CLINICAL AND LABORATORY STUDIES WITH CASTOR OIL

by

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CONTENTS.

CHAPTER 1

INTRODUCTION: PRODUCTION, PROCESSING, INDUSTRIAL AND MEDICAL USES OF CASTOR OIL

Castor oil is obtained by extracting the seed of the castor plant, which grows wild in nearly all tropical and subtropical countries. It is cultivated in India, mainly as a border to cotton or sugar plantations, and also in Brazil and East Africa. In recent years American production has been rising and the average per acre yield of castor beans in the United States increased from 252 pounds in 1952 to 2,078 pounds in 1958. In 1959 the United States produced about 23,000 tons of castor beans or about 17 per cent of their own requirement in that year. The United States currently consumes about one quarter of the world's production.

A United States Department of Agriculture circular (FFO-2-59) stated that the total apparent disappearance of castor oil in the United States in 1958 was 120 million pounds. The world production of castor beans in 1955 was approximately 450,000 tons.

EXTRACTION AND PROCESSING

The castor plant varies in size from a small shrub to a tree reaching about 20 feet in height, and the seeds, which have a characteristically mottled appearance, are the size of small beans, containing 22 per cent of husk and 78 per cent of oil-bearing kernel/

kernel. The average oil content is about 50 per cent, which is liberated by cold pressing and solvent extraction. The precise method of extraction is dependent on the grade of oil required. The British Oil and Cake Mills specify three grades - pharmaceutical, "firsts" and "seconds".

Medicinal, or pharmaceutical castor oil is obtained by hydraulic pressing at a temperature of between 90-100°F. The resultant oil is of a pale straw colour and after passing through a purification process is bleached by exposure to ultraviolet light. First grade castor oil, "firsts", is also obtained by hydraulic pressing, but at a much higher temperature. The expressed oil is bleached with Fuller's earth to ensure a product complying with the British Standards Specification. To prepare second grade oil, "seconds", the residual cake left after the expression of the firsts oil is broken, rolled and then extracted with a suitable petroleum solvent. The removal of the solvent by distillation gives an oil which after clarification is known as "seconds". It is higher in free acidity and darker in colour than "firsts".

"Blown" castor oil is produced by passing a rapid stream of air through hot firsts castor oil until the required viscosity is attained, after which the oil is cooled rapidly. Viscosity requirements vary according to the purpose for which the oil is used.

Castor oil can be hydrogenated under carefully controlled conditions. This produces a hard, wax-like material, of high melting point which has numerous industrial uses. The hydrogenation conditions have to be so chosen and controlled, that the hydroxyl group of the natural ricinoleic acid, 12-hydroxy 9-octadecenoic acid, the chief fatty acid

of/

of castor oil, is not reduced. This hydrogenation produces the triglyceride of hydroxystearic acid, of which it contains about 85 per cent. This material is a hard wax-like solid of nearly white colour and is translucent in thin layers. It melts at temperatures approaching 90°C to a clear, yellow, pale liquid and has wide applications in the paint and allied industries, particularly for modifying alkyd resins as suspending agents in the zinc rich paints and in the manufacture of thixotropic paints. Other uses will be indicated later.

Castor oil itself remains liquid at low temperatures, its solidification point being -10°C to -18°C. It does not thin rapidly with rising temperature, and is remarkable for its high viscosity and solubility in alcohol. It has an iodine value of 82 to 86, and its fatty acid composition as determined by gas liquid chromatography varies slightly, consisting of ricinoleic acid 86-90 per cent, oleic acid 4-6 per cent, linoleic acid 1-2 per cent and palmitic and stearic acids together, 4-6 per cent.

It is used in the raw or "blown" condition either as a lubricant or plasticiser. Sulphonation of the oil gives a product known as Turkey Red Oil. Heating it with a suitable catalyst converts it into a drying oil, dehydrated castor oil, which is useful in the preparation of paints and varnishes.

Details of most of the known uses for castor oil in American industry and commerce are given in Tables I and II (Bolley and Domingo, 1959). Only 2 per cent of the castor oil used in the U.S. is taken up by the cosmetic and pharmaceutical industry. Examination of the Tables shows/

Table 1. Estimate of percentage consumption of main classes of products derived from castor oil.

Class of Products	Proportion (%)	Pounds (10^6)
Protective Coatings	45	56
Plastics and Plasticizers	19	24
Lubricants	13	17
Surfactants	7	9
Hydraulic Fluids	5	6
Cosmetics and Pharmaceuticals	2	2
Miscellaneous	9	11
TOTAL	100	125

Table II. Uses of castor oil and its derivatives listed under the various classes of product.

Protective Coatings

Oil paint
Varnish
Lacquer
Thixotropic agent
Grinding medium
Emulsion paint
Enamel
Alkyd resin
Caulking compound
Polyurethane coating

Plastics and Plasticizers

Nylon
Plasticizer for polyvinyl
Plasticizer for cellulose acetate
Plasticizer for rosin
Plasticizer for shellac
Epoxy resin
Electrical insulation
Asphalt tile
Adhesive
Stabiliser for polyvinyl chloride
Polyurethane elastomer
Polyurethane foam
Latex foam
Leather lacquer
Artificial leather
Factice rubber substitute
Linoleum
Oil cloth

Lubricants

Dioctyl sebacate for turbines and jets
Grease
Motor fuel additive
Cutting oil
Lubricating oil

Surfactants

Turkey Red Oil
Dye oils for fabric
Textile lubricant
Emulsifier

Hydraulic Fluids

Brake fluid
Recoil mechanism
Shock absorber fluid

Cosmetics and Pharmaceuticals

Laxative
Lipstick
Medical soap
Fly dope
Hair dressing
Tooth paste
Cosmetic solvent
Disinfectant
Fungicide
Spermicide

Miscellaneous

Printing ink
Duplicating stencil
Transformer oil
Condenser oil
Belt dressing
Brake lining
Gasket paste
Fly paper
Insecticide
Leather preservative
Polish
Candle

shows that castor oil is used predominantly as an industrial raw material, and no discussion of this subject is complete unless it is explicitly pointed out that the medical use that made castor oil famous accounts for only a very minor portion of its current consumption, probably about 1 per cent, and perhaps even less.

INDUSTRIAL USES

The single largest use of castor oil is as the dehydrated oil in paints and varnishes. Under plastics and plasticisers, sebacic acid, derived from castor oil for the manufacture of a special type of nylon, is the most important use in this class. Castor oil, again through sebacic acid and dioctyl sebacate, is incorporated into low viscosity lubricants for turbines and jet engines. For this reason castor oil was classified as an essential raw material during the Korean war. About an equal amount of the oil is used in its hydrogenated form in the newer types of greases. Turkey Red Oil is one of the oldest surf-actants and still finds a major use under the familiar name of sulphonated castor oil.

When hydraulic brakes were first developed it was found that castor oil, plus a solvent, gave an excellent fluid, and this is still an important use for the oil. There is no single large use of castor oil in cosmetics and pharmaceuticals, although it goes into a very large number of different products. Its medicinal use is the most important in this class, although sizeable quantities are used in lipstick. It is reported that about 80 per cent of all lipstick used contains some castor oil.

Urethane polymers are a recent development that offer a new use

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for castor oil, and a large number of companies are studying the preparation, properties and application of this interesting class of products. The polyurethanes are prepared by reacting a diisocyanate with a polyhydroxy compound, the general reaction for which had been studied and reported by organic chemists about one hundred years ago. The original technology was expensive and this led to experimentation with polyglycols and castor oil. Castor oil was an obvious choice since it is a natural polyhydroxy compound. Indeed, of all the common vegetable oils it is the only one with a hydroxyl group. The use of modified castor oil and its special derivatives permits the preparation of urethanes of different properties that the many and varied applications necessitate.

One of the first surfactants, Turkey Red Oil, was prepared by reacting the hydroxyl group of castor oil with sulphuric acid. Recently, a series of surfactants had been made by reacting the hydroxyl group of castor oil with ethylene oxide. The more ethylene oxide added, the more water soluble the castor oil becomes. Thus a series of surfactants can be prepared that range from varying degrees of water emulsifiability to complete solubility. These are used as emulsifiers in cosmetics and in a number of other fields.

The addition of an atom of oxygen to the unsaturated bond in a fatty acid, using hydrogen peroxide, produces an epoxy fatty acid. When this reaction is carried out on triglycerides, or vegetable oils, epoxidised oils result. Since castor oil has a point of unsaturation a number of epoxy castor products have been prepared and offered to the chemical industry.

For/

For every pound of oil obtained from the seed there is at least a pound of waste meal or castor pomace. It normally contains about 36 per cent protein, but since this material may contain some toxin and allergen that is present in the castor seed, its use has been mainly limited to fertilizers. If the toxin and allergen could be completely eliminated, a more valuable use for this protein could be found.

MEDICAL USES

In medicine, castor oil has been used not only for its purgative action. Unfortunately, its other clinical applications have not always been so rational.

At an early point in the history of its pharmacology it was clearly demonstrated by Hans Meyer (1890) that the active component of castor oil was its principal fatty acid, ricinoleic acid. Werner (1870) had shown that the purgative action was not due to the toxic substance ricin, which was contained in the bean, since this was destroyed by boiling heat, while the oil itself lost none of its activity. Meyer showed that the purgative action of the oil was not lost even by heating up to 300°C in a current of carbon dioxide. He went further and excluded the possibility that a substance which he found adhering to castor oil might be the purgative principle, by preparing various salts of ricinoleic acid, purifying them by frequent recrystallisation from ethanol and testing these pure salts on animals and humans. In addition, by coupling purified fatty acid with glycerin, he produced a neutral oil containing ricinoleic acid, and this was tested also. Both the free acid and the neutral oil proved to be equally active, the/

the free acid being perhaps more active than ordinary castor oil.

The main body of medicinal research on castor oil was carried out between the years 1920 and 1948, and much of this was basic and sound. But at the same time some dubious, and a few ridiculous, applications for castor oil were promulgated.

Cooper (1921) first raised the possibility that castor oil might have oxytocic activity. Soon after, there followed a number of papers (Adler, 1927; Guiroy, 1928; Mathieu and Sichel, 1931; Aye, 1932; Mathieu, 1933; Morton, 1933), advocating the use of castor oil, either by itself or with quinine sulphate and pituitrin, in the induction of labour. However, there has never been a controlled trial of this regime, and it has come to be regarded as an unsubstantiated obstetric cult rather than as a tested induction procedure.

But, even if not unequivocally effective, it seems to have been safe, for there is only one report in the literature of a case of foetal mortality attributed to the use of castor oil for this purpose (Torland, 1928), and a report of one case in the medico-legal literature in which castor oil was implicated with a number of other procedures in the production of an abortion (Imparato, 1938).

Castor oil was not regarded as being so harmless in other clinical fields. Tanner (1927) drew attention to the danger of castor oil purgation in patients with acute appendicitis, and Melgaars (1929) followed this up with illustrations of its abuse in paediatric practice. Miyasaki (1929) advocated the combined use of castor oil and male fern in the treatment of tapeworm infestation, but five years later Kuck (1934)/

(1934) reported on a series of unpleasant complications which had followed this therapy.

Other uses of the oil have been suggested from time to time and it is of interest to mention these in a review such as this, though at the same time drawing attention to the fact that none has become established, and that most, with the wisdom of hindsight, fall outwith the bounds of the broadest empiricism. Thus in 1930, Ingham reported favourable results following the use of castor oil in the treatment of schizophrenia. Macphee (1934) recommended that the inhalation of castor oil vapour would result in adequate purgation and avoid the unpleasant taste. Pugh (1935), with perhaps less naivity, suggested that castor oil might be of value in eradicating chronic urinary infection where some intestinal bacterial intoxication was an aggravating or perpetuating influence.

Schoch (1939), pursuing this theme of chronic intestinal intoxication as a cause of disease, recommended the use of castor oil and sodium ricinoleate for the elimination of intestinal toxins where they might be causing, or contributing to, some of the chronic dermatoses. Skulskiy, Polonskaya and Granovskaya (1937) proposed the use of cod liver oil and castor oil in the treatment of inflammation of the cervix and vagina, and Smith and Baier (1939) claimed that castor oil and magnesium sulphate were of value in the treatment of upper respiratory diseases such as coryza and acute bronchitis.

Finally, and perhaps not altogether irrationally, Tisseuil and Guilhaumou (1938) claimed some action for castor oil, cod liver oil and methylthionine chloride in the treatment of tuberculoid spots.

