which involves increased catabolism of this sterol to bile acid.

The efficacy of intravenous CSO in increasing cholic acid synthesis and reducing the serum cholesterol is of interest in identifying the site of action of those vegetable oils which share these effects, assuming that they possess a common mode of action. Orally-administered oils could conceivably exert their effect in the alimentary tract itself, by a choleretic effect not shared by saturated fats. That parenteral administration of an oil should also stimulate bile acid secretion diverts attention from the intestine to the level of hepatic cellular metabolism; it is felt that the existing data favour the view that cholesterol is in some way rendered more susceptible to catabolism, or that the catabolic system is directly stimulated by the oils administered. Further studies on these lines appear in Chapter 4.

Conversely, the elevation of serum cholesterol levels by HCO was not associated with significant reduction of bile acid secretion as assessed in the fistula experiments and the studies of faecal bile acids. The rising serum cholesterol following ingestion of this saturated fat was not explicable on the basis of decreased cholesterol catabolism. It is generally believed that dietary saturated fats increase cholesterol synthesis; such fats are split by β-oxidation

to acetyl coenzyme A, and the latter is known to be converted to cholesterol in vitro by many tissues. Van Bruggen et al [25] showed that fasting decreases cholesterol synthesis. However, Hellman et al [29] have claimed that butter oil elevates the serum cholesterol by reducing its intestinal excretion.

It is simple to demonstrate that far more bile acid is secreted during administration of SSO and CSO than is necessary to account for the cholesterol leaving the circulation, in fact a ten to fifteen-fold excess. It will be recalled that in comparable studies on the faecal bile acid excretion of normal subjects, the increased elimination of bile acid was equivalent to 115% of the cholesterol removed from the blood. This discrepancy is not readily explained. Bile acid secretion in fistula subjects is probably far higher than normal [3], even on a low-fat diet, and one is reluctant to postulate an excessive responsiveness of such subjects to dietary stimuli. The absence of bile salt reabsorption may be relevant; while it is known that (over short periods of time) bile salts are choleretic, their behaviour over a longer time course may be different. It is not easy to find another explanation for the marked increase above normal in bile acid secretion which occurs in the 3 or 4 days following establishment of a bile fistula [3]; if one accepts that this increase is an attempt at compensation for the loss of bile acid normally readsorbed by the intestine, the corollary is an assumption that bile acid secretion is normally held in check by a negative feedback mechanism. Such considerations might account for the excessive increase in bile

acid secretion when SSO or CSO are administered to fistula subjects, but the many assumptions necessary render this line of argument exceedingly tenuous.

Even in intact subjects, however, the increment in faecal bile acid excretion is greater, on a molar basis, than the loss of cholesterol from the circulation. There are two possible inferences:

a) Cholesterol is being mobilized from the tissues; it is known that the cholesterol fo plasma and the tissues (particularly the liver) are in dynamic equilibrium [26].

b) Synthesis of cholesterol is increased; the evidence for and against this effect has been discussed.

It seems probable that both synthesis and catabolism of cholesterol are accelerated by feeding certain oils, the latter effect being greater than the former. A notable analogy may be drawn with cholesterol metabolism in hyperthyroidism; a tendency to a low serum cholesterol is evident despite increased cholesterol synthesis (Kurland and Lucas [27]). Weiss and Marx [28] have demonstrated in animal experiments that thyroid hormone stimulates the degradation of cholesterol to acid derivatives.

The foregoing discussion shows that further experimentation is needed, notably long-term studies on normal and bile fistula subjects which would decide whether mobilization of cholesterol from the tissue or increased synthesis is responsible for the discrepant figures for bile acid excretion and loss of cholesterol from the blood. The use of isotopic labelling of cholesterol will hasten a fuller understanding of

the action of dietary oil on cholesterol metabolism; Hellman et al [29] have made a useful start in this direction (see Chapter I). Identification of the constituent or constituents of SSO, CSO and related oils, responsible for their action on cholesterol metabolism, will greatly facilitate our approach to the biochemistry of this sterol.

SUMMARY.

Further investigation into the mode of action of certain dietary oils in reducing serum cholesterol levels took the form of short-term feeding experiments in subjects with bile fistulae, to facilitate the study of bile acid secretion without intervening variables. The use of such subjects was justified by showing constancy of bile acid production after a marked rise during the immediate postoperative period, and by demonstrating adequate and fairly constant fat absorption. Hydrogenated coconut oil failed to alter bile acid secretion, but oral sunflower seed oil and intravenous cottonseed oil (both highly unsaturated) led to an immediate 3 or 4-fold increase in cholic acid secretion and produced a fall in the serum cholesterol level. Bile cholesterol excretion did not show significant changes. The findings, obtained by use of a highly selective analytical method for bile acid determination, confirmed those in comparable studies of faecal bile acid excretion (Chapter I) and the theory was proposed that certain dietary vegetable oils reduce the serum cholesterol by a mechanism involving accelerated catabolism of this sterol.

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CHAPTER 4.

Isolation and Analysis of cholesteryl esters; the effect of dietary fat on plasma cholesteryl ester composition.

The foregoing studies suggested that administration of certain vegetable oils promotes the catabolism of cholesterol to bile acid. The present experiments describe an attempt to identify an intervening link in the causal sequence. As our understanding of the mode of action of such oils is hampered by ignorance of the nature of their active constituents, a resumé of current views on this topic is necessary by way of introduction.

Sunflower seed, cotton seed and corn oil are composed mainly of triglycerides in which the predominant fatty acid is linoleic (54 - 65%; 50% and 54% respectively [1]); oleic and saturated (mainly palmitic) acids are present too. In the unsaponifiable matter, (0.3 - 0.5%, 0.5 - 2.0% and 0.5 - 3.0%) sitosterol is conspicuous, but other phytosterols, and tocopherols and pigments, are also found.

Trilinolein and ethyl linoleate have been shown to lower the serum cholesterol in man [2,3,4]. Massive doses of β -sitosterol have the same property [5]. (Serum cholesterol-elevating properties were found in the unsaponifiable fraction of butter oil [5]). In interpreting such experiments one must bear in mind how difficult it is to remove the unsaponifiable material completely from natural oils [6].

It is likely that both the polyunsaturated fatty acid and

unsaponifiable fractions of certain oils are able to reduce serum cholesterol levels. Wilken's data[7] on assay in rats indicate that roughly half the serum cholesterol-lowering effect of sunflower seed oil resides in the unsaponifiable fraction.

This eclectic point of view is not an isolated instance of a metabolically related nutrient and micronutrient being found in the same foodstuff. Most natural carbohydrate foods contain members of the vitamin B group, which are largely coenzyme constituents required in carbohydrate metabolism.

Little is known of the biochemistry of sitosterol. Partly absorbed by the intestinal mucosa[8], and partly excreted in the faeces as sitocoprostanol[9], it blocks cholesterol absorption; this is presumably by receptor competition.[10,11].

The relation of certain polyunsaturated fatty acids to mammalian metabolism, on the other hand, has lately received great attention. Saturated acids and oleic acid are synthesized from acetate and therefore from fat and carbohydrate; amino-acids readily form the branched-chain fatty acid precursors of cholesterol[12]. But acids with more than one double bond are "essential" in that they cannot be synthesized, at least by rats, mice, guinea pigs, dogs, hamsters and chickens[13-16]; they are interconverted to a limited extent. Linoleic acid may be further desaturated to arachidonic acid, while linolenic acid is the source of pentaenoic and hexaenoic acid[17,33]. They are essential also in that an inadequate supply produces a recognisable deficiency syndrome in certain animals. This was first described in 1929 by Burr and Burr[18-20], and lately reviewed by Deuel[21]. Rats develop skin lesions, caudal necrosis,

renal tubular damage and myocardial fibrosis. Capillary permeability is increased. Growth rate is reduced and the reproductive process is impaired. Resistance to noxious stimuli such as X-irradiation is reduced. Requirements are increased by a high intake of saturated fat, which may lead to a conditioned deficiency.[22] It is noteworthy, too, that cholesterol feeding may precipitate essential fatty acid deficiency [23]. A high degree of stereochemical specificity is another characteristic of these fatty acids.

From this interesting aspect of animal nutrition, essential fatty acids have recently entered the medical limelight. Their rôle in human disease has been speculated upon by Sinclair [24-26]; coronary heart disease, bronchial carcinoma, leukaemia, hypercalcaemia of infants, and eczema are suggested to be, at least in part, aspects of essential fatty acid deficiency. Bronte-Stewart et al [27] contended that the serum cholesterol level was largely determined by the ratio of dietary saturated to unsaturated fats; they suggested that the rising incidence of coronary heart disease might be related to an increase in this ratio due to changing culinary habits. Such views have been challenged by Yudkin on demographic grounds [28]; Keys [29] has pointed out that atheroma has not been observed in rats deficient in essential fatty acids.

In order to lower the serum cholesterol in experiments on man, it has generally been necessary to feed quantities of oil providing a dietary supplement of 20-30 g. per day of linoleic acid; Kinsell [30] is alone in claiming an effect from only 2 g. of the acid. This contrasts with the few milligrams needed by the rat to prevent the deficiency syndrome. Although 25 years have elapsed since Hansen [35] first claimed

that corn oil feeding was often effective in reducing infantile eczema, (and that such patients have low serum linoleate and arachidonate levels [36]), no definite proof of the existence of a human essential fatty acid deficiency syndrome has been adduced. It is true that the serum cholesterol of White subjects on "Westernized" diets responds more dramatically than that of Bantu subjects to administration of unsaturated oils [37]. Yet it is unwise at present to assume that the action of vegetable oils in reducing serum cholesterol levels in man is necessarily related to essential fatty acid deficiency as seen in animals.

The human body contains an appreciable amount of polyunsaturated fatty acids, of chain length C18 to C22 and possessing 2 to 6 double bonds:

(a) These are present largely in the element constant (Terroine), that part of the body lipid often referred to as structural lipid and believed to be an essential constituent of protoplasm [38]. These fatty acids are found in all cells - in mitochondria, microsomes, nuclei, and the cell membrane; in the intracellular organelles, the fatty acids include 20% of tetraenoic acids [31]. They are present mainly in the form of phospholipid, but also as cerebroside and cholesteryl ester [32]. Prolonged exercise has been shown by Bloor [45] to increase the phospholipid and cholesterol content of muscle, pointing to a possible functional rôle of the element constant.

(b) Of great interest in the present context is the fact that the fatty acids of plasma cholesteryl esters (plasma CEFA) are highly unsaturated. In bovine plasma, Kelsey and Longenecker [39] showed that 62% of the CEFA was linoleic acid: Bloor [40] found a mean iodine value

of 158 in CEFA of normal human plasma. Using paper chromatography, Michalec [41] showed human plasma CEFA to contain linoleic acid; there was also a spot of Rf value corresponding to that of palmitic and oleic acids. By contrast, Keegan and Gould [42], offering somewhat inconclusive analytical data, claimed to have crystallized cholesteryl oleate in amounts which would indicate that this formed more than half the plasma cholesteryl ester.

If the polyunsaturated fatty acid content of the human body is limited by diet, it would be reasonable to expect changes in plasma CEFA composition on feeding fats of different degrees of saturation. The nature of the dietary fat is reflected in the composition of liver CEFA [34].