Castor/

Castor oil does act on the skin as a mild drying agent, and its use by local application produces visible effects in certain circumstances. It was this local effect which led to its use by Smelyanskiy (1940) in the treatment of squamous blepharitis.

The established ethical use of castor oil is as a purgative. Much of the early research tried to show how it acted, and a few of these key studies will now be considered.

Ogata and Okasaki (1923) studied the isolated small intestine of rabbit, a piece of which was suspended in 100 ml. Lock's solution at 40°C. In order to determine what chemical group in ricinoleic acid was responsible for the cathartic action they prepared alpha-benzalsuberic acid, alpha-benzalazelaic acid, alpha,alpha'-dibenzalsuberic acid and alpha,alpha'-dibenzalazelaic acid and added the salts of these acids to the water bath. The experiments showed that these acids had no effect on peristalsis and relaxation of the intestine, but produced immediate paralysis, an effect contrary to that produced by ricinoleic acid. As a result of their work they could be no more specific about the differences on action of these substances compared with ricinoleic acid, than that they were due to the absence of the OH group, the size of the molecule, the position of the double bond, the number of COOH groups or to the introduction of an aromatic group.

Bauer (1925) compared the type of effect on intestinal movement produced by certain oils, colocynth, gamboge and calomel. Using the isolated/

isolated guinea pig intestine, differences in the actions of these substances were observed comparable to those noted in the intact animal. Castor and croton oil at times caused increased peristalsis with an accelerated transport of material. With croton oil, irritating effects were often so strong that they led to closure of the intestinal lumen and to the arrest of peristalsis.

Heiduschka and Kirsten (1930) showed that castor oil contained a limited number of fatty acids, namely ricincleic, oleic, linoleic, stearic and dihydroxystearic. The last named was never found in any of the castor oil samples analysed in this study and may have appeared in their samples as a technical artefact. They went on to show that the action of castor oil was not due to free ricinoleic acid in the crude oil but to acid released by the process of digestion.

Grandall, Holinger and Walsh (1931) demonstrated the tissue irritability of the free fatty acids in general by observing their effect when injected intrapleurally and intrapericardially into dogs. Triglycerides were less irritant than their free fatty acids and there was no difference in irritant effect between animal and vegetable oils. Castor oil was one of the substances investigated in their study.

Lecoq and Savare (1934) showed that castor oil had no effect on the intestinal excitability of pigeons when it was fed with the diet and kept to a small proportion of the meal. Two years later Lecoq and Carel (1936) studied the liberation of ketones following the digestion and absorption of castor oil, and showed that the formation of ketone compounds was similar to that after the ingestion of other

fats/

fats and did not explain the intestinal disturbance caused by the castor oil.

Valette and Salvanet (1936a) confirmed that ricinoleic acid was the purgative principle in castor oil and in the same year (Valette and Salvanet, 1936b), in a study on one subject, they suggested that even when the oil produced purgation some of it was absorbed. Their conclusions were not based on direct recovery of castor oil or ricinoleic acid, but on a general fat balance in which they compared the amount of faecal lipid in the fasting state with the lipid recovery following the administration of known amounts of castor oil.

In experimental work (Valette and Salvanet, 1936c) they showed that sodium ricinoleate had no influence on the intestine deprived of its nervous and vascular connections. They felt that the purgative action was exerted on the intestinal mucosa and was independent of the absorption of any of the constituents of the oil. They thought it possible that the action of the alkaline ricinoleates could be attributed to their high cytolytic properties, the latter perhaps caused by a dissolving effect on the cellular lecithin.

Lecoq (1937) disagreed with this. Following up his earlier work on pigeons, he stated that the purgative action of castor oil was not limited to a local effect on the intestinal mucosa, but was supplemented by a systemic effect caused by certain absorbed factors. What these were he did not specify, although ricinoleic acid was clearly regarded as the likeliest.

Valette and Salvanet were supported in their contention by the work/

work of Yamamoto (1937) who studied the rabbit and dog intestine in situ and the isolated rabbit intestine. He showed that castor oil, ricinoleic acid and peristaltin, in the presence of bile acids, acted directly on the musculature of the intestine.

Hartwell (1938) studied the effect of pancreatic lipase on the digestibility of a large group of fats. Of these coconut oil was digested more rapidly than any other, while palm kernel oil and castor oil were digested more rapidly than butter, which in turn was digested more rapidly than any of the 17 other fats and oils tested. This group of 17 included lard, olive oil, peanut oil, bacon and beef fat. Ahmad and Bahl (1946) repeated this work and in many cases reversed the order of digestibility given by Hartwell. In particular, and with reference to an experiment to be described in a later chapter, they gave olive oil a much greater digestibility rating than castor oil.

In an interesting radiological study Bruck and Fruchter (1942) investigated intestinal motility following a single dose of some of the commonly used laxatives. Those studied were cascara, liquorice powder, magnesium citrate, magnesium sulphate and castor oil. Three normal individuals were treated with each laxative, and a control radiological examination was made on each patient about 7 to 10 days before the laxative was administered. Films were made immediately, and at 1, 3, 4, 6, 8, 24 and 48 hours after the laxative-containing barium had been given.

Fluid extract of cascara and compound liquorice powder produced no alteration in gastric evacuating time as compared to the control.

The/

The magnesium salts and castor oil produced delayed gastric motility. All laxatives produced an initial irritability of the proximal jejunum lasting one to three hours. With magnesium sulphate there was definite delay in the passage of the meal through the small intestine. The other laxatives showed moderately increased small intestinal motility. Fluid extract of cascara showed a slightly increased emptying of the colon.

Compound liquorice powder and castor oil, of all five laxatives used, produced the most satisfactory and complete emptying of the colon. The two magnesium salts showed more retention at 48 hours than the control, in spite of numerous bowel movements. The authors concluded that castor oil and compound liquorice powder were the best laxatives for colonic cleansing in preparation for X-ray examinations. Many radiologists still concur with this opinion and one of the main uses of castor oil today is in the preparation of patients for intravenous pyelography.

A possible mechanism of action of castor oil was proposed by Erspamer and Paolini (1946). In experiments on rats they showed that the administration of an antihistamine drug (dimethylaminoethyl-benzylaniline) slowed down the purgative action of castor oil, colocynth and senna. They concluded that the purgative action of these agents was due at least in part to the release of histamine. There has been no subsequent work which has either confirmed or disproved their opinion.

It can be seen from this survey of the miscellaneous medical literature/

literature on castor oil that there has been no single, broad study of this interesting vegetable compound. The work which follows is an attempt to investigate in a systematic way, the metabolism of castor oil.

It has not been possible to follow all of the possible lines of investigation. There are no data in this thesis on the effect of ricinoleic acid on the in vitro animal intestine preparation. Some preliminary studies were carried out, but it was soon apparent that the large number of variables, such as pH, calcium, sodium and potassium ion concentration, which required separate control and investigation were so many and so critical, that this in itself was a major undertaking.

The structure of the thesis is straightforward. Chapter 2 is a preliminary account of the basic methods in constant use. Chapters 3 to 5 describe the laboratory studies carried out in the rat, and Chapters 6 to 8 the studies carried out on human volunteers and patients. The final Chapter surveys what has been achieved and indicates where further investigation might proceed.

CHAPTER 2

GENERAL METHODS AND MATERIALS

Special techniques will be described with the experimental work for which they were especially devised, or to which they particularly apply. Some techniques were in regular use throughout the work. A detailed account of these is given now and also the sources and properties of basic materials.

CASTOR OIL

Castor oil was obtained from two sources, in America from the Baker Castor Oil Company, Bayonne, New Jersey, and in this country from the British Oil and Cake Mills, Hull. The fatty acid composition of the oil from these different sources is given in Table III. The chemical notation for the fatty acids is that suggested by Dole, James, Webb, Nizack and Sturman (1959). There are minor differences in the percentage amounts of the various fatty acids, but there is no major qualitative difference between them. Both oils were of pharmaceutical or medicinal quality.

I^{131}-LABELLED CASTOR OIL

I^{131}-labelled castor oil was prepared as required by the method of Rutenberg, Seligman and Fine (1949). Since the method was adapted for the special purposes of this study the actual procedure will be described in detail.

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Table III Percentage fatty acid composition of samples of castor oil from both supply sources, obtained from combined data of GLC on silicone gum and ethylene glycol adipate columns.

Fatty Acid	Baker Co.	British O. and C. Mills
Palmitic (16:0)	1.0	3.0
Palmitoleic (16:1)	trace	trace
Stearic (18:0)	1.0	3.0
Oleic (18:1)	3.3	5.2
Linoleic (18:2)	4.7	2.8
Ricinoleic (OH-18:1)	90.0	86.0

The first step is the preparation of radioactive iodine-monochloride ($I^{131}Cl$). Potassium iodide (0.36 g) and potassium iodate (0.25 g) are dissolved together in 0.62 ml distilled water. One millecurie of carrier-free iodide^{131} in negligible volume is added and mixed. Next, concentrated hydrochloric acid (0.72 ml) is added slowly and stirred vigorously until the resulting heavy dark precipitate of iodine-I^{131} redissolves. A small amount of iodine vapour appears in the flask during this procedure. The final solution is clear and orange coloured.

The second step is the labelling of the oil and subsequent removal of unbound radioactive iodine. The iodine-monochloride solution is cooled to 4°C and added in 3 portions, with continuous stirring, to a cold solution of 6 ml castor oil in 10 ml of ether. The mixture is mechanically shaken for 60 to 90 minutes at room temperature. During this time the colour of the aqueous solution is discharged.

The straw-yellow solution of oil in ether is washed once with water and twice with 5 per cent sodium sulphite in 0.1 N sodium hydroxide solution. It is then washed with water, dilute hydrochloric acid and finally three times with distilled water. These washings, which are carried out in a separating flask, have to be done gently. If the flasks are shaken too vigorously a fine emulsion forms which may take a long time to disperse.

The ether solution is dried with anhydrous sodium sulphate and the ether removed by distillation under reduced pressure and at a temperature/

temperature between 40-50°C. The yield of residual pale yellow oil is about 5.5 g and contains 80 to 90 per cent of the initial radioactivity.

ANIMALS

Animal studies at the National Institutes of Health were made on Sprague-Dawley and Wistar rats bred at the Institutes' own farm. In this country Wistar rats were supplied commercially.

EXTRACTION OF SERUM AND TISSUE LIPIDS

The method used is essentially that of Bragdon (1960) which is based on Sperry and Brand (1955).

Materials

1. 50 ml calibrated conical centrifuge flasks with ground glass stoppers, supplied by the Kimble Glass Company in America. A satisfactory equivalent was made by a local supplier in this country from Quickfit B29 gauge tubing.

2. Reagent grade chloroform and methanol, redistilled, mixed in the ratio of 2 parts chloroform to 1 part methanol.

3. 0.05 per cent aqueous sulphuric acid.

Method

About 46 ml of the chloroform - methanol (C/M) mixture is added to the extraction flask. Using a calibrated 2 ml syringe and a fine needle, 2 ml of serum is injected into the mixture, which is allowed to stand for 10 minutes and then made up to the 50 ml mark. When tissue, for example liver, is being extracted the piece of tissue is placed in 48 ml of the C/M mixture and the volume made up to 50 ml with/

with distilled water or isotonic saline. With tissue the extraction should continue overnight.

Next, 10 ml aqueous sulphuric acid is added and the flask inverted gently 10 times. It is allowed to stand for 10 minutes and then centrifuged, preferably in a refrigerated centrifuge, at 2000 rpm for 20 minutes. The lower, chloroform phase, is removed using a 50 ml syringe and a long needle, transferred to another flask and dried over anhydrous sodium sulphate. The volume of the wet chloroform phase is 36 ml. Most of the extracted lipid is present in it and only traces of very polar phospholipids remain in the upper phase.

The method may be used with different amounts of reagents, provided the same ratios are retained.

ESTIMATION OF TOTAL LIPIDS

The theory of the following method and the proof of its validity are fully discussed in the original paper (Bragdon, 1951). Only working details are given here.

Materials

1. Oxidising reagent, or "Nicloux solution". Potassium dichromate (reagent grade) is finely powdered in a mortar, and 20 g dissolved in 1000 ml concentrated sulphuric acid. Slight heat may be used for this. Protracted mixing is necessary to achieve solution. If protected from light and contamination the reagent is stable for one month at room temperature.

2. Palmitic acid standard. Palmitic acid (125 mg) is made up to 250 ml in a volumetric flask with chloroform:methanol (2:1). This is/

is stored in the refrigerator in a glass bottle.