Sinclair [24] has suggested that in "essential fatty acid" deficiency, cholesterol becomes esterified with unusual fatty acids, and is consequently "less readily disposed of in the tissues". He has shown deposition of cholesteryl esters in the epidermis of rats maintained on diets free from fat and sterol [43].

Highly unsaturated esters may be more readily transported, or more susceptible to catabolism to bile acid, than less unsaturated ones. Increase in cholesteryl ester unsaturation is therefore a possible link in explaining the action of SSO on the serum cholesterol level. Cholesteryl esters obtained from rabbits fed saturated fats are less readily hydrolysed than those from animals given unsaturated fat [44].

To study the effect of dietary fats on plasma CEFA composition, feeding experiments were carried out on two subjects. The analyses provided many technical difficulties and were laborious in the extreme. The procedure is discussed in Appendix 3.

Experimental.

The subjects were studied in the Metabolism Ward, and were free from active disease by the criteria listed in Chapter 2. Energy expenditure was reasonably constant. They received a basal diet containing 5-8 g. fat mainly of vegetable origin; hydrogenated coconut oil (HCO), I.V.6, was substituted isocalorically for carbohydrate, and was later replaced by sunflower seed oil (SSO), I.V.136. In the first experiment the dose of each oil was 100 g. per day, and in the second 150 g.

Blood was drawn before breakfast for CEFA analysis and for total serum cholesterol determination by the method of Anderson and Keys. Plasma was separated within 30 minutes, and the analyses commenced immediately, as described in Appendix 3.

Results.

Experiment 1, (Figure 4, 1; Table 4, 1;)

Only five observations were made, a flaw in the analytical technique vitiating some data.

Initially low, the serum cholesterol increased by 35% when HCO was fed, and fell 30% below the basal level during SSO administration.

Reciprocal changes took place in the plasma CEFA iodine value: cholesteryl esters became somewhat less unsaturated during HCO feeding; in the SSO period the iodine value increased to 46% above basal. This was due mainly to variations in the percentage of dienoic (linoleic) and tetraenoic (arachidonic) acids in CEFA; the trienoic (linolenic), pentaenoic and hexaenoic acid percentages showed little variation. Fatty acids with 5 and 6 double bonds are exceedingly unstable, and as pure samples could not be obtained to verify the isomerization technique, it is possible that the analytical data for these acids were unreliable.

	Basal	Basal + HCO		Basal + SSO	
Number of days on each diet	5	2	5	8	11
Serum cholesterol	131	154	170	107	96
CEFA iodine value	139	120	107	144	204
% linoleate	41	38	32	48	66
% linolenate	3.6	2.8	2.9	3.1	4.2
% arachidonate	11.5	7.3	5.7	12.0	16.5
% pentaenoate	1.1	2.1	1.8	0.2	1.7
% hexaenoate	1.4	1.7	1.3	1.3	2.4

Table 4, 1.

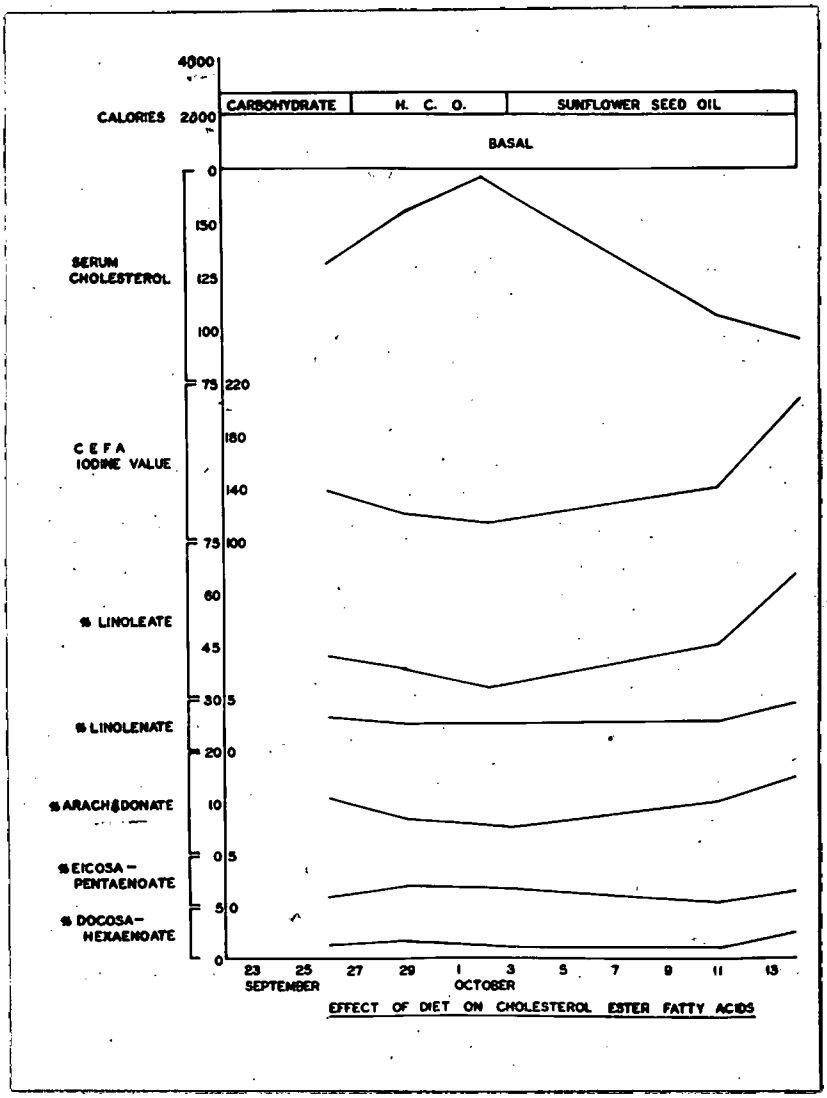

Figure 4, 1. Plasma cholesterol ester fatty acid composition during administration of hydrogenated coconut oil and sunflower seed oil.

Experiment 2, (Figure 4, 2; Table 4, 2;)

Two basal observations were made, and three during each fat-feeding period.

Both total and ester cholesterol levels rose steeply when HCO was given, and fell rapidly in the SSO period; the ester fraction appeared far more responsive than free cholesterol to dietary change.

Variations in the CEFA iodine value were due entirely to changes in the percentage of linoleic and arachidonic acids. These fell somewhat gradually on HCO administration, and rose abruptly when SSO was substituted. There was thus a negative correlation between the plasma ester cholesterol and the CEFA iodine value.

It is worthy of note that in the SSO period the increase in unsaturation preceded the fall in cholesterol levels (day 16); when HCO was introduced they appeared to change simultaneously.

	Basal		Basal + HCO			Basal + SSO		
Number of days on each diet	4	6	2	4	8	2	4	10
Serum cholesterol	135	132	139	159	180	170	122	101
Plasma ester cholesterol	82	75	84	107	125	123	72	61
CEFA iodine value	154	150	143	135	122	159	186	199
% linoleate	52	48	45	40	36	45	55	58
% linolenate	2.6	2.8	2.6	2.8	2.1	2.0	2.4	3.5
% arachidonate	9.1	9.9	8.7	7.9	6.3	13.1	16.5	16.8
% pentaenoate	1.2	1.8	1.6	2.0	1.9	1.6	2.2	1.9
% hexaenoate	1.7	1.6	1.0	1.2	1.3	1.1	1.9	2.4

Table 4, 2.

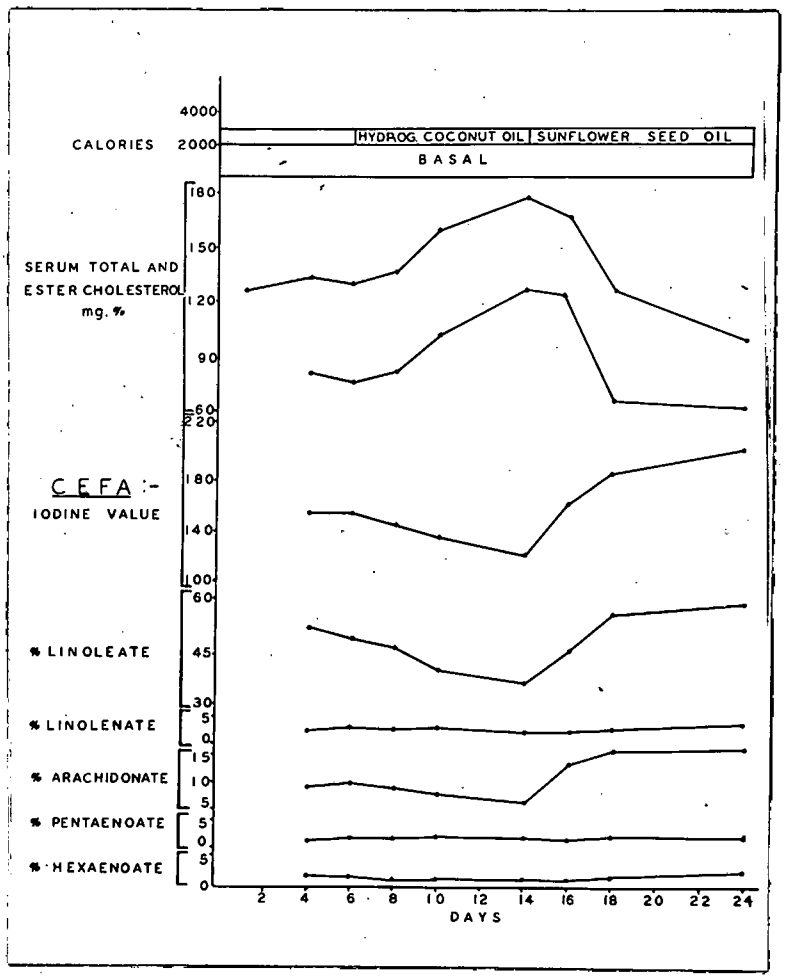

Figure 4, 2. Plasma cholesterol ester fatty acid composition, and total and ester cholesterol levels, during administration of hydrogenated coconut oil and sunflower seed oil.

DISCUSSION.

The most important observation in these studies is that dietary oils alter the quality as well as the quantity of cholesterol compounds in the blood. Plasma CEFA composition is remarkably sensitive to the type of dietary fat. On a diet providing 60-70 g. of linoleic acid per day, its percentage and that of its derivative arachidonic acid rose steeply. In fact the plasma CEFA are more readily influenced by the diet than total plasma fatty acids appear to be [3], and reach iodine values far higher than that of the dietary oil.

CEFA iodine values during the basal period were somewhat less than those observed by Bloor in normal subjects; this is probably due to the low intake of polyunsaturated fatty acids during that period. The further reduction in iodine value on feeding HCO is readily explained by increased cholesterol synthesis at a time when linoleic acid intake is particularly low; this is analogous with the precipitation of essential fatty acid deficiency in rats by feeding saturated fat [22], or cholesterol itself [23]. Perhaps all this may be accounted for by the law of mass action, esterification of cholesterol most readily taking place with the most plentiful substrates.