3. Ground glass stoppered 25 ml volumetric flasks.

4. Water baths at 60°C and 100°C.

5. Rack for holding flasks.

6. Nitrogen.

Method

5 ml of the chloroform phase of the lipid extract obtained by the previous method is placed in a 25 ml flask. To similar flasks 5 ml of palmitic acid and 5 ml of C/M are added as standard and blank respectively. The flasks are evaporated to dryness at 60°C under a stream of nitrogen. Immediately 10 ml Nicloux reagent is added to avoid spontaneous oxidation or loss by evaporation. The flask is stoppered, a good seal made by touching the stopper with a drop of the reagent, and boiled for 30 minutes. It is important to make sure that the entire inner surface of the flask is wetted with the reagent. The stopper, although making a good seal, should be loose and the water level in the bath should completely cover the flask to the base of the stem.

The flasks are cooled in a water bath at room temperature. About 10 ml of distilled water is added to each flask and, for safety, the orifice of the flask is kept turned away from the face, rotated gently and kept cool. Water is added to the mark after all bubbles have stopped rising.

The optical density is read at 580 mμ between 15 and 90 minutes after dilution. The blank is used to adjust the photometer to zero.

Calculation/

Calculation

The standard contains 2.5 mg palmitic acid. Since the theoretical dichromate reducing capacity of palmitic acid is 17.6 mg $K_2Cr_2O_7$ per mg $C_{15}H_{31}COOH$, the standard is equivalent to 2.5 x 17.6 = 44 mg $K_2Cr_2O_7$.

The theoretical dichromate reducing capacities of different types of tissue lipids expressed as mg dichromate per mg of lipid are:-

palmitic acid and other fatty acids	17.6
cholesterol, free and esterified	19.1
phospholipid	15.2
triglyceride	17.7

When the total mixed lipids of a serum of tissue are required, the factor equivalent of 17.7 is taken as being a good average.

Under the conditions in the laboratory where this method was first developed and later used by this author, the factor combining the value for the mixed-lipid dichromate equivalent and the optimal density of the standard, was 109. The equation for the calculation is therefore:-

$$\text{mg lipid per ml chloroform extract} = \frac{109}{17.7} \times \frac{1}{5} \times \text{O.D. unknown}$$

TITRATION OF FREE FATTY ACIDS

The method used was the modification by Trout, Estes and Friedberg (1960) of the original method by Dole (1956). However, since the method was used not for the titration of free fatty acids in serum, but of those released by the incubation of rat epididymal fat pads and during the in vitro lipolysis of castor and olive oils, the actual procedure/

procedure employed is given in detail.

Materials

1. Extraction mixture. Isopropanol 40 parts, n-heptane or iso-octane 10 parts, and normal sulphuric acid 1 part. It is possible to obtain reagents of sufficient purity that redistillation is not necessary, but this should be tested by running blanks.

2. Indicator. A stock aqueous solution of commercial Nile Blue A in a concentration of 0.2 g per litre is prepared and washed with octane or heptane until free of extractable coloured impurities. Working indicator is prepared fresh each day by mixing 1 part of this stock solution with 9 parts of absolute ethanol.

3. Alkali. 0.02 N sodium hydroxide, standardised by any accepted procedure.

Method

The post-lipolysis sample was 1 ml in volume and the post-incubation sample was 3 ml. The method will be described as for the 1 ml sample. With the larger sample the reagents are added proportionately.

1 ml of sample is mixed with 5 ml of extraction mixture in a glass stoppered tube or flask, shaken vigorously and allowed to stand for 10 minutes or more. Then 3 ml heptane and 2 ml water are added and the mixture is shaken for at least 2 minutes. The system will separate into 2 phases and on standing form a sharp interface.

As much as possible of the upper "heptane" layer is removed to a glass stoppered centrifuge tube and shaken vigorously for 5 minutes with an equal volume of 0.05 per cent aqueous sulphuric acid. The

tube/

tube is centrifuged at 300 g for 5 minutes. Blanks and standards should also be washed in this way. When larger volumes of reagents are being used a 4 or 5 ml aliquot of the heptane layer is taken.

3 ml of the washed heptane layer is pipetted into a conical glass centrifuge tube, 1 ml of indicator is added and the mixture is titrated with 0.02 N sodium hydroxide. The alkali may be delivered from any suitable microburette. A stream of washed nitrogen serves to mix the two phases during titration and to exclude atmospheric carbon dioxide. At the end point the indicator will change to a pink colour. It is necessary to turn off the nitrogen intermittently to allow the droplets of the indicator to coalesce completely, and then again disperse them through the hydrocarbon phase before deciding finally that the end point has been reached.

Calculation

$$\text{Free fatty acids } (\mu.\text{eq/ml of sample}) = \frac{20 \times V}{F}$$

where 20 is the concentration of alkali in μ.eq/ml,

V is the volume of alkali used (ml),

and F is the aliquot factor for the heptane phase.

The aliquot factor is not always the same, as it depends on the actual hydrocarbon solvent used and on the room temperature. The volume of the upper phase from which the 3 ml aliquot is taken, when minimal volumes are used, is usually 4.1 to 4.2 ml. A reagent blank must be run with each group of analyses and its result subtracted from the fatty acid value obtained for each sample.

Notes/

Notes

Samples should ideally be analysed without delay, but if necessary they can be stored in the frozen state.

The modification of acid washing of the heptane layer minimises interference by an acetone insoluble lipid which may be a phospholipid.

GAS LIQUID CHROMATOGRAPHY (GLC)

Columns

Much of the work reported here was made possible by the fact that the silicone gum column, first developed by Vanden Heuvel, Sweeley and Horning (1960) for the separation of steroids proved to be ideal for the fatty acid analyses of tissues containing ricinoleic acid. Our published work contains the first account of the silicone gum column for the rapid separation of hydroxy fatty acids (Watson, Gordon and Karmen, 1961; Watson and Gordon, 1962). It is appropriate at this point to give a brief account of the preliminary observations which led to the adoption of this type of column. The method was initially developed for the Burrell GLC, which contains a 6 foot glass column, but was easily adapted to the Pye apparatus, which has a 4 foot column.

Ricinoleic and 12-hydroxystearic acids in lipid extracts of tissues were analysed by gas chromatography of their methyl esters. The retention times of methyl ricinoleate, on three different kinds of column, compared with those of methyl stearate as a reference, are listed in Table IV, and an example of a record of an analysis of mixed standards, on the silicone gum column, is shown in Figure 1. The silicone gum column clearly separates the ricinoleic acid ester from that of 12-hydroxy-

stearic/

Table IV. Retention times in minutes for methyl ricinoleate and methyl stearate on three types of GLC column

Column Conditions	Me. Ricinoleate	Me. Stearate
Silicone gum (Dow Corning 400) 3% Chromosorb W* 97% Temp. 200°C Argon 23 p.s.i.	16.7**	10.0
Ethylene glycol adipate polyester 10% Chromosorb W 90% Temp. 190°C Argon 23 p.s.i.	51.7	8.4
Ethylene glycol isophthalate polyester 10% Chromosorb W 90% Temp. 180°C Argon 23 p.s.i.	96.0	13.8

* Trademark for the diatomaceous earth product of the Johns-Manville Corporation.

** Retention time of methyl hydroxystearate under these conditions was 19.5 minutes.

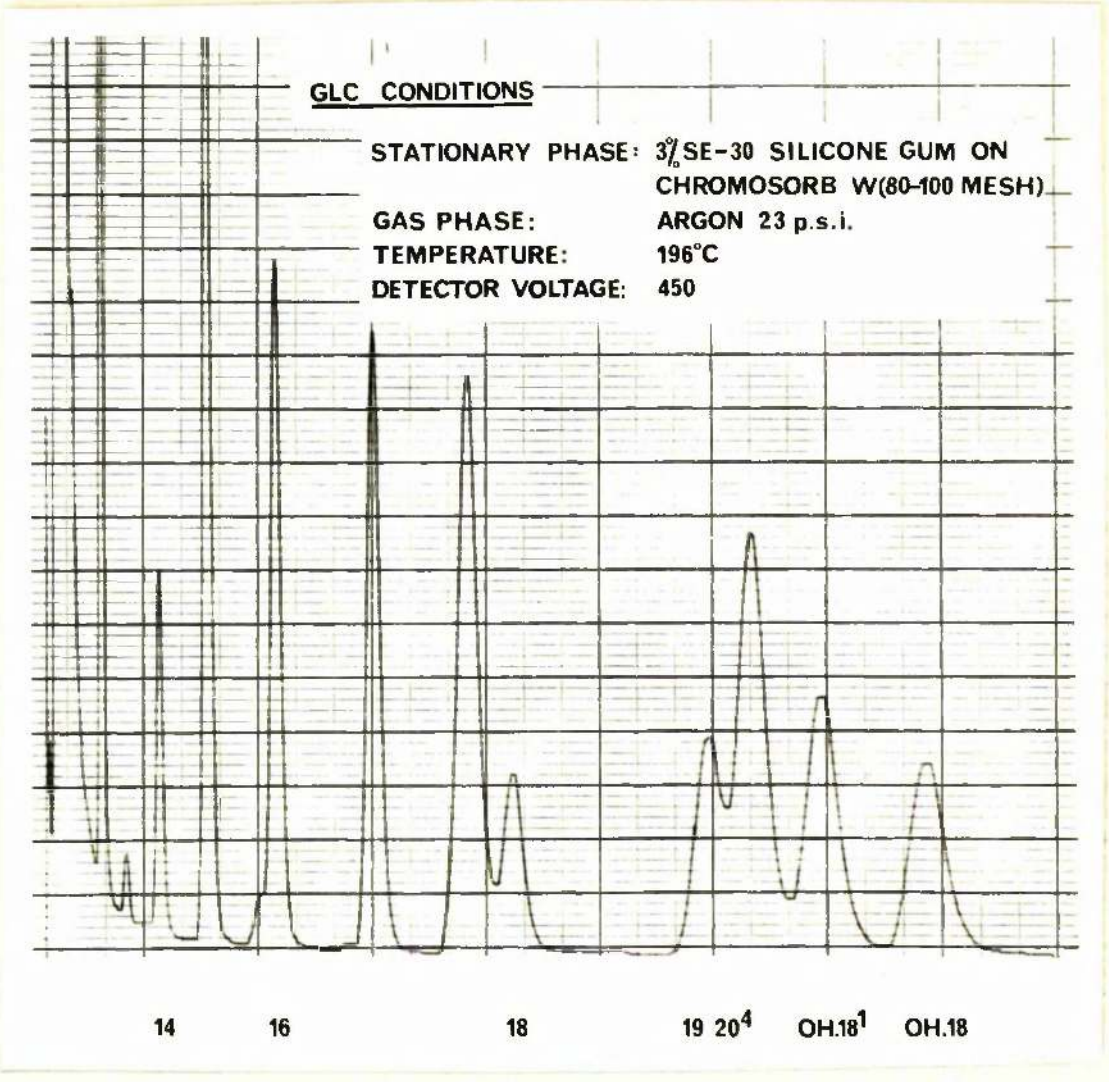


Figure 1. Example of GLC analysis of a mixed fatty acid standard on the silicone gum column. There is clear separation of nonadecanoic (19), arachidonic (20:4), ricinoleic (OH.18:1) and hydroxystearic (OH:18) acids. Time to ricinoleic acid is 18 - 20 minutes.

stearic acid and both of these from other fatty acid esters of interest, namely arachidonic (20:4) and nonadecanoic (19:0). In practise, however, because of the special conditions of this study, methyl ricinoleate was the predominant fatty acid ester found in this region of separation. Oleic, linoleic and linolenic acid esters are not separable on this column.

Use of the polyester columns (see Table IV) permits complete separation of the unsaturated acid esters. The retention times of the hydroxy acids relative to that of methyl stearate varied from one polyester to another, demonstrating that these columns, which differ only slightly in their behaviour toward straight chain saturated and unsaturated acids, have distinctive behaviours when other functional groups are present. Although the two hydroxy acids were well separated from each other and were well separated from the other acids of interest, the hydroxy acids were retained on these columns for too long to permit their use to be convenient. Indeed, on the 22 per cent ethylene glycol adipate column, which was used in preference to the 10 per cent column because of its generally superior resolving power, the hydroxy acids were not recovered from the column until the following day. Each sample was therefore analysed on both silicone gum and ethylene glycol adipate columns, the latter for the non-hydroxy fatty acids only, and a complete analyses obtained from the combined data.

Detectors

Both instruments used during this work (Burrell and Pye), incorporated detectors based on the principles described by Lovelock (1958).

The/

The detector in the Burrell apparatus contained 80 microcuries of radium D as the source of ionising radiation, while the Pye machine was fitted with a 20 microcurie Strontium 90 radioactive source, sealed inside gold foil.

The detectors were calibrated by injecting the same volume of a series of dilutions of the methyl esters of corn and castor oils. The detector voltage chosen was that at which the areas of the peaks were linearly related to the quantity of ester injected. For the Burrell instrument 450 volts was the optimum selected, while 1000 volts was best for the Pye machine. Argon was always used as the carrier gas.

Quantitation

Since the response of the detector to the molecules of gaseous ester passing through it, at the correct voltage and temperature, is directly proportional to their number, the areas of the peaks on the recording chart are related quantitatively to the fatty acids which they represent. These areas are measured by "triangulation", as shown in Figure 2. The percentage amounts of the various fatty acids are calculated by summation and fractionation of the areas for each fatty acid.

Preparation of Esters

Methyl esters of all fatty acids can be prepared by the process of transmethylation. At the correct temperature, and given a suitable length of time, fatty acids will react with anhydrous methanol thus:-

fatty acid + methanol -> fatty acid methyl ester + water

$$R.COO(H + OH)CH_3 \rightarrow R.COOCH_3 + H_2O$$

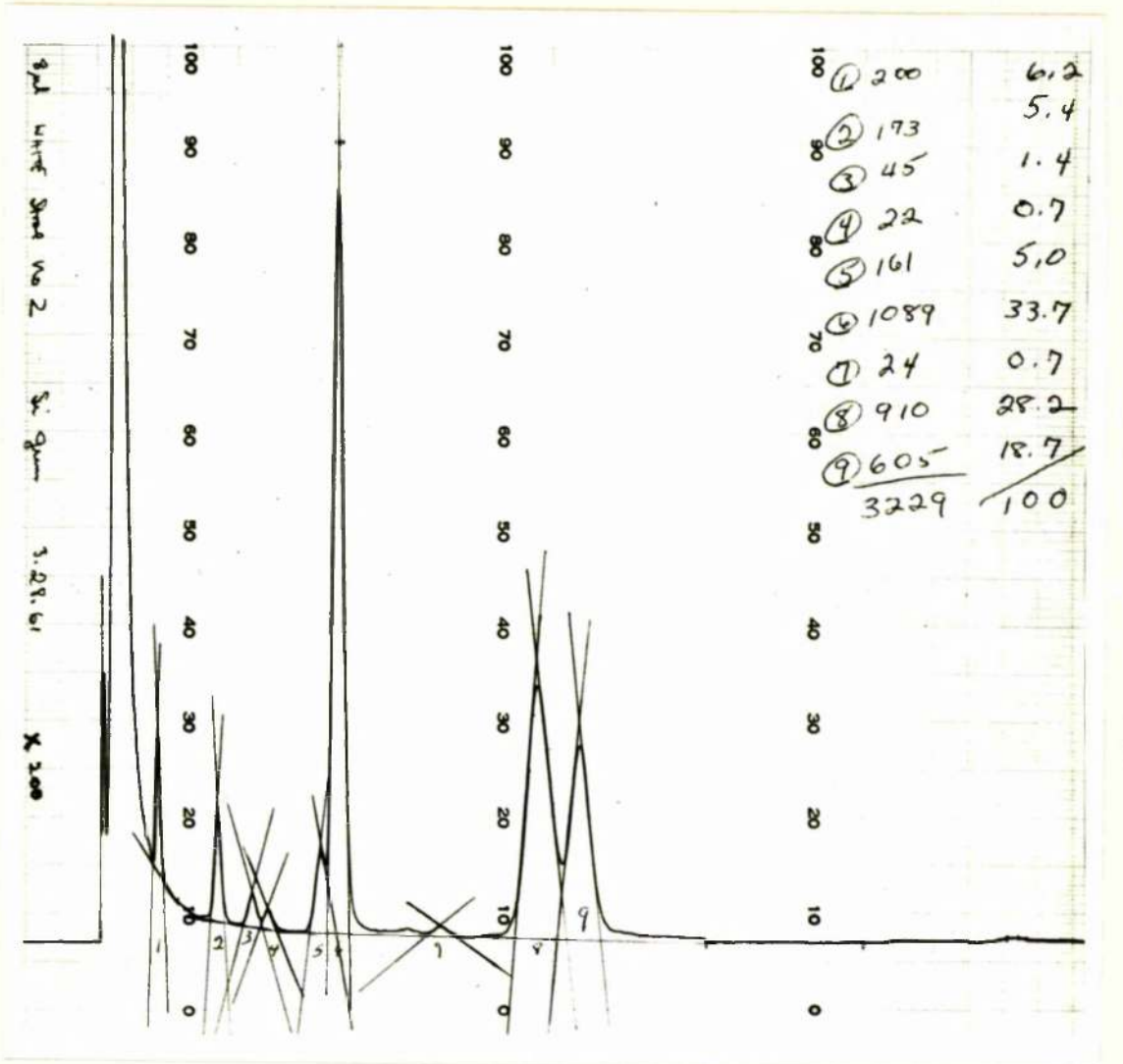


Figure 2. Reproduction of a working record showing the technique of measurement of fatty acid peaks by triangulation.

This reaction which proceeds readily with free fatty acids will also take place with the fatty acids of phospholipids, cholesterol esters and glycerides, if they are first hydrolysed by a suitable agent. The methylating mixture was:-

anhydrous methanol (100 parts)

benzene (10)

concentrated sulphuric acid (2), the hydrolysing reagent.

If phospholipid predominates in a lipid mixture, the proportion of sulphuric acid is increased to 7, or even 9 parts.

A suitable amount of the lipid to be esterified, preferably about 10 mg. is taken to dryness under nitrogen in a clean test tube, threaded to take a screw-cap lid. The test tubes, which can be re-used, are cleaned in chromic acid and washed repeatedly in distilled water. The sealing caps, each of which is lined with a teflon washer, are discarded after one use. 1 ml of the methylating mixture is added to the dry lipid in the tube, the cap firmly screwed on, the tube shaken to assist solution of the lipid and then placed in an oven at 60°C. After 1 hour the tube is removed from the oven and the cap tested for tightness. Usually it requires a further slight turn to make a good seal. The tube is replaced in the oven and left overnight.

Next morning the tube is removed from the oven, allowed to cool to room temperature and the seal broken. 1 ml of distilled water is added to the tube and the fatty acid esters extracted by 3 x 1 ml aliquots of petroleum ether (boiling range 40-60°C). The petroleum ether extract is transferred to a small glass vial, evaporated under nitrogen/

nitrogen and the esters redissolved in as small a volume of n-heptane or iso-octane as possible. The methyl esters are very stable and can be retained for repeat analysis over a period of months.

It is imperative when esters are being prepared in batches that cross contamination of samples is scrupulously avoided. It is essential therefore to use individual, disposable pasteur pipettes for all transfers, and to discard these after use.

This completes the account of basic methods in use throughout this study, and it is now possible to proceed to an account of the experimental work.

CHAPTER 3

THE ABSORPTION AND ASSIMILATION OF CASTOR OIL IN RATS

The work of this chapter is divided into three sections. In Section 1 direct proof of castor oil absorption was sought and obtained by demonstrating the presence of ricinoleic acid in thoracic duct chyle following the ingestion of the oil. In Section 2 proof of absorption was corroborated by showing that ricinoleic acid had become incorporated into the adipose tissue triglyceride. In Section 3 an attempt was made to show that ricinoleic acid could participate in the in vitro synthesis of triglyceride.

SECTION 1

Recovery of orally administered ricinoleic acid from rat chyle

Faecal balance methods using I^{131}-labelled fats give only indirect proof of the absorption of a lipid substance. Incomplete recovery may be due to chemical or isotopic degradation during intestinal transit. Nor is the presence of a substance in the gut mucosa proof of absorption, since it could be re-excreted into the intestinal lumen without emptying either into the portal venous system or into the lymphatic channels.

Therefore it seemed that the most direct proof of absorption of castor oil would be to show the presence of ricinoleic acid in thoracic duct chyle after the administration of the oil. The experiment was

also/

also planned to discover whether the degree of absorption, if any, was affected by feeding or fasting.

Methods

For this experiment, and others reported elsewhere, it was necessary to learn the technique of thoracic duct cannulation in the rat. This is quite the most difficult research technique the author has had to acquire and it needed much practice before a pair of rats could be successfully cannulated on the same day.

The procedure was essentially that described by Bollman, Cain and Grindlay (1948). The approach was through a midline abdominal incision, and not through the left upper trans-lumbar incision advocated by the original authors. Fine polyethylene tubing was used for the cannula instead of the plastic tubing which was the best material available in 1948. Before insertion the cannula was rinsed with a dilute solution of heparin to inhibit the formation of chyle clots, which usually form if the animal does not drink well after the operation and the flow of chyle is meagre. Visualisation of the cysterna chyli and thoracic duct is enhanced if olive oil is added to the food given to the animals on the previous day.

After operation the rats were placed in special restraining cages constructed according to the description of Bollman (1948). These cages are small in order to prevent the rat from turning round, but they do permit some forward and backward movement.

The experiments were carried out on 4 pairs of young male Sprague-Dawley rats, one pair at a time. Each rat was allowed unlimited 0.5 N saline/

saline, but only one of each pair was given chow. On the morning after cannulation, provided both animals were in good condition and the cannulae were flowing freely, each animal was lightly anaesthetised with ether by placing it, still in its restraining cage, inside a square metal wastepaper basket containing cotton wool soaked in ether. The open top of the basket was covered with a hand towel. 1 ml of castor oil was administered by stomach tube and chyle collected for the next 24 hours.

An aliquot (A) of the total volume of chyle (V) was extracted with chloroform:methanol (2:1) for the estimation of total lipids (L) and for the preparation of methyl esters for gas-liquid chromatography. The percentage amount of ricinoleic acid in the chyle fatty acids was obtained by analysis of the fatty acid esters on the silicone gum column.

Calculation

The calculation to determine the proportion of the dose of ricinoleic acid absorbed requires one assumption and one or two minor approximations.

Borgstrom (1952) showed that following the feeding of corn oil to rats the composition of the intestinal lymph fat recovered during the subsequent 24 hours was, glyceride fatty acids 88 per cent, phospholipid fatty acids 10 per cent, and cholesterol fatty acids 2 per cent. On the assumption that a similar distribution of chyle fatty acids will follow the feeding of castor oil it can be shown that the chyle fatty acids account for approximately 90 per cent of the weight of the chyle lipid. The rest of the weight is accounted for by glycerol, cholesterol and phosphate. It is unlikely that any significant amount of the chyle

lipid/

lipid will be free fatty acid.

The equation for the calculation is therefore derived as follows:-

I. Weight of ricinoleic acid in chyle (mg) =

weight of 24 hour chyle lipid (mg) x 0.9 x R

where 0.9 is the factor derived from the above paragraph, and

R is the percentage ricinoleic acid in the lymph fatty acids as determined by GLC.

II. Dose of ricinoleic acid administered (mg) =

$$1.0 \times S.G. \times 0.9 \times \frac{896}{988} \times 1000$$

where 1.0 is the volume of oil administered (ml),

S.G. is the specific gravity of castor oil,

0.9 is the factor accounting for the fact that ricinoleic acid is 90 per cent of castor oil fatty acids, and

$\frac{896}{988}$ is the factor accounting for the glycerol component of castor oil.

Then the recovery of ricinoleic acid = $\frac{I}{II} \times 100$ per cent.

Since the volume of castor oil was the same for each rat, factor II is a constant and equal to 734 mg.

Results

The results are shown in Table V. They prove two things. Firstly, that ricinoleic acid, which is never present in the intestinal lymph of the normal rat, has been absorbed in varying amounts by each of the experimental animals, and secondly, that there is a significantly greater degree of absorption by the fed animals ($p < 0.01$).

Comment/

Table V. Recovery of ricinoleic acid in the chyle of fed and fasted rats following the intragastric administration of 1 ml of castor oil.

Dietary state	Rat No.	% Recovery of ricinoleic acid
Fasted	1	4.6
	2	9.3
	3	2.7
	4	10.4
	Mean	6.8
Fed	5	18.5
	6	19.7
	7	27.5
	8	31.1
	Mean	24.2

Comment

Experiments in which intestinal lymph has been collected have been of fundamental importance to the study of fat absorption. During earlier development in this field, investigators were generally limited to obtaining lymph from anaesthetised animals. As normal fat absorption is dependent on normal intestinal mobility such experiments sometimes gave incomplete and erroneous results because intestinal mobility is profoundly influenced by anaesthesia and lymph flow from the intestine is only a fraction of the normal.