There was a striking reciprocal relationship between the plasma CEFA iodine value and the serum cholesterol level, in particular with that of the ester fraction. In Experiment 2 it appeared that SSO feeding led to a rise in the percentage of cholesteryl arachidonate and linoleate before the serum cholesterol fell. This was compatible with the hypothesis that increased CEFA unsaturation led to greater susceptibility of the ester cholesterol to catabolism. However, it is also

possible that a high intake of polyunsaturated fatty acids in some way facilitates such metabolic activities; arachidonic acid is rapidly deposited in the liver on feeding linoleic acid [17], and it is known that the former normally constitutes 20% of the fatty acids in the phospholipids of mitochondria and microsomes. These organelles are the sites of a vast number of metabolic processes.

Further analysis of this problem will necessitate the following experiments:-

1) Observation of the time relations between the rises in CEFA iodine value and bile acid secretion on administering SSO or a similar oil. If suggestive:-

2) A study of the rate of clearance from the circulation of parenterally administered esters of cholesterol with various fatty acids, at the same time following bile acid secretion.

3) Studies of the rate of cholesterol catabolism in liver slices obtained from animals maintained on various diets.

Kinsell et al [4] have recently described studies in which CEFA iodine value was elevated by feeding safflower oil, trilinolein, ethyl linoleate, and a phospholipid rich in arachidonic acid; it was reduced by triolein and ethyl oleate. On replacing ethyl linoleate by ethyl oleate in the diet, the CEFA iodine value fell before the plasma cholesterol, phospholipid and neutral fat rose. Kinsell regarded these results as supporting the notion that linoleic acid is essential in human nutrition.

SUMMARY

Controlled feeding experiments have demonstrated that a high intake of hydrogenated coconut oil (which is almost saturated) reduces the iodine value of plasma cholesteryl ester fatty acids; sunflower seed oil, which contains about 60% of linoleic acid, has the reverse effect. Thus reciprocal changes take place in the serum cholesterol and the unsaturated fatty acid content of its esters. The fatty acids accounting for this change in iodine value are linoleic and arachidonic acids.

Diet-induced changes in the total serum cholesterol, in a single short-term study, were due almost entirely to variations in the level of the ester fraction.

The rise in unsaturation of the esters is thought to precede the change in the serum cholesterol level, and a possible causal relationship between these events is discussed.

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CHAPTER 5.

A comparative study of plasma cholesteryl ester composition in Bantu and White subjects and in patients with coronary heart disease.

The nature of dietary fats has been shown to influence the composition of plasma cholesteryl ester fatty acids (CEFA). As numerous workers have considered the quantity and more recently the quality of dietary fat to be important aetiological factors in coronary heart disease, it was decided to study plasma CEFA in patients with this condition. Furthermore, the virtual freedom of the Bantu from coronary heart disease [1], which has been attributed to their dietary habits [2,3], suggested an investigation into CEFA composition in that group.

The aetiology of atherosclerosis is almost certainly multiple. Current interest is largely focussed on the hypothesis that excessive quantity [4,5,6] or incorrect quality [3,7,8] of dietary fat produces an abnormality in the blood lipids which causes the arterial disease. This may be true; but the evidence is circumstantial. It is all too easy to extrapolate from epidemiological data and feeding experiments to "the cause of coronary heart disease". Aetiological theories will be discussed later, in relation to the present findings. For the moment it should be said that belief in a causal connection between abnormal blood lipids and atherosclerosis rests chiefly on the observation that the latter is predisposed to in diseases associated with hypercholesterolaemia-diabetes, myxoedema, xanthomatosis, and familial hypercholesterolaemia. Progeria has recently been added to this list [9]. In patients with established coronary heart disease, secondly, the serum

cholesterol [6,10], β-lipoproteins [11] and low-density β-lipoproteins of flotation rate Sf 12-20 and 35-100 are commonly high [10]. This applies also to the cholesterol/phospholipid ratio [12], (which correlates closely with the serum ester cholesterol). Such evidence based on association is far from proving that the relationship is causal; the correlation between total blood and arterial lipid concentrations is poor, if one is to judge from the small volume of published work. Paterson and Derrick [13] have lately described their failure to find a relation between the total serum cholesterol (serially determined in people in the age group 60-89 years), and the total lipid subsequently extracted from large segments of their coronary arteries. The arterial wall is capable of synthesizing cholesterol [38], and it is conceivable that this process is influenced by some of the same factors (dietary and otherwise) as affect the blood lipids.

In the present study, CEFA isolated from atheromatous coronary arteries were analysed, to determine the qualitative relationship between them and the circulating cholesteryl esters in patients with coronary heart disease.

The survey to be described was a collaborative project; several biochemical, haematological, social and clinical modalities were investigated. Analyses of plasma CEFA were carried out "blind", and the author therefore made no direct contribution to the arduous task of selecting the subjects of the study, apart from assisting initially in the design of the experiment.

One's indebtedness must be expressed to Dr. C. Merskey, Dr. V. Schrire, Dr. H. Gordon and Dr. H. Nossel for making this survey possible.

Experimental.

The analytical techniques were those described in Appendix 3. Fractionation of plasma lipids and CEFA analyses were performed in batches of 6 samples, identified by code number alone. Each run required eight working days, including calculation and the preparation of reagents. The number of analyses was therefore smaller than would otherwise have been desirable.

The three groups studied were as follows :-

a) Twelve White patients who had had one or more episodes of cardiac infarction. The diagnoses were verified electrocardiographically. At the time of the survey none were receiving anticoagulants, and all were ambulant. Dietary histories were obtained, and no patient was included whose dietary habits appeared to have changed significantly since the coronary occlusion. The mean age of the group was 40 years. No subject had complicating or intercurrent disease, diabetics in particular being excluded.

b) Twelve Bantu subjects. Clinically they were in good health, and this was confirmed radiologically, by blood counts, measurement of serum proteins thymol and zinc turbidities, urine analysis and electrocardiography. Their mean age was 35.5 years.

Bantu men commonly spend periods of several months in the larger towns of the Union, to which they are attracted by the prospect of employment; they return at intervals to their families and tribal ways of life in the "Native Reserves". This migratory labour system has resulted in the partial adoption of Western habits during their sojourn in the towns. To minimize the effect of urbanization on their dietary customs, the Bantu were chosen from new arrivals to Cape Town.

3) Eight white subjects, clinically normal and similarly investigated served as controls. The mean age of this group was 48 years.

Only male subjects were investigated.

Samples were collected between 8.30 and 9.30 a.m., the White subjects having been instructed to avoid major deviation from their normal dietary habits during the preceding 48 hours.

Results (Table 5, 1; Figure 5, 1).

Differences were observed between the three groups in almost every modality investigated. Not all of these achieved significance by the t test, owing perhaps to the small number of subjects.

1) Total plasma lipid concentrations were lowest in the Bantu, differing significantly at the 5% level from the other groups. Though the figures for patients with coronary heart disease were higher than for normal White subjects, the difference failed to achieve significance.

2) The same pattern applied to the total serum cholesterol levels, significantly lower in the Bantu than the other groups, as established by previous work [2]. Unimportant differences were seen between affected and normal White individuals, though large-scale surveys have detected significantly elevated levels in patients with coronary heart disease [6,10].

3) Ester cholesterol levels too were lowest in the Bantu, showing significant differences at the 1% level, and only slightly elevated levels distinguished coronary patients from White controls.

4) Analyses of CEFA showed some marked variations, though the ranges always overlapped. The iodine value was highest in the Bantu (the mean approximating to the I.V. of pure linoleic acid). In the coronary

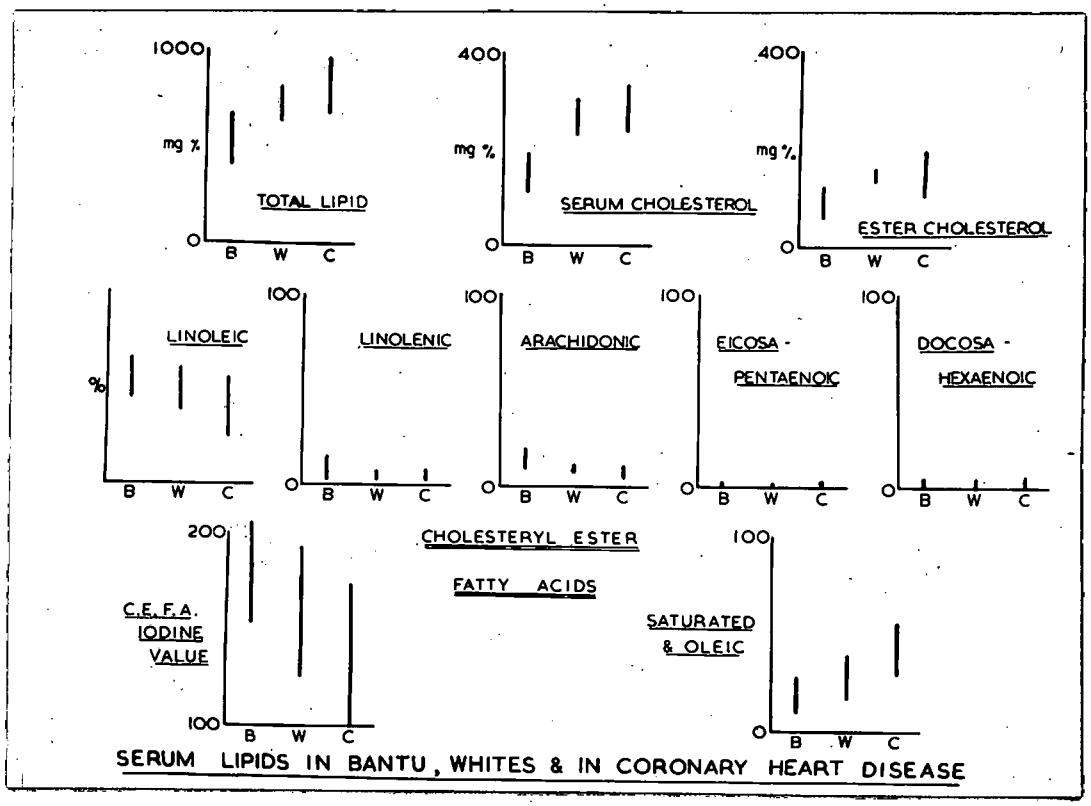

Figure 5.1. A comparison of the circulating lipids of normal Bantu and White subjects, and of patients with coronary heart disease, (B, W and C respectively). The vertical bars indicate the mean $\pm$ the standard deviation for each lipid fraction.

Table 5, 1.

	Plasma						Coronary Arteries
	Bantu		White		Coronary		
Total plasma lipids, mg.%	550	(131)	738	(94)	837	(150)	-
Serum cholesterol, mg.%	154	(42)	263	(40)	280	(51)	-
Plasma ester cholesterol mg.%	91	(32)	141	(12)	151	(31.5)	-
CEFA iodine value	179	(26.5)	160	(34.5)	136	(35.6)	64
CEFA % linoleate	55.4	(10.8)	53.2	(10.7)	40.6	(15.5)	11
CEFA % linolenate	8.6	(4.3)	5.1	(2.5)	5.4	(2.8)	3.3
CEFA % arachidonate	13.6	(4.9)	9.5	(2.3)	7.6	(3.0)	3.6
CEFA % pentaenoate	1.24	(0.83)	1.43	(0.46)	2.10	(1.04)	0.31
CEFA % hexaenoate	3.16	(1.99)	2.43	(2.70)	2.80	(2.65)	0.44
CEFA % saturated plus oleate	18.0		28.3		41.5		81.3

Table 5, 1. Plasma lipid analyses expressed as mean and standard deviation in normal Bantu and White subjects and in patients with coronary heart disease. CEFA composition in atheromatous coronary arteries.

group CEFA iodine values were lower than in the controls, all groups differing significantly at the 5% level; that between the Bantu subjects and the coronary patients was all but significant at the 1% level.