The introduction of a technique for the collection of lymph from the thoracic duct of the unanaesthetized rat has therefore been of great importance. But although recovery of over 90 per cent of the absorbed fat is not uncommon in some of the reported work on rats, some caution has to be exercised in interpreting the results. Entirely normal pressure conditions and lymph flow cannot be expected when an open cannula has been inserted into the thoracic duct. The animal also loses large volumes of water, protein and salts that are difficult to replace entirely. These factors probably contribute to the large individual variations that have been recorded by different investigators.

Nevertheless, in spite of these practical and theoretical difficulties there can be no doubt of the proof of absorption of ricinoleic acid as shown by the experiments described here. Further, since the experiments were carried out on paired animals it seems reasonable to accept the statistically supported evidence of the data that the absorption is significantly greater in fed than in fasted animals. Why this might/

might be so, will be deferred for later consideration.

It is also clear, however, that the degree of absorption of ricinoleic acid is much less than the recoveries of 60 to 97 per cent of C^{14}-labelled palmitic, oleic, linoleic and stearic acids reported by Bloom, Chaikoff, Reinhardt, Entenman and Dauben (1950), Bloom, Chaikoff, Reinhardt and Dauben (1951), and Bergstrom, Blomstrand and Borgstrom (1954), using similar quantities of oil. This difference reflects the metabolic uniqueness of ricinoleic acid. It will be shown later, however, that the degree of absorption of ricinoleic acid is inversely related to the dose and the implications of this fact will be explored.

Having demonstrated that ricinoleic acid was absorbed and that its absorption was enhanced by giving the oil in the post-prandial state, the next experiment was planned and begun.

SECTION 2

The deposition of ricinoleic acid in adipose and other tissues

Methods

Seven weanling rats were given a diet of ground Purina rat chow mixed with 20 per cent by weight of castor oil. The diet was acceptable and they gained weight, though less well than a control group on an olive oil supplemented diet.

After 4 weeks of the diet the right epididymal fat pad was removed from each animal in both groups through a small right lower suprapubic paramedian/

paramedian incision, and after a further 4 weeks the animals were killed and the left pad removed. The initial incisions were closed with Michel clips and there was no trouble with wound healing in any of the animals.

The fat pad lipid was extracted with chloroform:methanol (2:1) and methyl esters prepared. Fatty acid analyses were carried out on the silicone gum column.

At various times throughout the feeding random stool samples were analysed for faecal fatty acids, and at the end of the feeding period when the animals were killed, fatty acid methyl esters were prepared from the extracted lipids of brain, muscle, heart, kidney, liver, retroperitoneal and subcutaneous fat of one of them.

Results

The weights of the castor oil fed rats during the first 4 weeks of the experiment are shown in Table VI, and the ricinoleic acid content of the fat pads in Table VII.

Clearly ricinoleic acid is readily incorporated into the triglyceride fatty acids of adipose tissue. The maximum degree of incorporation seems to be about 10 per cent. Individual animals continued on the diet for longer periods achieved no greater deposition of ricinoleic acid. Analyses of the other tissues showed similar amounts of ricinoleic acid in perinephric, subcutaneous and muscle fat. It was not present in heart, liver or brain.

Random analyses of rat faeces showed the presence of hydroxystearic acid in amounts up to 6 per cent of the total fatty acids. This fatty acid/

Table VI. Weights (g) of 7 rats fed on the Purina Chow: 20% castor oil diet during the first 4 weeks.

Rat no.	Weeks on Diet				
	0	1	2	3	4
1	155	195	225	230	260
2	160	200	225	240	275
3	130	155	185	200	220
4	165	205	235	255	275
5	164	205	230	235	255
6	145	185	215	235	255
7	150	180	220	240	260
Mean	153	189	219	234	257

Table VII. Amount of ricinoleic acid in adipose tissue of 7 rats on 20 per cent castor oil diet.

Rat No.	% Ricinoleic acid in fat pad	
	After 4 weeks	After 8 weeks
1	9	8
2	11	10
3	7	10
4	7	10
5	11	10
6	10	11
7	9	9
Mean	9.1 ± 1.7	9.7 ± 1.0

acid is not present in the faeces of rats on normal diet, and the likeliest explanation for its presence is the hydrogenation of ricinoleic acid in the lumen of the gut, by intestinal bacteria. This problem is examined in detail in Chapter 8.

At this point, having shown that ricinoleic acid was incorporated into lymph and tissue triglyceride in the intact animal, it was of some interest to see whether it would participate in triglyceride synthesis in vitro.

SECTION 3

Incorporation of ricinoleic acid during the in vitro synthesis of triglyceride

The experimental techniques for the in vitro synthesis of triglyceride from fatty acid were initially developed by Kornberg and Pricer (1953) and later by Stein and Shapiro (1958) who used rat liver microsomes as the source of activating enzymes. The principles of their methods were subsequently adapted by Steinberg, Vaughan and Margolis (1961) for use with rat adipose tissue microsomes, and it is the method of these authors which was used in the following two experiments.

Methods

For the next two experiments the following aqueous solutions were prepared:-

potassium ricinoleate	0.3 mM/ml
fat free albumin (Goodman, 1957)	5.0 %
adenosine triphosphate (ATP)	0.1 M

coenzyme/

coenzyme A(CoA)	0.01 M
magnesium chloride	0.3 M
cysteine hydrochloride	0.5 M
potassium hydrogen phosphate (pH 7.0)	1.25 M
sodium fluoride	0.625 M
alpha glyceryl phosphate (aGP)	0.1 M

To complete the system, fatty acid activating enzymes are necessary and these were obtained by the separation of rat adipose tissue microsomes as follows.

Fed male rats weighing 150 to 250 g were decapitated, and the epididymal fat pads were removed and homogenised in 4 ml of 0.15 M potassium chloride, for each gram of adipose tissue. Defatted homogenates were prepared by removing the thick layer of congealed fat formed at the top of the tube during centrifugation at low speed in the cold (Steinberg, Vaughan and Margolis, 1961). Mitochondria were then separated by centrifugation for 15 minutes at 10,000 x g in an International Centrifuge at 4°C. The supernatant fraction from this was centrifuged for 1 hour at 4°C in a Spinco Model L ultracentrifuge at 105,000 x g. The sedimented material from this second centrifugation is the microsome fraction. The microsomes were then resuspended in half the original volume of 0.15 M potassium chloride.

With adipose tissue microsomes freshly prepared for each occasion, and the other materials taken from refrigerated stock solutions, the following experiments were carried out.

Experiment 1/

Experiment 1

The systems shown in Table VIII were set up in 25 ml Erlenmeyer flasks. Microsomes were added last. The system in flask 1 was immediately inactivated by the addition of 10 ml alkaline Dole's reagent, while the other 3 flasks were incubated at 37°C for 30 minutes in a shaking incubator.

At the end of the incubation period Dole's alkaline reagent was added to flasks 2, 3 and 4. The total volume of mixture in flask 4 is 3.93 ml. A 3.0 ml aliquot of this was taken to keep the extraction volumes similar. The flasks were stoppered and vigorously shaken and the lipid extraction allowed to proceed for 1 hour. Thereafter the Borgstrom procedure for the separation of free fatty acids and triglycerides was carried out (Borgstrom, 1952). The triglyceride phase was washed three times, since it was imperative to avoid contamination with free fatty acid. Methyl esters of the triglyceride fatty acids were prepared and analysed on the silicone gum column.

Results

The percentage amounts of ricinoleic acid in each of the four triglyceride extracts were:-

flask 1 0.4: flask 2 2.6: flask 3 3.4: flask 4 1.8

These figures show that while there is some contamination of the triglyceride fraction by free fatty acid in the control system (flask 1), the concentration of ricinoleic acid in the triglyceride of the other flasks is 4 to 8 times greater, indicating definite incorporation of/

Table VIII. Recipe for the incubation mixtures used in the first triglyceride synthesis experiment (expt. 1, sect. 3). The numbers are volumes in ml. The concentrations of the various substances are given in the text (p. 35).

Reagent	Flask number			
	1	2	3	4
Ricinoleic acid	2.0	1.2	1.2	2.0
Fat free albumin	0.4	0.2	0.4	0.4
A.T.P.	0.05	---------->		
CoA	0.02	---------->		
Magnesium chloride	0.01	---------->		
Cysteine	0.05	---------->		
Pot. hydr. phosphate	0.10	---------->		
Sodium fluoride	0.20	---------->		
aGP	0.10	---------->		
Microsomes	1.0	---------->		

of ricinoleic acid. Although there is no statistical proof from these figures that the system of flask 3 is the best for ricinoleic acid incorporation, it is the system which was used in further experiments of this type, and it seemed to give consistently satisfactory results.

This experiment, however, does not prove that ricinoleic acid participates in triglyceride synthesis. It could be incorporated into any available triglyceride by the process of transesterification, and a certain amount of preformed triglyceride is available in the adipose tissue microsome preparation.

Experiment 2

In this experiment an attempt was made to exclude the possibility of exogenous triglyceride acting as a substrate for transesterification by ricinoleic acid. The number 3 system of the previous experiment was used as the basic model, but was varied as shown in Table IX. By omitting ATP and CoA from one flask, and aGP from another an attempt was made to define the role of the energy-cofactor component on the one hand, and that of the glycerol substrate on the other.

Flask 1 was treated as in the previous experiment. That is, it was immediately inactivated by Dole's reagent, while the remaining 3 flasks were gently agitated for 30 minutes at 37°C. The triglyceride fraction was separated and the fatty acid methyl esters prepared and analysed as described above.

Results

The percentage amounts of ricinoleic acid in each of the four triglyceride/

Table IX. Recipe for the incubation mixtures used in the second triglyceride synthesis experiment (expt. 2, sect. 3). The volumes of reagents used are those of flask 3, experiment 1. The important variants here are the omission of aGP from flask 3, and of ATP and CoA from flask 4.

	Flask number			
	1	2	3	4
Ricinoleic acid	+	+	+	+
Fat free albumin	+	+	+	+
A.T.P.	+	+	+	-
CoA	+	+	+	-
$MgCl_2$	+	+	+	+
Cysteine	+	+	+	+
KPO_4	+	+	+	+
NaF	+	+	+	+
aGP	+	+	-	+
Microsomes	+	+	+	+

triglyceride extracts were:-

flask 1 0.5: flask 2 3.2: flask 3 2.7: flask 4 2.2.

These figures do not clarify the issue. Although there is a slightly higher percentage of ricinoleic acid in the triglyceride recovered from the whole system (flask 2), it is not so much greater as to establish that synthesis, in addition to transesterification, is taking place. The insertion of ricinoleic acid into the triglyceride fraction without the necessary presence of ATP, CoA or aGP is against synthesis as the exclusive explanation for its incorporation.

DISCUSSION

The work described in this chapter has shown that ricinoleic acid is absorbed from the alimentary tract of the rat, though less efficiently than other fatty acids of similar chain length, and rapidly laid down in the adipose tissue depots to a maximum of about 10 per cent of the adipose triglyceride fatty acids.

The mere presence of a fatty acid in chyle does not necessarily imply its absorption, because there is always a fraction of chyle triglyceride, however small, derived from endogenous fatty acids. This objection does not arise with ricinoleic acid which is absent from the tissues of rats (and all other animals for that matter), unless it has previously been incorporated into their diet.

The main lipid classes were not fractionated from the chyle lipids in this study. Where this has been done by other workers they have found that about 90 per cent of the lipid is triglyceride, 8 per cent is/

is phospholipid (chiefly lecithin) and the remainder cholesterol esters (Whyte, Karmen and Goodman, 1963; Borgstrom, 1952). Whyte and his co-workers have shown in studies using C^{14}-labelled palmitic, stearic, oleic and linoleic acids, that whereas these four fatty acids are incorporated into chyle triglyceride nonpreferentially, there is a definite order of preference for incorporation into lecithin, with stearic being most readily utilised and oleic least.

As will be shown later, the pathways for triglyceride and phospholipid synthesis are very similar and yet, although ricinoleic acid was readily deposited in the adipose tissue lipids which are mainly triglyceride, it did not appear in brain lipid, which is largely made up of the specialised phospholipids of the nervous system which have a very slow turnover of fatty acids. This finding is in keeping with the earlier observations of Stewart and Sinclair (1945) that the feeding of castor oil to rats was not followed by the appearance of ricinoleic acid in the phospholipids of either small intestine or liver. Why ricinoleic acid should find its way into adipose tissue triglyceride with such apparent ease and yet be so completely excluded from tissue phospholipid is of considerable biochemical interest.