As in the feeding experiments, variations were due chiefly to changes in the percentage of linoleic and arachidonic acids; the Bantu had rather more linolenic acid than the White groups. In the coronary group over 40% of the CEFA was computed to be saturated or monoethenoid (oleic); these acids comprised 28.3% of the CEFA in normal White subjects and only 18% in the Bantu. Estimating oleic acid from the difference between the observed iodine value and that calculated from the isomerization data for polyethenoid acids, figures of 10-15% were obtained; this is clearly most approximate. Paper chromatography of samples from each group showed spots corresponding to linoleic acid, small ones of stearic acid, and intermediate-sized spots with the Rf value of palmitic or oleic acids (which have the same mobility).

The percentage of arachidonic acid differed significantly at the 5% level between the three groups, and the linoleic acid percentage was lower in the coronary heart disease patients than in the normal subjects at the same significance level.

No differences were detected in pentaenoic and in hexaenoic acid percentages.

CEFA of atheromatous coronary arteries

Segments of coronary arteries containing moderate to gross atheroma were obtained at four autopsies, from patients 48-73 years of age After drying *in vacuo* over phosphorus pentoxide they were extracted with ethanol followed by ether (6 hours of each) in a continuous hot extractor (Figure 5, 2). Hydroquinone was used to prevent oxidation of the extract. The procedure for plasma analysis (Appendix 3), was then used for isolation and determination of CEFA.

Results (Table 5,1) showed a mean iodine value far lower than that of plasma CEFA even from patients with coronary heart disease.

DISCUSSION

To place these findings in perspective it is necessary to review some contemporary opinions on the aetiology of coronary heart disease.

1. Epidemiology

(a) The steeply rising incidence of deaths certified as being due to coronary heart disease is one of the major problems presently facing medical science. More accurate diagnosis has probably contributed to this trend, as emphasized by Lew [14]; but the clinical picture has been well known for many years. Greater awareness of the disease is unlikely to explain either the threefold rise in coronary mortality between 1940 and 1955 in the United Kingdom [15] and South Africa [16], or the increasing penetration of the disease into younger age groups (in males) dramatically shown by autopsies on young American soldiers killed during the Korean War [34]. Ryle and Russell [17] and Keys [18] have adduced an overwhelming case for a real increase in incidence.

Figure 5, B. Continuous hot extractor used in analysis of cholesterol esters from atheromatous plaques.

(b) Racial incidence: At Groote Schuur Hospital, 5004 patients suspected of having coronary heart disease were electrocardiographed by Vogelpoel and Schrire [1], with positive findings in 448 Whites, 100 Coloured patients and 2 Africans. Patients of each group present at the hospital in the ratio of approximately 2:3:1.

(c) Sex incidence: The disease is far commoner in males than females in the fourth decade, the sex disparity dwindling with increasing age and disappearing in the seventh.

(d) Obesity: Although insurance data have indicted a body weight above accepted norms [19], there is little difference in weight distribution between young people with and without coronary heart disease [20,21].

It is of course true that life expectancy in general is less in overweight individuals; and the course of established heart disease is adversely affected by obesity.

(e) Social class and physical activity: The disease is roughly twice as common in professional men as in manual labourers, its incidence correlating in striking fashion with income [15]. Whether this is due to diet [6], reduced energy expenditure [22], smoking or "stress" is obscure.

Within given occupations remarkable variations in incidence occur: the disease is commoner in general practitioners than consultants, bus drivers than conductors, dock labourers than building labourers [23].

(f) Smoking: Not only is the disease commoner in smokers, but its incidence rises with the number of cigarettes etc. smoked [24]. As likely as a causal connection is the possibility that smoking and coronary heart disease are promoted by similar factors.

(g) Emotional tension: It is rash to disregard psychosomatic mechanisms in any disease. Evidence based on the fall in incidence of coronary disease in Norway during the war [25] is open to other interpretations, e.g. reduced fat intake or smoking. On the other hand, the social incidence of the disease - its predilection for the "Managerial class" - has been regarded as implicating mental stress; that executives undergo greater tensions than the poor is a view prevalent only among the former.

(h) Familial incidence: Despite the commonness of a positive history, inheritance has not been established as an important aetiological factor; clearly many environmental factors may contribute to a familial incidence.

One's faith in a multiple aetiology of coronary heart disease is based on the remoteness of the possibility that any one mechanism would explain this variegated epidemiology.

2. Dietary Fat.

(a) Many lines of circumstantial evidence have pointed to fat intake as a major cause. Keys [4,5,6,26] has attributed the temporal, geographical, racial and social incidence of the disease to the quantity of dietary fat, expressed for some reason as "the % calories derived from fat". Yudkin [15] has shown that these claims are only partly true, e.g. coronary deaths correlate with fat intake in some countries but not in others. His findings prove that fat intake is not the only factor in the aetiology of the disease, a view which all responsible workers would accept. Keys [49] found that the correlation between fat intake and coronary heart disease did not exist for cerebrovascular disease.

There is no general agreement that the rising incidence of coronary atherosclerosis has in fact been accompanied by greater fat consumption. Katz [27] believed it had; Stare [28] reached the opposite conclusion.

(b) Quality of dietary fat. Many unsaturated fats are metabolised differently from saturated ones; for example they may have opposing actions on the serum cholesterol level [3,29,30]; and the present investigation has shown qualitative as well as quantitative changes in the circulating cholesteryl esters. Sinclair's views on the role of dietary insufficiency of polyunsaturated fatty acids were reviewed in Chapter 4 and find support in the survey described above. Though "essential fatty acid" intake by the population of the U.S.A. had not changed in the past fifty years [31], Sinclair has asserted that inactivation of such acids by food processing (hydrogenation and additives which may oxidise fatty acids) may have led to a decreased intake of the active foodstuff. Yudkin [32] quotes Deuel as stating that despite hydrogenation, margarine provides five times more essential fatty acid than butter on biological assay. Schroeder [33] has implicated trace element inhibitors of pyridoxal-containing enzymes necessary for arachidonic acid synthesis from dietary linoleic acid.

It is of interest, in relating the essential fatty acid hypothesis to epidemiological data, that the requirements of male animals is 5 times greater than that of females.

Despite Kinsell's assertion [8] that low fat diets are unphysiological, depriving the body of polyunsaturated fatty acids, Morrison [48] obtained distinct prolongation of life in a controlled

study of the effect of such a diet on patients with coronary heart disease.

3. The thrombotic hypothesis.

Duguid [35,36], has argued for a return to the Rokitansky concept of atheroma as due to mural thrombosis with endothelial growth over the thrombus, conversion of the fibrin to fibrous tissue and ultimate lipid deposition. He has observed such a sequence of events in fibrin fragments injected into rabbits to form pulmonary emboli. When distended by pressures approximating to normal arterial blood pressure, atherosclerotic vessels were found to be dilated not narrowed [37], reduction of the lumen being due (by inference) to thrombosis.

To relate this concept to epidemiological facts there are as yet few positive arguments. Merskey and Nossel [39] were unable in scrupulously controlled studies to demonstrate an effect of dietary fats on most aspects of the coagulation process. Robinson and Poole [40] described increased coagulability during the lipaemia following a fatty meal, which they ascribed to ethanolamine phosphatide present in the chylomicra.

There is every reason to believe that the prevalence of coronary heart disease may be explained by environmental factors to which increasing numbers of people are being exposed. And there is nothing in the foregoing discussion which is incompatible with the hypothesis that the quantity and quality of dietary fat are among these factors. This hypothesis has some pragmatic value too, in that if

borne out by future work, rectification of dietary habits will be a practicable if difficult undertaking.

Variations in the content of saturated and unsaturated fats in the diet have been shown in the present investigation and elsewhere to produce different patterns in serum total and ester cholesterol, and in plasma CEFA composition. Similar differences have been demonstrated in the survey described above. Is there reason to believe that these patterns bear any causal relationship to coronary heart disease, (and freedom from it)?

The answer at present is clearly negative. Further studies are necessary and will probably extend over several years. Purely as a line of thought in planning such studies, the following speculations are submitted.

Sinclair [7] has suggested that in "essential fatty acid" deficiency, plasma cholesteryl ester composition may alter, producing compounds "less readily disposed of from tissues". In the light of present studies one offered the hypothesis that when cholesterol is esterified with highly unsaturated fatty acids its catabolism to bile acid is more readily accomplished, with reduction in the serum level. Curran demonstrated that cholesteryl esters, obtained from rabbits fed saturated fat, are less readily hydrolysed than those from animals fed unsaturated fat [41].

A second hypothesis concerns the "solubility" properties of cholesteryl esters: highly unsaturated cholesteryl esters are more readily transported in the plasma than less unsaturated esters; the latter tend to be deposited in the tissues.

Plasma and tissue cholesterol are in dynamic equilibrium [42]. Present studies on CEFA from coronary atheromatous plaques have shown the predominance of saturated fatty acids; this is in agreement with Schoenheimer's observation [43] that the fatty acids in aortic plaques are mainly stearic, palmitic and oleic; Tuna et al [44] reported contradictory findings, in abstract, to the effect that they could not distinguish between the lipids of atherosclerosis and plasma.

Some support for the "solubility" hypothesis comes from animal studies. Rats fed a fat-free sterol-free diet deposit cholesteryl esters in the epidermis [45]. Alfin-Slater et al [46] showed that a fat-free diet reduced the serum cholesterol but led to cholesterol and fat deposition in the liver. Results from the same laboratory [47] showed further that under these conditions the liver cholesterol becomes esterified with saturated acids instead of oleic acid.

SUMMARY.

Plasma cholesteryl ester fatty acid (CEFA) composition is affected by the nature of the dietary fat. Because the quantity and quality of the latter have been considered by many to be important aetiological factors in coronary heart disease, the plasma CEFA of patients with this condition have been compared with White and Bantu controls; the latter group possesses an almost complete immunity to the disease. Significant differences between plasma CEFA iodine values were observed, Bantu subjects having the highest degree of unsaturation and coronary patients the lowest; this was mainly due to variations in the percentage of linoleic and arachidonic acids. CEFA from atheromatous

coronary arteries had a far lower iodine value than those from plasma.

The aetiology of coronary heart disease is discussed, and is considered to be multiple. The type of dietary fat consumed could explain the differences between the plasma CEFA of coronary and other groups.

Two explanatory hypotheses are submitted. One suggests that cholesterol is more readily catabolized to bile acid when esterified with highly unsaturated fatty acids than with less-unsaturated acids. The other regards highly unsaturated cholesteryl esters as having the greatest "solubility" in plasma; saturated esters tend to be deposited in the tissues, a view compatible with the finding of a low iodine value in the CEFA of coronary atheromatous plaques.

Addenda.