There are three observations which may partly explain this disparity. Firstly there is the discrimination against oleic acid incorporation into phospholipid during its synthesis, as shown by Whyte and his colleagues. Ricinoleic acid is of course hydroxy-oleic acid and it may be that it inherits the biochemical disability of oleic acid for phospholipid incorporation. Secondly, there is the suggestion from the experiments/

experiments of Section 3 of this chapter that transesterification rather than synthesis may account for the inclusion of ricinoleic acid in the adipose tissue triglyceride. When this possibility is considered with the further observation of Whyte et al (1963) that the preparation of phospholipid fatty acid methyl esters is peculiarly difficult, and we remember that this is a transesterification procedure, another theoretical reason for the non-incorporation of ricinoleic acid emerges.

In summary, it can be stated that ricinoleic acid, whether by a process of synthesis or transesterification, is readily laid down in adipose tissue. On the other hand, either because there is discrimination against it during synthesis, or because there are peculiar difficulties in the transesterification of phospholipid, it is not incorporated in this lipid class.

CHAPTER 4

FACTORS AFFECTING THE ABSORPTION OF CASTOR OIL

Knowledge about the digestion and absorption of fat in the human and animal intestine is incomplete, in spite of a large amount of research. Until about 1940 it was generally accepted in accordance with the Pfluger-Verzar hypothesis that complete hydrolysis of fats was an essential prerequisite for their absorption (Verzar and McDougall, 1936). The views, current during the latter part of the last century, that finely emulsified neutral lipids could be absorbed as such, were again brought forward by several authors in the thirties (Mellanby, 1927; Channon and Collinson, 1929; Drummond, Bell and Palmer, 1935), and subsequently developed by Frazer and collaborators in a series of papers (Frazer, 1938; Frazer, 1943; Frazer, Schulman and Stewart, 1944; Frazer and Sammons, 1945).

According to the "partition theory" as outlined in these publications, triglycerides were only partially hydrolysed in the intestine, the largest amount being absorbed in particulate form and passed on via the lymphatic system, while the free fatty acids were absorbed via the portal vein. The theory went on to state that when free fatty acids were fed alone they were absorbed as such via the portal vein and mainly deposited in the liver, whereas the glycerides were mainly deposited in the fat depots.

These/

These views were based on chylomicron counts and the feeding of glycerides and fatty acids mixed with Sudan, a lipid stain, methods that have been vigorously criticised (Verzar, 1948; Frank and Ingelfinger, 1950). Nevertheless, they have not altogether been discredited. Recent work in the bile fistula rat (Saunders and Dawson, 1963) seems to show that in the absence of bile a large fraction of free oleic acid may be absorbed directly into the portal vein. The elegant electron microscope studies of Palay and Karlin (1959) support the idea of transfer of particulate fat across the epithelial membrane of the intestinal villi.

Current concepts of Fat Absorption

Present views on the subject might be summarised as follows. Under the influence of bile salts, intestinal and pancreatic lipases at the optimum pH, and in an intestinal milieu which permits the continuing re-esterification and absorption of fatty acids, dietary triglycerides are progressively hydrolysed until about 20 to 40 per cent exists as monoglyceride, 30 to 60 per cent as free fatty acid and the remainder as diglyceride and triglyceride. The subsequent fate of the glyceride fractions is not clear. The higher glycerides may be directly absorbed through the intestinal epithelium by the process known as pinocytosis, while the fate of the monoglyceride fraction is virtually unknown. It is possible that it may play a role similar to that of alpha-glycerophosphate in the re-esterification of the free fatty acid. The fate of the free fatty acid on the other hand is much better understood.

Fate of Free Fatty Acid

In a series of papers from 1953 to 1958 it was shown by three groups of authors that the synthesis of fatty acid to triglyceride in the liver required the presence of Coenzyme A(CoA), adenosine triphosphate (ATP) and mitochondrial or microsomal enzymes (Kornberg and Pricer, 1953 a and b; Weiss and Kennedy, 1956; Stein, Teitz and Shapiro, 1957; Stein and Shapiro, 1958). Later a similar synthesis system for the incorporation of intestinal long chain fatty acids into triglyceride, and utilising homogenates of rat and human intestinal mucosa, was demonstrated by Dawson and Isselbacher (1960). In a later paper, Senior and Isselbacher (1960) showed that rat gut mucosa contained a fatty acid activating thiokinase enzyme, most active in the microsomal fraction obtained from duodenal and upper jejunal tissue. We had discovered this in the course of our own studies before the preliminary report of these authors came to our attention.

A suggested scheme for the total synthesis of triglyceride from fatty acid is shown in Figure 3. This scheme combines the theoretical concepts and experimental findings of the above authors. Free fatty acid, its potassium or sodium salts, in the presence of ATP, CoA, magnesium ions and the appropriate thiokinase enzyme (from liver, adipose tissue or intestine), is "activated", becoming a fatty acyl-CoA complex. The fatty acid is now in a physico-chemical state which enables it to combine with aGP to form phosphatidic acid. This in turn loses its phosphate radical by a process of dephosphorylation to yield a 1:2-diglyceride, which thereafter combines with another fatty acyl complex/

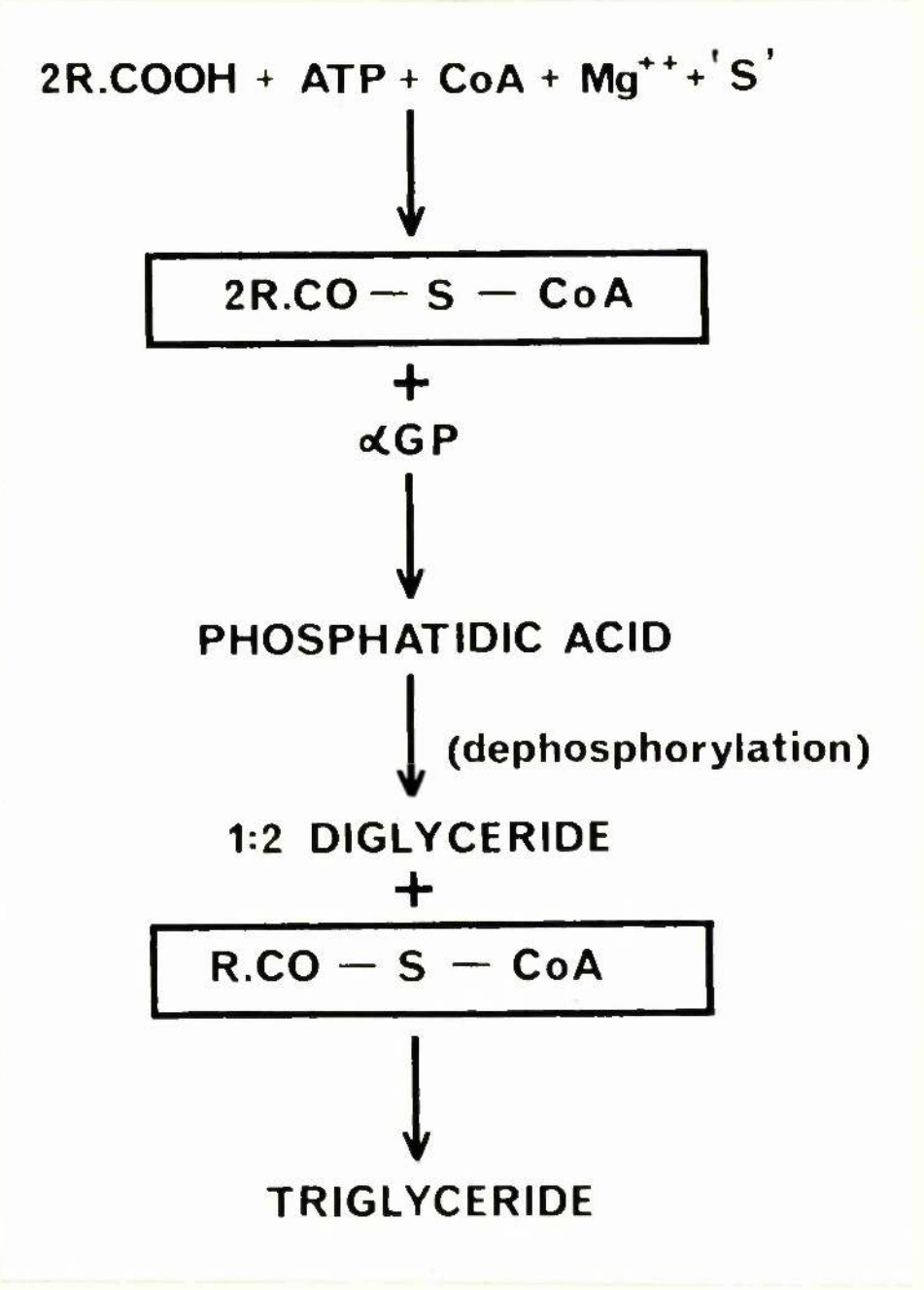


Figure 3. Suggested pathway of triglyceride synthesis showing the conjugation of the 'activated' fatty acid with a-glycerophosphate. Phosphatidic acid formed by this process is dephosphorylated to diglyceride, which then combines with one activated fatty acid molecule to give triglyceride.

complex to give triglyceride. The thiokinase enzyme and the CoA are not consumed during this synthesis. Following the "deacylation" of the fatty acid they are liberated and become available for further fatty acid activation.

It is uncertain where all this activity goes on. It is likely that the final synthesis of triglyceride takes place within the epithelial cells of the gut mucosa. But it is not clear where the fatty acid activation occurs, whether in the epithelial cells or at the luminal or cellular surface of the cell membrane. Certainly during the absorption of fat there is a considerable increase of neutral fat and of free fatty acids that can be extracted from the wall of the small intestine. If the beautiful electron micrographs by Palay and Karlin of fat absorption in the rat are studied critically, it is curious that the fat particles which they so clearly demonstrate within the epithelial cells, and whose presence they explain by the process of pinocytosis, are never seen in contact with the luminal surface of the cell membrane. On the basis of chance, considering the vast numbers of fat particles which appear in the preparations, one would expect that if these do represent triglyceride in actual physical progression from intestinal lumen through the cell substance, some of them ought to be seen in the juxta-membranous zone. That this is not so, suggests that this is the zone on which fatty activation takes place or through which fatty acids, already activated, move to their final incorporation in triglyceride, at a point more deeply within the cell.

Role/

Role of Mucosal Phospholipids

Although the neutral fat and free fatty acids of the small bowel mucosa increase during fat absorption, the amount of phospholipid stays relatively constant (Sinclair, 1929; Barnes, Miller and Burr, 1941; Schmidt-Nielsen, 1946). However, even if the amount of phospholipid is not affected, there are pronounced changes in its fatty acid composition depending on the fat being absorbed.

This was first demonstrated by Sinclair (1929), who found that the iodine value of the fatty acids of the phospholipids of the wall of the small intestine changed with that of the fat fed. He suggested that phospholipids were involved as intermediates in the resynthesis of triglycerides. Similar results were later obtained from experiments with elaidic acid and iodized fatty acids. More recent experiments with labelled saturated acids of different chain length indicate that the intestinal phospholipids have a very rapid turnover of fatty acids, and that there are differences in the extent to which different acids are incorporated into the phospholipids of the intestinal wall.

When triolein that had been transesterified with 5 per cent saturated C^{14}-labelled fatty acids was fed, it was found that the incorporation into the phospholipids of the intestinal wall was larger the longer the chain (Bergstrom, Borgstrom and Bottenberg, 1952). Other workers (Favarger, Collet and Veraguth, 1949) have found indications that choline containing phospholipids turn over more fatty acids than the non-choline containing. There is also a certain amount of net/

net synthesis of phospholipids during fat absorption, since it has been shown that labelled phospholipids are given off to the lymph while the amount in the mucosa stays constant. This amount, however, is generally well below 10 per cent of the triglycerides simultaneously given off to the lymph.

The experimental work to be described was planned to explore how far the digestion and absorption of castor oil conformed to the above account of fat absorption in general, while at the same time particular divergences from it which might explain the purgative action of the oil were sought.

The work is divided into 5 sections. The first is a study of the hydrolysis of castor oil by pancreatic lipase. The second is an investigation into ricinoleic acid activation by intestinal enzymes. The third section considers the role of intestinal phospholipid in the absorption of the oil. The fourth endeavours to clarify the relation between the amount of oil ingested and the degree of absorption or purgation. The fifth and final section is a study of the rate of absorption of the oil compared with other 18 carbon chain fatty acids.