1) Keegan and Gould [50] have demonstrated cholesteryl palmitate, stearate and oleate in atherosclerotic human aortae; this is in agreement with the present finding that CEFA of atherosclerotic coronary arteries include very little polyunsaturated acid.
2) Hammond and Lundberg [51], in a small series, studied the total plasma fatty acids in a similar survey to the present one; they observed lower percentages of linoleic and arachidonic acids in atherosclerotic patients than blood bank donors; Guatemalans (whose fat intake is low and mainly of vegetable origin) had even higher percentages of these acids than blood donors. Investigating Bantu mine labourers, White blood bank donors and patients with coronary heart disease, Antonis [52] found a similar pattern, the Bantu having by far the highest

percentage of linoleic and arachidonic acids. On the other hand James et al [53] were unable to demonstrate an abnormality of serum fatty acids in patients with coronary heart disease.

In interpreting such studies, it should be noted that the various components of the plasma lipid respond differently to some dietary and endocrine factors, and do not represent a homogeneous physiological entity.

It is not easy to compare these interracial surveys with the present one, in view of the small numbers studied in each; the differences in CEFA composition were somewhat more pronounced than those in total fatty acids.

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CHAPTER 6.

DISCUSSION.

The foregoing experiments have been discussed individually. It remains to correlate the findings with one another and with certain existing concepts in sterol metabolism.

The serum level of cholesterol is one conveniently-measured aspect of its metabolism, and there is an impressive body of information about the factors influencing its concentration, and that of related lipids and lipoproteins. But if the relation of cholesterol to coronary heart disease is to undergo fundamental analysis we need far more detailed knowledge of its handling by the body. The influence of diet on such processes is of particular interest in view of present aetiological hypotheses.

It is important to know that some vegetable fats not only reduce the serum cholesterol level but do so by promoting catabolism of this sterol (Chapters 2 and 3). The possibility becomes remote that their action is due to increased deposition of cholesterol in the tissues. One is more hopeful (at the speculative level), that feeding such oils may render atherogenesis a reversible process, by mobilizing cholesterol from affected arteries. Kinsell believed that certain unsaturated fatty acids reduced cholesterol synthesis; reference was made in the introduction to the lack of evidence for this concept. No direct information was obtained in the present study about the mode of action of dietary saturated fats. There is nothing incompatible with the view that such fats promote cholesterol synthesis.

The composition of circulating cholesteryl esters has been shown to be influenced profoundly by the nature of the dietary fat. This may be the primary event whereby sunflower seed oil increases the catabolism of cholesterol, and the data support certain of Sinclair's views outlined in Chapter 4. In the light of these feeding experiments, qualitative differences were sought in plasma cholesteryl esters in coronary heart disease and in two control groups. Feeding a saturated fat reduced the proportion of highly unsaturated fatty acids in plasma cholesteryl esters; it led to the pattern found in patients with coronary artery disease. A high intake of sunflower seed oil had the converse effect. Normal White subjects had a distribution of fatty acids intermediate between that of patients with coronary heart disease and normal Bantu subjects.

The indigenous Bantu do in fact eat relatively little animal fat (which contains mainly saturated fatty acids); this supports the theory that their dietary habits are conducive not only to a low serum cholesterol [1] but also to a qualitative difference from White subjects in cholesteryl ester composition. The latter eat large and increasing amounts of fat derived from animal sources [2]; this might be expected to reduce the iodine value of their circulating cholesteryl esters. Of the White subjects, those with coronary heart disease had the lowest content of unsaturated fatty acid in plasma cholesteryl esters.

These findings are compatible with the view that dietary fats contribute to the aetiology of coronary heart disease. A larger survey of this type is certainly necessary. But observations based on association alone cannot prove a hypothesis: they can only indicate the appropriate definitive experiment.

One possibility is a prospective survey of cholesteryl ester composition in normal subjects. The prediction of coronary heart disease (on a statistical basis), would further the dietary hypothesis considerably. It would be of interest to know the biological half-life of labelled cholesterol administered to normal and atherosclerotic subjects.

The ranges of cholesteryl ester composition in subjects with and without coronary artery disease overlapped considerably; only a statistical difference was observed. Its determination would have little value in diagnosing or predicting the disease in any individual.

The utmost conservatism is necessary in interpreting or extrapolating from the experiments constituting this thesis, but despite their technical limitations, one hopes that the present experiments will prove to have contributed to our understanding of cholesterol metabolism in health and disease.

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APPENDIX I.

Reagents

Ethanol 95% distilled over sodium hydroxide. Potassium hydroxide 2N. Zinc sulphate 40% w/v (10 ml. should be neutralized by 10.8-11.2 ml. of the 2N KOH using phenolphthalein as indicator). Sodium hydroxide 5N. Sulphuric acid 60% v/v. Petroleum ether 40-60° B.P. distilled. Chloroform AR distilled weekly. Sodium hydroxide 0.02N in ethanol standardized against potassium hydrogen phthalate.

Procedures

Faeces are thoroughly homogenized, if necessary with the addition of saline. An aliquot of 5 g. (or up to 20 g. if the water content is high) is weighed into a 100 ml. centrifuge tube with a B19 socket; 50 ml. of ethanol is added and the mixture refluxed gently for 1 hour. The residue is separated by centrifugation and extraction repeated once.

The combined extracts are taken to dryness in a boiling water-bath under a stream of air. To the tarry residue is added 2 ml. of 2N potassium hydroxide. Solution is aided by warming, and the flask contents are transferred quantitatively to a 40 ml. centrifuge tube with repeated hot-water washings, the last of which should be colourless. The total volume should not exceed 15 ml. To this solution 2 ml. of 40% zinc sulphate is added drop by drop, with continuous shaking; after 15 minutes it is centrifuged at 3,000 R.P.M. for 5 minutes, and the supernatant transferred to a wide-necked 100 ml. Erlenmeyer flask. The precipitate is washed 3 times with 5 ml. of ethanol, the washings being separated by centrifugation and added to the flask. The flask

contents, which should be straw coloured, are evaporated down to 1-2 ml. in a boiling water bath under an air current and transferred to a glass-stoppered 40 ml. centrifuge tube. Two 2 ml. quantities of 5N sodium hydroxide are used to wash in the remainder of the flask contents. The solution is acidified with 60% sulphuric acid, Congo red being used as indicator.

Two extractions with 20 ml. of petroleum ether remove fatty acids, fats and sterols, after which 4 extractions with 20 ml. of chloroform are performed. The combined chloroform extracts are washed once with 10 ml. of water, transferred in stages to a 50 ml. Ehrlenmeyer flask and taken to dryness on a steam bath. The residue is dissolved in 10 ml. of boiled-out ethanol. An aliquot (usually 0.025 ml.) may be taken for paper chromatography. Two drops of thymol blue are added to the flask, the contents of which are titrated with 0.02N ethanolic sodium hydroxide to a blue-green end-point which persists for at least 30 seconds. A blank titration is performed; the blank value should not exceed 0.05 ml. Results are expressed as mg. of desoxycholic acid. Analyses are carried out in duplicate.

Paper chromatography is carried out as described by Sjövall [19], but spots are best visualized by the antimony trichloride reagent (Carey and Bloch [20]), which is more informative than the phosphomolybdic acid or rhodamine procedures.

Comments.

(1) The extraction technique was found to be satisfactory, provided that a large excess of ethanol was employed. Some groups of workers

have carried out further extraction of the faecal residue in the Soxhlet apparatus; this time-consuming operation did not appear justified in the following experiment:-

After extraction by refluxing with ethanol for two hours as described, the faecal residue was dried at 100°C. for one hour, then transferred to a continuous hot extractor containing ethanol 95% for 24 hours. The extracts were carried separately through the analytical procedure; the yields are tabulated below (Table 8, 1).

Table 8, 1.

Solvent extraction for 2 hours (mg.)	Further yield after 24 hours (mg.)
7.1	0.3
12.5	0.55
8.7	0.62

(2) The decolourizing procedure, (Harwood [1]), is efficient only if the reagents are prepared with precision. Adequate removal of bile pigments is essential; these amphoteric substances may otherwise contribute to the titration figure. Reversed-phase column chromatography as applied to bile acid analysis by Bergström & Sjövall [2] does not separate bile acids from faecal pigments; these migrate diffusely, at least in the methanol-water/chloroform-heptane system; and this procedure is at present semi-quantitative, giving 70-90% recoveries

of bile acids. (However a quantitative modification of this procedure by Mirvish [3] has been of the utmost value in bile analysis (Appendix 2)).

(3) The determination of bile acids in bile necessitates saponification of the glycine and taurine conjugates of these acids, an operation requiring vigorous reaction conditions. It has been suggested that faecal bile acids are not conjugated, an observation confirmed as follows:

The extract from samples of normal faeces was decolourized in the usual way, and to part of the bile acid solution, 33% sodium hydroxide was added to a final concentration of 5N. The bile salts were saponified in an autoclave at 15 lbs./sq.in. for 4 hours; bile acids were determined in this and the unsaponified solution by the procedure outlined (Table 7, 2).

Table 7, 2.

Unsaponified (mg.)	Saponified (mg.)
8.7	9.8
11.4	12.0
10.1	10.3

Results indicated that a small and inconstant amount of bile acid conjugate is present in faeces. Saponification did not appear to be necessary (though this would probably not apply under conditions of intestinal hurry, nor during the administration of broad-spectrum antibiotics).

(4) Titrimetric determination was compared with more selective methods of quantitation. In five experiments the method of Doubilet [4] was applied to aliquots of the final extracts as prepared for titration, bile acids being precipitated from buffered aqueous solution as their ferric salts. The latter were determined by their iron content.

The mean results were 12% lower than those obtained by direct titration, but were scattered somewhat widely from -30 to +18% of the titrimetric figure.

The aqueous supernatant after separating the ferric bile salts by centrifugation was acidified and extracted with chloroform, the extract being washed and titrated. Results did not differ significantly from the solvent blank, indicating that in the technique described, virtually all the material titrated formed insoluble ferric salts.

Another optical method for determination of bile acids, that of Mosbach et al [5], was applied to aliquots of the extracts prepared for titration. This sensitive procedure depends on the characteristic absorption spectra of certain bile acids after heating in solution in 65% sulphuric acid, and gave figures for deoxycholic acid -40 to -4% of those obtained by titration.

A peak was also observed at 300 m μ. This is not the wavelength of any of the lesser absorption maxima of deoxycholic or cholic acids. Eriksson and Sjövall [8] have determined the spectra of 27 substituted cholanic acids after the Mosbach procedure (heating for 15 minutes in 65% sulphuric acid); and they found that absorption

maxima in this vicinity were given by two ketonic acids, 3α-hydroxy, 12-ketocholanic acid (302 m μ) and the unsaturated Δ^{9-11}, 12-ketocholenic acid (297 m μ). The demonstration of carbonyl compounds in the bile acid fraction of faeces has been referred to in the present study, and further spectrophotometric analyses are described in Chapter 2, Experiment 3.

Paper chromatography of further aliquots of the same extracts in the system of Sjövall [6] were developed by the antimony trichloride procedure of Carey and Bloch [7]. The largest spot had the Rf value and fluorescent colour of deoxycholic acid; fainter spots with the properties of cholic acid and chenodeoxycholic acid were noted in three of the five samples. A spot running near the solvent front (Rf 0.7-0.8) and emitting red-purple fluorescence was noted in all chromatograms, varying in extent from about 25 to 50% of that of the deoxycholic acid spot; the properties suggested the presence of one or more ketocholanic acids of low polarity. The presence of ketones in all samples was indicated by their precipitation from solution in 70% ethanol by ethanolic 2:4 dinitrophenylhydrazine. The paucity or absence of cholic acid was noteworthy, and in agreement with Carey and Watson's findings. The cholic acid peak occasionally shown by the spectrophotometric procedure had no counterpart in the chromatograms of the same samples and probably indicated an impurity in the extracts.

Recoveries

To one of duplicate samples of faeces, deoxycholic, dehydrocholic or cholic acids were added in quantities of 5 or 20 mg. The

acids, kindly supplied by Merck and Co., were recrystallized twice from ethyl acetate. Recovery of deoxycholic acid was found to be 94% ± 6, in ten experiments. In five determinations each, dehydrocholic acid recovery was 97% ± 5, and cholic acid recovery 83% ± 8. It would appear that the more polar the acid the poorer its recovery, the measurement of cholic acid being relatively unsatisfactory; the drawback is not serious in view of the paucity of this acid in human faeces. Deoxycholic acid, and the keto-acid dehydrocholic acid are virtually quantitatively determined.

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Appendix 2. Determination of Bile Acids in Bile.

Method

Bile was collected in 24 hour batches using thymol as a preservative; it was stored at -15°C and worked up within 4 days. Aliquots of 1 ml. were saponified in sealed ampoules at 120°C for 4 hours, using 2 ml. 2 N sodium hydroxide. The unsaponifiable fraction was extracted with petroleum ether 40 - 60°C, B.P., cholesterol being determined by the Liebermann-Burchard reaction. The aqueous phase was then acidified, using Congo Red as indicator; fatty acids were removed by petroleum ether extraction, and bile acids extracted with four 4-volume quantities of ether; the ether extract was washed with 0.2 volumes of water, then taken to dryness. In this form it was stored at -15°C. until analysis was completed. Chromatography was carried out as described by Mirvish [1].

This modification was based on the observation that the mobile phase, in columns which had stood for some hours, became alkaline due to an impurity leached out of the kieselguhr. This could not be removed by previous washing, but addition of a minute quantity of hydrochloric acid to the solvent system improved recoveries from an irregular 60 - 75% to 92 - 98%.

The chromatographic method need not be described in detail. It was our practice to prepare columns from a single batch of kieselguhr, distilling the solvents on the day of the experiment; each column was used once only. The mobile solvent was 58% methanol containing 0.04% hydrochloric acid, and the stationary phase chloroform-heptane 100:28 v/v.

This system does not separate chenodeoxycholic and deoxycholic acids. Wooton [2] has stated that human bile contains mainly cholic and chenodeoxycholic acids. The figures quoted for deoxycholic acid may therefore include the former substance. Purely optical methods, depending on spectrophotometry of bile acids in solution in 65% sulphuric acid, suffer from the same limitation.

The syphon delivered 1.21 ml., and the titration cell was essentially as described by Howard and Martin [3]. The end-point was detected electrometrically at pH 9.0. Bile acids were titrated with 0.01 N ethanolic sodium hydroxide, standardized against potassium hydrogen phthalate and delivered from a microburette in a carbon dioxide-excluding system.

The apparatus is shown in Figures 8, 1 and 8, 2.

Overall recoveries for the extraction procedure and chromatography were determined, using duplicate samples of bile. Cholic, deoxycholic and glycocholic acids of analytical grade were recrystallized from ethyl acetate, dried in vacuo at 80°C., and stored in vacuo over phosphorus pentoxide. Five recovery experiments were carried out for each acid; the results were 85.0 - 97.5% recovery of cholic acid, mean 91.2; 82.0 - 100.2% of deoxycholic acid, mean 86.0; 82.8 - 94.4 of glycocholic acid, mean 88.6.

Refs. (Appendix 2).

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Figure 8, 1. Apparatus for quantitative chromatography of bile acids.

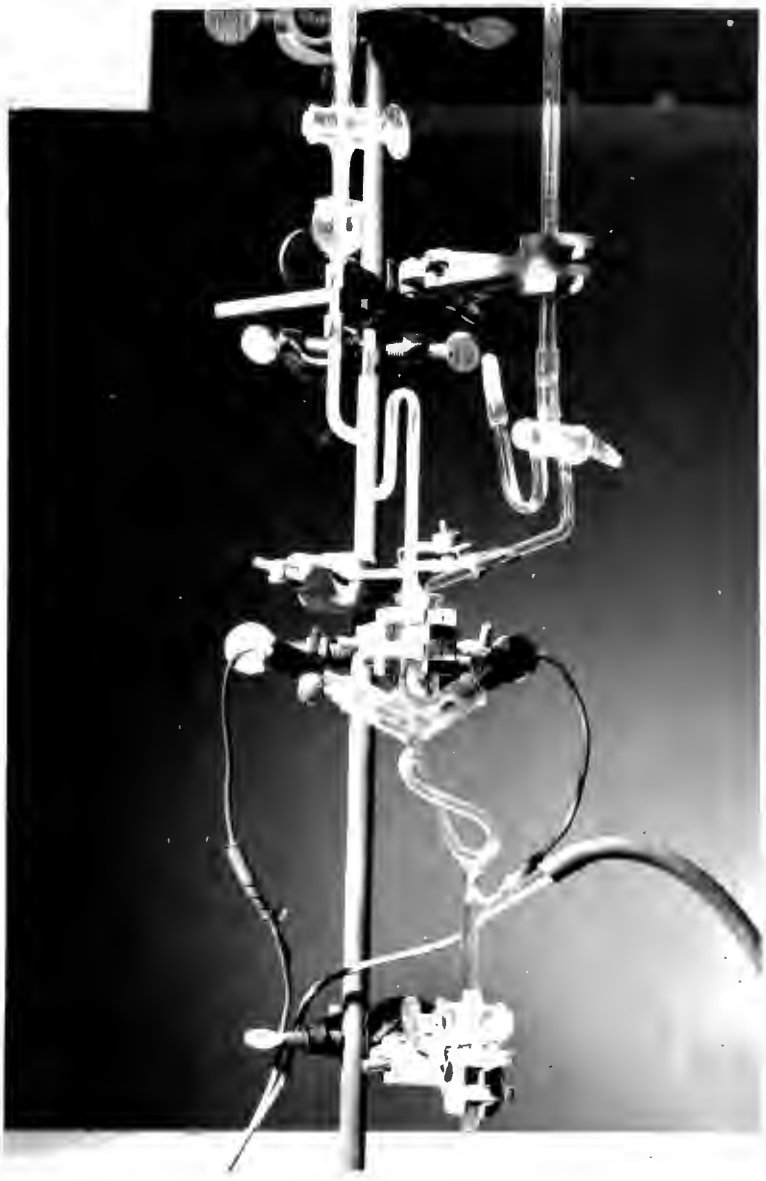

Figure 8, 2. Apparatus for quantitative chromatography of bile acids. Detail of syphon and titration chamber.

APPENDIX 3.

The isolation and analysis of plasma cholesteryl ester fatty acids.

Non-chromatographic methods have been used for the separation fo cholesterol esters from the plasma lipid [1,2], depending on fractionation by differential solubility in various organic solvents, and on the selective action of a soybean lipase which hydrolyses triglyceride but not cholesteryl ester. The recently-available chromatographic method of Fillerup and Mead [3] was a considerable advance: in one procedure all the major classes of the blood lipid were claimed to be separable; it was clear that several samples could simultaneously be analysed if appropriate apparatus were constructed.

This technique depends on the 'stepwise' elution of the lipid fractions from a silica gel column by solvents of increasing polarity; the authors obtained quantitative recoveries. The adsorptive activity of silica gel depends on its degree of hydration. In many centres it has fallen into disrepute as a chromatographic adsorbent on account of variability of results, no doubt because of its sensitivity to moisture. Particularly in the humid atmosphere of the Cape it was necessary to test the validity of the method with care.

The provisional method, in essence, was to extract plasma with methylal-methanol (Delsal [4]), chromatograph the extract on silica by the method of Fillerup and Mead [3], saponify the cholesterol ester fraction in a boiling water bath with 20% methanolic potassium hydroxide [5] and determine the fatty acids by the alkali isomerization procedure of

Herb and Riemenschneider [6]. The degree of unsaturation of the fatty acid mixture was estimated by a micro-modification of Kaufman's bromine addition technique [7].

The first analyses of lipid from normal human plasma were disappointing. The iodine value of the cholesterol ester fatty acids (CEFA) was inconstant in duplicate analyses, and far lower than expected (90-120). To explain these results one postulated

(a) Decomposition of the highly unsaturated acids, which are notoriously unstable; oxidation or isomerization were possible, or

(b) Failure to isolate cholesteryl esters from relatively saturated plasma lipids, or

(c) Elution of saturated cholesteryl esters, the unsaturated ones remaining on the column due to their slightly higher polarity.

Assessment of separate stages in the procedure showed that:- 1) The 'cholesterol ester' fraction, (i.e. the material eluted by 1% ether in petroleum ether from columns prepared as described) contained an excess of fatty acid over cholesterol on a molar basis. The order in which the plasma lipids are eluted from silica gel is cholesteryl ester - triglyceride - free cholesterol - free fatty acid - phosphatide. As the triglyceride fraction is known to be relatively saturated, it was likely that cholesteryl esters and triglycerides were incompletely resolved.

Activation of the silica was tried, by heating at 600°C. for 4 hours, packing the columns under conditions as nearly anhydrous as possible before washing them as described by Fillerup and Mead. Determination of cholesterol in 20 ml. fractions of the 1% ether eluate showed very slow elution by this solvent, the sterol still being present in the 25th cut.

Using activated columns and 2% ether in petroleum ether, however, most cholesterol was eluted in the second and third 20 ml. cuts; the sixth contained none. Continued elution showed a second peak (measured gravimetrically), appearing in the 10th fraction and presumably containing triglyceride; no cholesterol was present in this material.

The original chromatographic method was therefore modified in three ways:

(i) Silica gel was activated before use.

(ii) Cholesteryl esters were eluted with 2% ether in petroleum ether.

(iii) Elution of this component was taken to be complete when 150 ml. solvent had been collected; the published method involved weighing the residue of each fraction, and continuing elution with a given solvent until no more material appeared. It was evident that more polar lipids than cholesteryl esters migrated in 1% and 2% ether; it was therefore preferable to limit the eluate volume.

2) The saponification method was recommended for total plasma lipid, vigorous conditions being necessary to split phosphatides. Cholesteryl esters are relatively easily saponified, and in view of the possibility of isomerization as well as oxidation, lower temperatures were preferable. To ensure completeness of the reaction

(i) A large excess of alkali was used.

(ii) The reaction time was increased to 14-15 hours.

(iii) Potassium tert-butoxide in tert-butanol was used instead of methanolic potassium hydroxide, the former being a more efficient saponifying agent on account of the greater proton affinity of the tert-butoxide group in comparison to methoxide.

3) The extinction coefficients of linoleic and linolenic acids were determined by the alkali isomerization procedure of Herb and Riemenschneider [6]. The acids were obtained from the Hormel Institute by courtesy of Dr. R. Holman. There were unimportant differences from the E values reported by these authors (Table 9, 1.)

Table 9, 1.

	Linoleic	Linolenic
Wavelength.	234	268
Specific extinction coefficient (Herb and Riemenschneider).	91.6	90.5
Specific extinction coefficient (present experiment)	90.4	89.0

4) Hilditch [8] has referred to the problem, when using silica gel for chromatography, of oxidation of samples by adsorbed oxygen.