SECTION 1

In vitro hydrolysis of castor oil by pancreatic lipase

The hydrolysis of castor oil was compared with that of olive oil in two ways, firstly by direct titration of the free fatty acid released by enzyme hydrolysis in vitro, and secondly by GLC analysis of/

of the free fatty acids after hydrolysis of a mixture of equal volumes of the oils.

Experiment 1

Materials and Methods

The following reagents were prepared:-

1. Calcium chloride (45%), prepared by dissolving 45 g anhydrous calcium chloride in 100 ml water.

2. Ammonium chloride (1 M), prepared by dissolving 5.35 g anhydrous ammonium chloride in 100 ml water and adding ammonium hydroxide drop by drop until the pH is 8.0. This is checked by pH meter.

3. Pancreatic lipase. This material is actually a crude mixture of enzymes, but it is adequate for the present purpose. It is prepared by extracting 50 mg steapsin in 10 ml 0.025 M NH_4OH for 30 minutes at 0°C. The extract is centrifuged for 15 minutes at 10,000 x g, and the supernate recovered. Ammonia of the required molarity is obtained by making up 1.7 ml 28 per cent ammonium hydroxide (S.G. 0.9) with water to 1 litre.

4. Triglyceride substrate. This was prepared by dissolving 200 mg of castor and olive oils in separate 10 ml amounts of ethanol. The oil solutions were finely emulsified by injecting 0.5 ml into 1 ml of hot water in a graduated centrifuge tube. This is done by injecting forcibly through a fine needle, the tip of which is held just below the surface of the water. The ethanol was evaporated by boiling the mixture for about 10 minutes to give a final volume of 0.8 ml, containing 10 mg/

10 mg of lipid.

To each graduated tube was added 2.5 ml of the 1 M ammonium chloride and 0.25 of the calcium chloride, and the volume made up to 4.3 ml with water. Four 1 ml aliquots of this mixture were pipetted into conical centrifuge tubes. Lipase (0.01 ml) was added to each tube. Immediately tubes 2, 3 and 4 were incubated at 37°C for 15, 30 and 60 minutes respectively. Tube 1 was the control to which 5 ml of Dole's reagent was added before the addition of the enzyme.

The reaction was terminated by the addition of 5 ml Dole's reagent and the free fatty acids extracted and titrated as described in Chapter 2, except that to each tube 0.25 ml N sulphuric acid was added.

Results

The results are given in Table X. They show that castor oil is hydrolysed at least as well as and perhaps slightly better than olive oil. But any difference that there may be is certainly not gross.

The experiment is a true measure of the rate of partial hydrolysis of the respective oils, for even after incubation for 60 minutes less than 10 per cent of the oil has been split. There is approximately 2.3 mg of oil in each tube and 0.35 μ.eq. of ricinoleic acid represents about 0.22 mg of hydrolysed oil.

It seemed that the best estimate of the relative capacities for hydrolysis, of the two oils, would only be obtained if they could be hydrolysed under absolutely identical conditions. The next experiment was planned with this aim in mind.

Experiment/

Table X Amount of free fatty acid (μ.Eq) released during the enzymatic hydrolysis of castor and olive oils. (Duplicate experiments using 0.01 ml enzyme)

		15 min	30 min	60 min
Castor oil	I	0.20	0.31	0.36
	II	0.21	0.32	0.31
Olive oil	I	0.20	0.25	0.33
	II	0.16	0.24	0.27

Experiment 2

Methods

200 mg of a mixture of equal parts of castor and olive oils was dissolved, emulsified and hydrolysed as described in the previous experiment. The procedure was carried out in two sets of tubes. 0.1 ml of enzyme was added to one pair of tubes and 0.02 ml to the other. The freed fatty acids were separated by the combined Dole-Borgstrom procedure and their methyl esters prepared. These were analysed on both the silicone gum and ethylene glycol adipate columns.

Results

The analytical data from the two GLC columns are combined in Table XI.

The fatty acid composition of the castor-olive oil mixture is shown in the first column of the Table. If enzymatic splitting of the fatty acids from the two oils were to occur in an entirely random and nonpreferential way, this is the fatty acid composition of the mixture of freed fatty acids which would be expected at any stage of the hydrolysis with any amount of enzyme. But it is quite clear from the Table that this is not the case. With the smaller amount of enzyme there is a considerably greater release of ricinoleic acid, about 25 per cent more than expected, while oleic acid has been released to a degree about 35 per cent less than expected. The palmitic and stearic acid components have also been preferentially split, while linoleic acid has not.

Comment/

Table XI Percentage fatty acid composition of free fatty acids after enzymatic hydrolysis of castor/olive oil mixture. Only major fatty acids are listed.

Fatty acid	Oil Mixture	15 min	30 min	60 min
		0.02 ml enzyme		
16	7.8	13.0	14.8	13.2
16:1	1.1	0.6	2.0	1.5
18	1.6	4.7	6.7	3.3
18:1	39.4	26.7	25.7	24.6
18:2	8.4	5.3	5.0	5.5
18:1:OH	41.7	49.7	45.8	52.0
		0.10 ml enzyme		
16		13.0	18.5	14.4
16:1		0.8	1.5	1.4
18		4.0	4.5	4.0
18:1		31.3	31.8	35.2
18:2		5.8	6.0	7.0
18:1:OH		45.1	37.6	38.0

Comment

On the basis of these experiments, and particularly from the results of the latter, there is evidence for the more efficient hydrolysis of castor oil compared with olive oil. The results are particularly convincing for the reaction using the smaller amount of enzyme. At the most conservative estimate it is clear that castor oil is no different from olive oil at this first stage of its digestion.

SECTION 2

Activation of ricinoleic acid by rat gut mucosal enzymes

Since, as the previous section has shown, there is no block to the hydrolysis of castor oil, the next step in the study of its absorption pathway seemed to be an investigation of the fatty acid activation mechanism as related to ricinoleic acid. The most direct way to do this was to compare the degrees of participation of ricinoleic and oleic acids in the activating system. At the same time the opportunity was taken to compare the saturated fatty acids, stearic and hydroxystearic.

The method used for the estimation of activated fatty acids depends on the following reactions.

$$R.COOH + ATP + CoA \xrightarrow{(Mg^{++} + \text{'S'})} R.CO\_CoA + AMP + PP + H_2O \qquad (I)$$

$$R.CO\_CoA + NH_2OH \longrightarrow R.CONHOH + CoA \qquad (II)$$

In reaction I fatty acid, usually as its sodium or potassium salt/

salt, is activated to form a fatty acyl-CoA complex, with the production of adenosine monophosphate, pyrophosphate and water.

In reaction II the fatty acyl-CoA is esterified, or "trapped", with hydroxylamine to give the fatty acid hydroxamate. This in turn is made to react with ferric chloride and the resulting ferric-hydroxamic acid complexes are estimated photometrically at 520 mμ. This condensation reaction of fatty acids with hydroxylamine was first introduced by Lippman and Tuttle (1945 a and b: 1950), used by Kornberg and Pricer (1953) in their studies on fatty acid synthesis by animal liver enzyme systems, and later adapted by Senior and Isselbacher (1960) for their preliminary work on fatty acid activation by rat gut microsomes.

If C^{14}-labelled fatty acids are available the labelled fatty acid hydroxamates can be separated and counted directly in a liquid scintillation counter. When labelled substances are not available the colorimetric technique, though less refined, is invaluable.

Methods

In a series of preliminary studies to determine the optimum substrate conditions for fatty acid activation it was discovered that the maximum enzyme activity was in the microsomal fraction of the rat gut mucosal extract. This was later found to be in agreement with the preliminary communication of Senior and Isselbacher (1960).

Preparation of microsomes. The microsomes were isolated as follows. Young rats were killed by decapitation. The gut was removed, rinsed through three times with ice-cold potassium chloride (0.15 M), cut open and spread out on blotting paper with the epithelial surface facing/

facing up. The mucosal layer was then scraped off, using the side edge of a metal spatula, and homogenised in a teflon homogeniser, in phosphate buffer (0.1 M, pH 7.4). The ratio of buffer to wet weight of tissue was about 4 to 1. All stages in the preparation of the microsome fraction were carried out at 0°C.

The microsomes were harvested by centrifuging the homogenate first at 2,000 x g for 15 minutes to throw down the coarse cellular debris, secondly at 10,000 x g for 10 minutes to separate out the mitochondria, and finally at 104,000 x g for 60 minutes to recover the microsomes. The microsomes, which can be seen as a reddish-brown pellet at the bottom of the tube, were resuspended in the phosphate buffer and the protein concentration of the preparation estimated by the Biuret method (Layne, 1957).

Fatty acids were prepared as potassium salts in a 5 per cent aqueous solution of lipid free bovine albumin, "defatted" by the method of Goodman (1957).

The reaction system contained 0.5 ml enzyme suspension (6-10 mg protein/ml), 0.5 ml 2 N hydroxylamine (prepared by mixing equal volumes of 4 N hydroxylamine hydrochloride and 4 N KOH), 20 μM ATP, 30 μM cysteine, 50 μM sodium fluoride, 20 μM magnesium chloride and 1 mg CoA. Fatty acid and CoA were omitted from the control. After incubation at 37°C for 30 minutes the reaction was stopped by the addition of 3 ml methylene chloride and the fatty acid hydroxamate extracted by two washings with this solvent.

After evaporation of the solvent, 1 ml of Hill's reagent A (Hill/

(Hill, 1947) diluted to 1 part per 100 in 95 per cent ethanol, was added, the colour developed for 20 minutes and read at 520 mμ in a Beckman spectrophotometer. 1 μM stearhydroxamate, kindly supplied by Dr. Roy Vagelos of the National Heart Institute, was used as the standard.

At first it was difficult to get this in vitro system to work, and about 4 weeks was spent rehearsing it before consistent results began to emerge. For example, unless the magnesium chloride solution was added last, a precipitate would form which meant that some component of the system was no longer in solution. The data reported here are in a sense the best data, but they do represent the fairly uniform trend of the trial experiments.

Results

The results of duplicate experiments comparing the hydroxamate production of increasing concentrations of paired fatty acids are given in Table XII and illustrated in Figures 4 and 5.

They show that ricinoleic acid is less readily activated to its fatty acyl CoA than oleic acid, and that a similar, though perhaps lesser difference exists between stearic and hydroxystearic acids. Although the amount of hydroxamate production increases with the increase in amount of fatty acid substrate, the proportion of available fatty acid activated reduces. This corresponds with the experience of Kornberg and Pricer (1953) who suggested that increasing concentrations of fatty acid might inhibit the activating enzymes.

Table XII Results of duplicate experiments comparing fatty acid hydroxamate production of paired fatty acids.