Higher iodine values were obtained when duplicate samples of plasma lipid were run on columns previously washed with methanolic hydroquinone than on columns washed with methanol alone. The appearance of the yellow colour of quinone during washing was ample justification for this manoeuvre.

The modified procedure, carried out in duplicate, gave iodine values of 208 and 197, 168 and 181, and 156 and 161 in analyses of normal human plasma, and was regarded as reflecting plasma CEFA composition satisfactorily. Formal recovery experiments were not carried out, owing to the prohibitive cost of commercially-prepared cholesteryl linoleate and palmitate; the syntheses published by Page and Rudi [9] appeared excessively time-consuming; an attempt at esterification of

cholesterol with linoleic acid using an acid-regenerated cation exchange resin, Amberlite IR 100 as catalyst, proved an unsuccessful "short cut"; Sussman [10] had had good results in synthesizing butyl oleate by this method.

The technique adopted for isolation and determination of cholesteryl esters will now be described:-

Reagents.

Methylal, B.D.H., distilled with collection at 41-44°.

Methanol, refluxed over KOH and distilled.

Petroleum ether, BP 68-69°, distilled.

Silica gel, Davison, 200 mesh. Washed 20 times by swirling with distilled water. Partially dried on Buchner funnel. Washed on funnel under slight suction with methanol, ether and petroleum ether. Finally dried in oven at 100°C.

Ether: allowed to stand over aqueous pyrogallol for 48 hours, then distilled, (still in the presence of pyrogallol), passed through a column of activated silica gel and used the same day.

Potassium tert-butoxide: Tert-butanol was partially dried by cooling to 23°, discarding the supernatant, melting and repeating twice more. Traces of water were removed by addition of metallic sodium, after which the tert-butanol was distilled. Carefully-cleaned metallic potassium was added, the reagent being adjusted to approximately 1.5 N.

Potassium hydroxide, 21% in ethylene glycol; as described by Herb and Riemenschneider. Exclusion of oxygen was essential; the conical flask shown in figure 9,1, facilitated preparation of the reagent under nitrogen, - the small round one was useful for preheating the

reagent when carrying out the isomerization, allowing 2.5 ml. quantities to be withdrawn by a syringe pipette.

Ethanol, for spectrophotometry, was prepared from 95% stock by refluxing for 12 hours with KOH and aluminium dust, distilling roughly, and redistilling the middle 60% fraction in an efficient column.

Method.

Oxalated blood is centrifuged within 30 minutes of collection. A sample of 10 ml. is added dropwise, with stirring, to 90 ml. methylal-methanol 4:1 v/v (Delsal [4]); the suspension is stirred vigorously for 10 minutes, then centrifuged at 3000 R.P.M. for 5 minutes. The supernatant is decanted, and the protein extracted with a further 50 ml. solvent. A few crystals of hydroquinone are added to the combined extract; the solvent is removed by heating in a water bath at 50° rising to 70° under nitrogen and at atmospheric pressure. The turbid aqueous residue (5-10 ml.) is extracted twice with equal volumes of petroleum ether, and the extract taken to dryness in a weighed tube under nitrogen, care being taken to avoid heating the dry residue. The sample is weighed after being stored overnight in a vacuum dessicator containing P_2O_5 and previously filled with nitrogen; plasma total lipids are computed. It is then taken up in 2 x 2 ml. petroleum ether for chromatography.

Silica gel 13 g. is heated in a muffle furnace at 600°C. for 4 hours, then allowed to cool in a closed container. The columns (10 x 2 cm.) are packed as a mull in petroleum ether, then washed successively with 50 ml. each of anhydrous methanol containing 0.1% hydroquinone, ether containing hydroquinone, and petroleum ether.

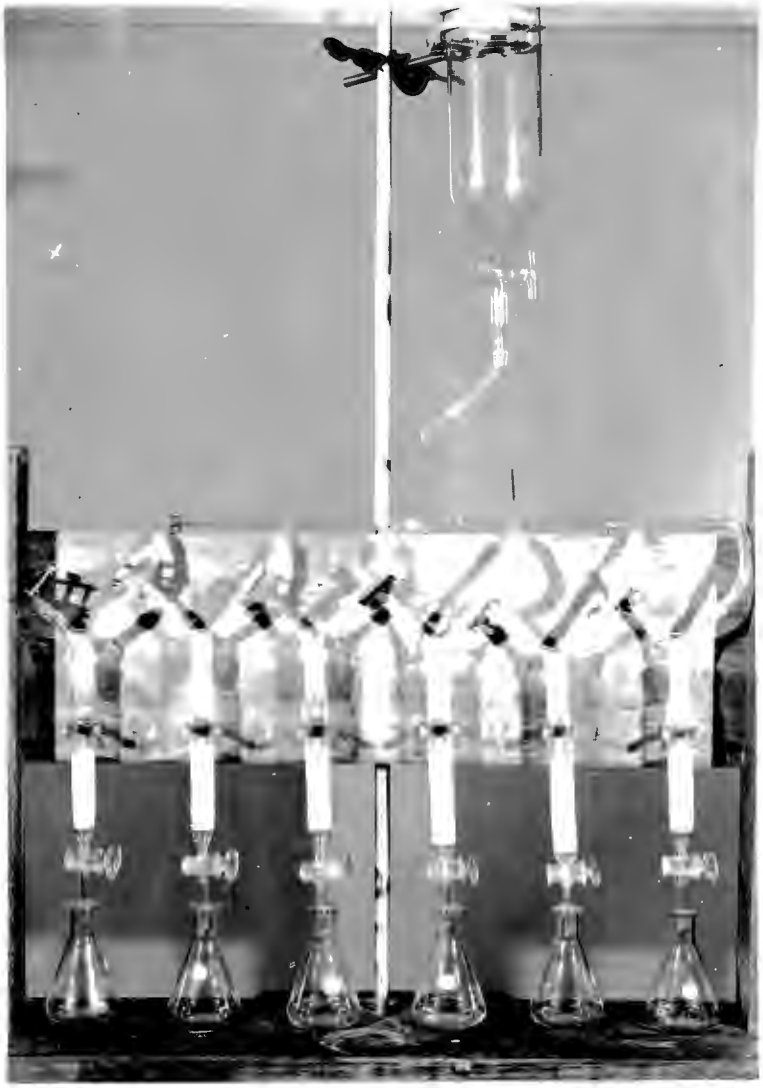

Figure 9. 1. Rack and manifold for six columns, used in chromatography of plasma lipids on silica gel.

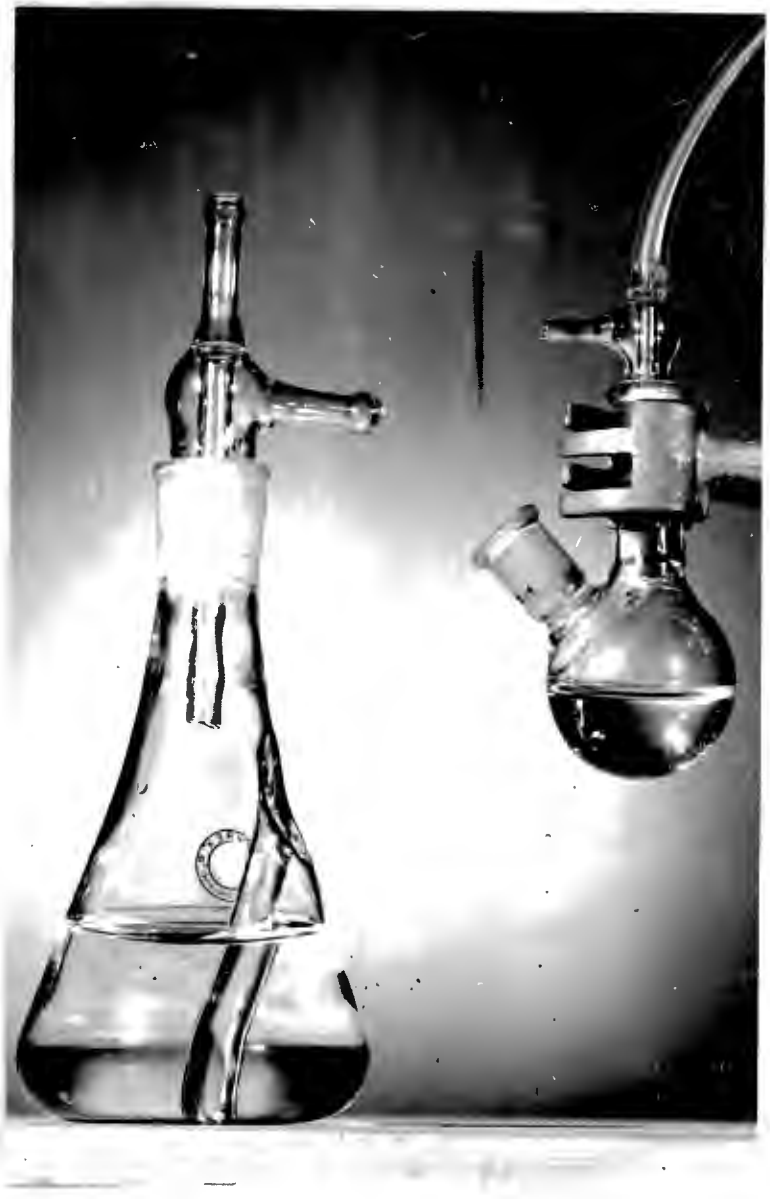

Figure 9.2. Flask for preparation of reagent for alkali isomerization (on left). Flask for preheating reagent. Exclusion of oxygen is essential to ensure low blank values.

The sample is applied to the column, and the surface allowed to sink just below that of the silica. The solvent, 2% ether in petroleum ether is then run at 20-25 drops per minute, 150 ml. being collected. Six chromatograms are run simultaneously, using the manifold and rack shown in figure 9, f.

The eluate is taken to dryness, in portions, in a 50 ml. glass-stoppered centrifuge tube, and under a nitrogen stream; a water-bath at 70-80° is used. The last traces of solvent are removed in vacuo.

Two millilitres of potassium tert-butoxide reagent are added, and a few crystals of hydroquinone. Air is displaced with nitrogen, and the tube stoppered. Saponification takes place overnight in a water bath at 35 °C. Two millilitres of water are then added, and cholesterol extracted with 2 x 5 ml. petroleum ether. The latter is made up to 25 ml. in a volumetric flask, and an aliquot of 1 ml. taken for cholesterol determination by the Lieberman-Burchard reaction, (i.e. ester cholesterol). The aqueous layer is acidified to Congo red with concentrated hydrochloric acid, and extracted with petroleum ether, 4 x 20 ml. The volume of the petroleum ether solution is reduced in a nitrogen atmosphere, and the CEFA transferred quantitatively to a 10 ml. volumetric flask.

An aliquot of 2 ml. is freed from solvent, and re-dissolved in 3 ml. boiled-out ethanol. It is transferred to a Howard and Martin titration cell (Figure 8, 2.) The fatty acids are titrated electrometrically to pH_9 with 0.01 N methanolic KOH, a stream of nitrogen mixing the reagents. The alkali is delivered from a microburette in a CO_2-excluding system. A mean molecular weight of 286 (that of linoleic acid)

is assumed in calculating the fatty acid content. This may introduce a very small error.

An aliquot of the petroleum ether solution containing 2-3 mg. fatty acid is pipetted into a 5 x ½" 'isomerization tube', bearing a B14 socket. The solvent is removed under a nitrogen stream at 70-75°, the process being completed in a P_2O_5-containing dessicator filled with nitrogen and then evacuated.

Alkali isomerization is carried out precisely as described by Herb and Riemenschneider [6], based on the addition of preheated 21% KOH in ethylene glycol to the sample and maintaining at 180 ± 0.2°C. for exactly 15 minutes, while ensuring a nitrogen atmosphere. Dilutions are made, using specially prepared absolute ethanol. Optical densities are measured in a Beckman DU spectrophotometer.

In the first few runs, a sample of Hormel linoleic acid was isomerized as a standard; consistent agreement with published specific extinction coefficients was obtained, and the practice was later carried out only when new batches of reagent were prepared.

Depending on the degree of unsaturation shown by alkali isomerization, an aliquot of the fatty acid solution containing 0.5-1 mg. is transferred to a 50 ml. glass-stoppered conical flask; the solvent is removed in vacuo and the iodine value determined by the micro-bromine addition procedure of Kaufman [7]. This is carried out in duplicate.

On some occasions samples have been chromatographed on paraffin-coated paper using 90% acetic acid as mobile phase (Kaufman and Nitch [11]). The additional information has proved very limited however.

DISCUSSION.

The obvious disadvantage of the procedure described is its length: analysis of a batch of six specimens took eight days. Some thought was devoted to the possibility of fractionating the cholesteryl esters themselves, but it seemed likely that the minor differences in polarity were unlikely to permit chromatographic resolution of such hydrophobic molecules. (It is conceivable that vapour-phase chromatography might separate these esters).

Alternative methods of determining the individual fatty acids were sought; the disadvantages of alkali isomerization, apart from technical difficulties, include failure to provide information about saturated and mono-ethenoid acids. There is no doubt that the ideal procedure for fatty acid analysis at the present time is vapour-phase chromatography, which handles fatty acids with up to 20 carbon atoms; one hopes to use this technique in future studies. Chromatography of fatty acid derivatives was another possibility:- the hydroxamic acid derivatives of long-chain fatty acids are readily chromatographed [12]. Chromatography of mercuric chloride addition compounds will resolve even palmitic and oleic acids [13], an achievement shared only by vapour-phase methods. Preliminary studies suggested that a great deal of work would be necessary to adapt chromatography of these addition compounds to routine quantitative use.

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PUBLICATIONS.

A. On cholesterol metabolism and related topics:-

1. "Determination of faecal bile acids".

   S.A.J.Lab.Clin.Med. 3, 316, (1957).

2. "Studies in cholesterol transport and catabolism".

   A paper read at a meeting of the Research Forum of the University of Cape Town, and abstracted in S.A.M.J.32,85,(1958)

To be published:-

3. "The effect of certain dietary oils on bile acid secretion and the serum cholesterol".

4. "Plasma cholesterol ester composition in the Bantu and in coronary heart disease, and its relation to dietary fat".

With Dr.H.Gordon, Professor L.Eales and Professor J.F.Brock :-

5. "Dietary fat and cholesterol metabolism".

   Lancet ii, 1299, (1957).

6. "Effect of different fats on the faecal end-products of cholesterol metabolism".

   Nature 180, 923 (1957).

B. Other published work:-

1. "The Plasma Corticosteroids".

   Thesis submitted for the degree of Doctor of Philosophy in the Department of Physiology, University of Cape Town.

2. "A paper-chromatographic technique for the determination of plasma corticosteroids".

   J.Clin.Path. 10, 148 (1957).

3. "Preparation of paper for quantitative chromatography of corticosteroids".

   Biochim.Biophys.Acta 20, 396, (1956).

4. "An elution apparatus for quantitative paper chromatography".

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Other published work.(contd.)

Addendum to a paper by Professor L.Eales and Professor G.C.Linder:

5. "Primary aldosteronism. Some observations on a case in a Cape Coloured Woman".

Q.J.M. (new series) 25, 539. (1956).

Studies in cholesterol metabolism - Lewis.

Chapter 1

Poses the problem of the relationship between serum cholesterol and dietary factors. Do changes in cholesterol reflect changes in synthesis, excretion a breakdown. Illustrates the regulation of cholesterol level with a diagram (p 5a) which makes the situation even more confused.

Chapter 2.

Shows that faecal bile acids (FBA) are a breakdown product of cholesterol, and describes the method of measuring them. Normal range 53 - 300 mgm / 24 hours. Mean 149 SD 51.

Previous studies in 7 post-menopausal white subjects - on good diet for a month before study. Does not say has he omitted the aid of the subjects, as the tests, involving prolonged dietary regimen manipulation, blood sampling, & the passage of Miller Abbott tube seem a little stringent for patient with planta wart, 18 month old forearm burns!!

A major drawback in that only one of the 7 subjects is over the age of 34, bloodletting

Conclusion

Shows that feeding saturated fat raises serum cholesterol, and reduces FBA output, while

unsaturated fat lowers the cholesterol & produces a rise in FBA excretion. These changes are not paralleled by an increased faecal neutral fat & so change not just due to malabsorption.

Shows that increased bile acid output is roughly proportional to the loss of cholesterol (fall in serum level × blood volume for calculating blood volume).

Giving saturated & unsaturated together produces little change in serum cholesterol, but increases output of FBA. This may imply increased synthesis of cholesterol

Annoying & persistent misprint throughout this

chapter - PINH instead of PNPH for phenyl
& hydrazine.

Chapter 3

Studies on patients with biliary fistulae, thereby eliminating effect of bile acid cycle on results. Unabsorbed fat feeding gave increased bile acid output & must imply increased production. Giving fat by i.v. route gave same result, discounting the idea that the action of the fat is a non-specific choleretic one.

Show that the changes in cholesterol are not related to changed absorption from the gut,

a to excretion of the cholesterol as such, but that the increased FBA output must imply an increased breakdown of cholesterol in response to unsaturated fat feeding.

Chapter 4

Mention that serum cholesteryl esters are of high iodine value (IV), and that about 50% is cholesteryl oleate.

Analyses fatty acids in the esters (CEFA) in 2 patients while on normal diet, and then on saturated & unsaturated supplements. Show that saturated fat produces more saturated esters, while the

unsaturated material produced ester of higher iodine value (higher in fact than the oil fed).

Chapter 5.

Describes studies on ester fatty acids in 12 white patients with ischaemic heart disease, 12 normal Bantus, 8 & 8 normal whites.

Shows that iodine value of Bantu ester higher than that of whites. Apart from this result was too much scatter to be meaningful.

Shows that iodine value of atherosclerotic plaques is lower than that of plasma & atheroma collected

in more saturated.

Remainder of paper recounts the new notation alone, but of coronary disease, and bears little relation to what has gone before.

In the appendices, however, the various methods are described, and my general impression is that in this section lies the real meat of the paper. The author has taken pains to evolve accurate & repeatable methods, and has then applied them to small groups of subjects.

The methods are very time-consuming, and have necessitated a good deal of hard work. The increased

bile acid output confirms previous workers' views

on the mode of action of unsaturated oils, but

on the whole ~~[illegible]~~ the author has ~~[illegible]~~

produced ~~[illegible]~~ ~~[illegible]~~ little ~~[illegible]~~ original

~~[illegible]~~ thought.

To sum up ~~[illegible]~~ :- valuable methods,

well worked out ; interesting results on bile acid

output and cholesterol ester composition, but little

in the way of original thought on what he

has found or would hope to do next

CHAPTER 7.

SUMMARY

The physiology of cholesterol and of related sterols and steroids is discussed. In a review of some aspects of its metabolism reference is made to the great attention presently devoted to its relationship to dietary fat on the one hand and to coronary heart disease on the other. Despite this interest, little information is available on the way in which dietary fats and oils influence the handling of cholesterol in the body. In view of the need for such data in investigating various factors in the aetiology of atherosclerosis, the studies described in Chapters 2, 3 and 4 were carried out. Reference is made to the possible mechanisms by which the serum cholesterol level may be altered by diet.

To investigate the effect of certain fats on cholesterol catabolism and excretion, a method was developed for determination of faecal bile acid excretion; bile acids are known to be the main end-products of cholesterol metabolism, both in bile and faeces. Faecal bile acid and neutral sterol excretion was measured in seven feeding experiments: administration of a highly unsaturated oil reduced the serum cholesterol level and increased the bile acid excretion to an extent which corresponded to 115% of the cholesterol leaving the circulation. Elevation of the serum cholesterol by a saturated fat was not related to noteworthy changes in the faecal lipids. Paper chromatography of bile acids obtained from faeces and from duodenal contents suggested that the increase in the former (due to the unsaturated oil) could be ascribed mainly to greater excretion of ketonic

bile acid derivatives. Support was obtained for the view that these arise from cholic acid. The precise nature of faecal bile acids being unknown, a relatively unselective method of determination had to be used.

Further investigations took the form of short-term feeding experiments in three subjects with bile fistulae, to facilitate the study of bile acid secretion without intervening variables. The use of such subjects was justified by showing constancy of bile acid production after a marked rise during the immediate postoperative period, and by demonstrating adequate and fairly constant fat absorption. Hydrogenated coconut oil failed to alter bile acid secretion, but oral sunflower seed oil and intravenous cottonseed oil (both highly unsaturated) led to an immediate 3 or 4-fold increase in cholic acid secretion and produced a fall in the serum cholesterol level. Bile cholesterol excretion did not show significant changes. The findings, obtained by use of a highly selective analytical method for bile acid determination, confirmed those in comparable studies of faecal bile acid excretion (Chapter I) and the theory was proposed that certain dietary vegetable oils reduce the serum cholesterol by a mechanism involving accelerated catabolism of this sterol.

Another aspect of cholesterol metabolism was then studied; it was prompted by the knowledge that plasma cholesteryl esters contained highly unsaturated fatty acids, particularly linoleic acid. This acid, which cannot be synthesized by mammals, predominates in all oils known

to reduce the serum cholesterol. Investigations into plasma cholesteryl ester fatty acid composition required the development of a method for their isolation and determination. In two feeding experiments, reduction of the iodine value of these fatty acids was produced by a saturated fat, and a pronounced increase followed the feeding of an oil containing 60% of linoleic acid. The altered iodine values were due mainly to varying percentages of linoleic acid and its derivative arachidonic acid. Thus reciprocal changes took place in the iodine value of plasma cholesteryl esters and the serum cholesterol level (in particular the ester cholesterol); dietary fats could produce qualitative as well as quantitative changes in circulating cholesterol compounds.

In view of the possible causal relationship between coronary heart disease and the type and amount of fat consumed, the plasma cholesteryl esters of patients with this condition have been compared with White and Bantu controls; the latter group possesses an almost complete immunity to the disease. Significant differences between plasma CEFA iodine values were observed, Bantu subjects having the highest degree of unsaturation and coronary patients the lowest; this was mainly due to variations in the percentage of linoleic and arachidonic acids. CEFA from atheromatous coronary arteries had a far lower iodine value than those from plasma.

The aetiology of coronary heart disease is discussed, and is considered to be multiple. The type of dietary fat consumed could explain the differences between the plasma CEFA of coronary and other groups.