	Fatty acid hydroxamate (μM)			
μM Fatty acid	Oleic		Ricinoleic	
	I	II	I	II
0.25	0.16	0.15	0.08	0.10
0.50	0.20	0.22	0.08	0.10
1.0	0.34	0.34	0.25	0.22
2.0	0.44	0.40	0.24	0.29
	Stearic		Hydroxystearic	
	I	II	I	II
0.25	0.22	0.23	0.20	0.18
0.50	0.24	0.24	0.13	0.19
1.0	0.36	0.37	0.30	0.28

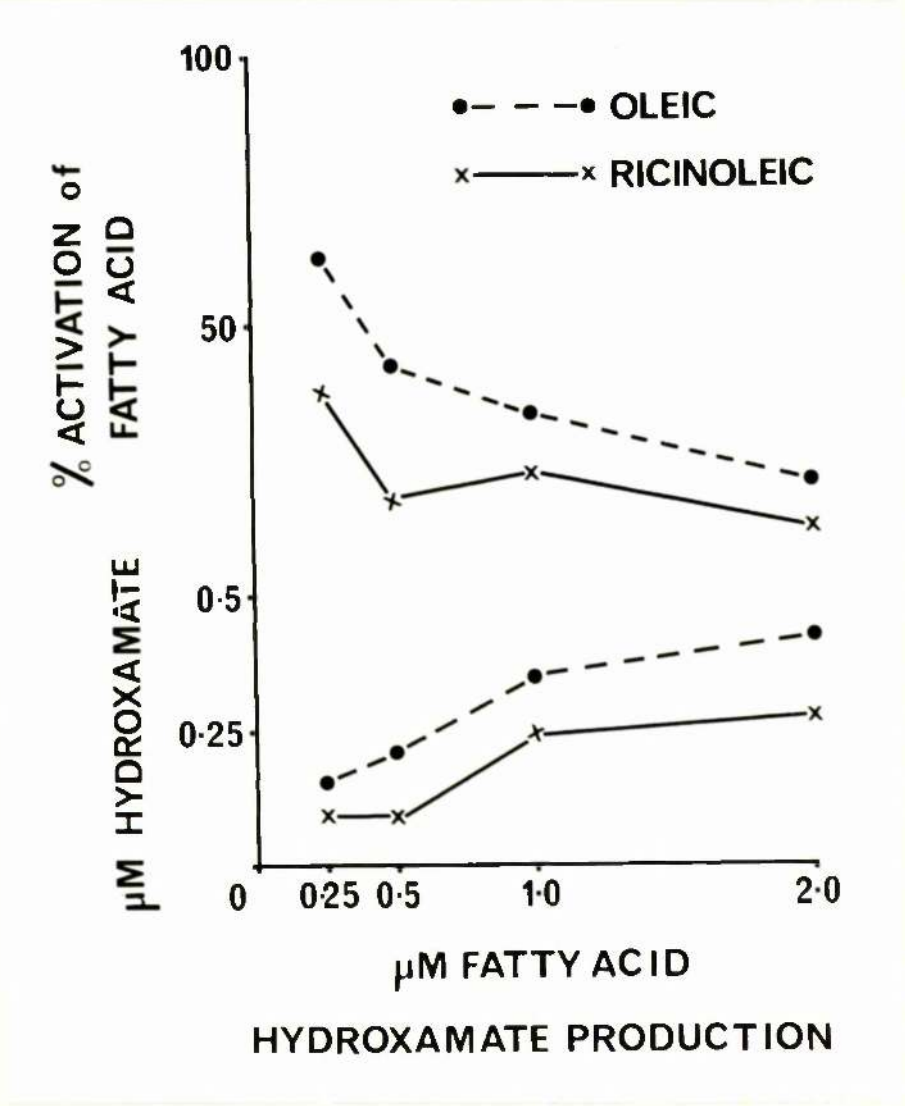


Figure 4. Graph illustrating the in vitro production of fatty acid hydroxamates of oleic and ricinoleic acids. While the amount of hydroxamate rises with increasing fatty acid concentration, the efficiency of activation falls.

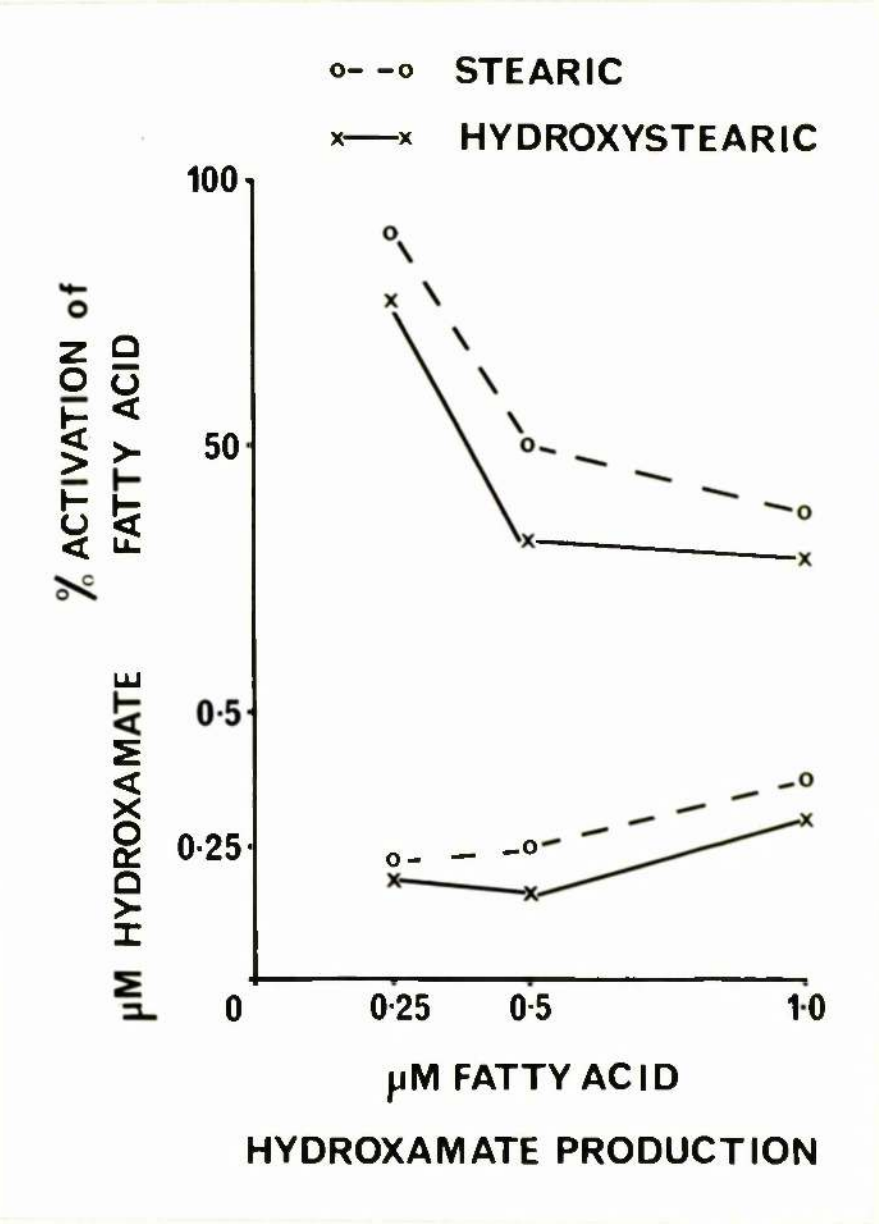


Figure 5. Graph illustrating the in vitro production of fatty acid hydroxamates of stearic and hydroxystearic acids. As in the previous figure the efficiency of activation falls with rising concentration of the fatty acid.

SECTION 3

Role of mucosal phospholipid in the absorption of ricinoleic acid

Stewart and Sinclair (1945) had published data which showed that ricinoleic acid did not appear in the phospholipids of rat gut mucosa or liver, and at first it seemed redundant to investigate this aspect of ricinoleic acid absorption. However, a closer study of their work suggested that a re-examination of their findings was pertinent, even if only to corroborate them, and particularly within the context of a comprehensive study such as this.

These workers gave castor oil at a concentration of 48 per cent of the diet. They claimed that this diet did not cause diarrhoea in the animals. But it contained more than twice as much castor oil as was used in the chronic feeding experiments of the previous chapter, and such a large concentration could adversely affect the normal absorption mechanisms.

Their methods for the identification of ricinoleic acid were non-specific. They do not state that ricinoleic acid does not appear in phospholipid, but that it does not appear in significant amounts, though they do not say precisely what a significant amount is. The most important criticism of their paper, however, is that there were no control observations that ricinoleic acid was being absorbed into the mucosal neutral lipids, or that other fatty acids were being incorporated into the phospholipid fraction. In the absence of such information it did not seem proven that the exclusion of ricinoleic acid/

acid from phospholipid was a true and peculiar event.

It is felt that the following experiment provides more satisfactory proof for the non-incorporation of ricinoleic acid into rat gut mucosal phospholipid.

Methods

To ensure the maximum absorption of ricinoleic acid the rats used in this experiment were fed overnight. Next morning each was given by stomach tube, 0.5 ml castor oil containing 0.05 μc C^{14}-palmitic acid. No purgation occurred and the animals were killed 1 and 2 hours later. The entire small bowel was removed, washed out repeatedly with cold saline, cut open on clean blotting paper and the mucosa scraped off with a metal spatula. The mucosal lipids were extracted in chloroform: methanol (2:1). The chloroform phase was separated by the usual method, dried over anhydrous sodium sulphate and evaporated under nitrogen. The lipids were redissolved in 1 ml benzene and kept at -20°C during the intervals in the various stages of the experiment.

As a standard for carbon^{14} counting, 0.1 ml of the oil mixture administered to the rats was dissolved in 0.9 ml benzene. 0.1 ml of each of the benzene solutions of the mucosal lipids, and 0.1 ml of standard, were spread in planchets and counted at 1200 volts in a Nuclear Chicago, beta-emitting, gas-flow scintillation counter. Each sample was counted to 10,000 counts.

When this procedure had shown that a reasonable amount of C^{14}-palmitic acid had been absorbed into the gut lipids the 1 hour(A) and 2 hour (B) samples were separated into neutral and phospholipid/

phospholipid fractions using the silicic acid column chromatographic method of Marinetti, Erbland and Kochen (1957). The neutral lipids, which are recovered in chloroform, were dried under nitrogen. The phospholipids, which are recovered in methanol, were dried under vacuum in a rotavapor apparatus, using gentle heat. The purity of the samples was checked by thin layer chromatography.

The neutral and phospholipid fractions were redissolved in 1 ml benzene and 0.2 ml of each was spread on planchets and counted as described above to a total of 5,000 counts. The remainder of each fraction was converted to methyl esters of the fatty acids by the usual procedure and analysed on the silicone gum column.

Results

The results of this experiment are given in Tables XIII and XIV.

Table XIII shows the amount of incorporation of the C^{14}-palmitic acid into the neutral and phospholipid fractions of the mucosal lipid. These figures are important. They establish the validity of the experiment, in that they show the incorporation of a fatty acid, administered at the same time as the castor oil, into both the neutral lipid and phospholipid of the mucosal lipid. 6.2 and 7.3 per cent of the doses of C^{14}-palmitic acid have been absorbed into the neutral lipid fraction, while 1.9 and 1.7 per cent of the doses have been incorporated into the mucosal phospholipid. If one remembers that these figures represent amounts of fatty acid in the intestinal epithelium at one moment of time, and not the total amount of fatty acid passed through the/

Table XIII Recovery of C^{14}-palmitic acid from the intestinal mucosal lipids 1(A) and 2(B) hours after administration of the labelled oil

	Recovery (% dose)	
Lipid fraction	A	B
Total lipid	8.1	9.0
Neutral lipid	6.2	7.3
Phospholipid	1.9	1.7

Table XIV Percentage fatty acid composition of neutral and phospholipid fractions in Table XIII.

	Neutral lipid		Phospholipid	
Fatty acid	A	B	A	B
16	12.5	12.4	21.8	23.5
18	11.7	11.8	21.0	22.1
18:1 and 2	31.7	26.3	40.8	41.7
18:1:OH	41.6	45.7	None	None
20:4	3.4	3.8	16.6	12.9

the mucosa during the complete process of absorption, then clearly they indicate satisfactory uptake of the administered fatty acid by the gut mucosa. The figures also show that the amount of palmitic acid in the phospholipid fraction is about 25 to 30 per cent of the amount in the neutral lipid fraction.

Table XIV shows the fatty acid composition of the two main lipid classes for the principal fatty acids only. The percentage of ricinoleic acid in the neutral lipid is considerable (42 and 46 per cent), while none at all is present in the phospholipid fraction. This absence of ricinoleic acid is total. Excessively large samples were deliberately analysed and no trace of ricinoleic acid could be found.

Comment

This experiment shows more conclusively than the work of Stewart and Sinclair that ricinoleic acid does not appear in the mucosal phospholipid of the rat gut. At a time when administered palmitic acid can be detected with ease in both the neutral and phospholipid fractions of the mucosal lipids, and when ricinoleic acid comprises the major proportion of the neutral lipid, none at all can be detected in the phospholipid. This absence is unlikely to be due to the rapid transfer of ricinoleic acid in and out of the phospholipid component, and almost certainly implies the absolute non-uptake of ricinoleic acid by phospholipid. It is curious that phospholipid should have this biochemical aversion for ricinoleic acid, and the fact presumably has some functional significance.

An incidental observation arising from this experiment, but one which/

which has particular reference to the work of a later chapter, is that in none of the samples analysed for fatty acid composition does hydroxystearic acid appear. This means that whatever may emerge from the subsequent studies it seems unlikely that any hydrogenation of ricinoleic acid occurs in the process of its activation, absorption and incorporation into the mucosal lipids.

SECTION 4

Effect of dose of castor oil on absorption

It was already apparent from human studies which were proceeding in step with the animal work that the absorption of castor oil was inversely related to the dose, the smaller the dose the greater the percentage absorption. The following experiment was designed to give information about the amounts of ricinoleic acid actually in the bowel wall and recoverable from the chyle of rats given big (purgative) and small (non-purgative) doses of castor oil.

Methods

Polyethylene cannulae were inserted into the thoracic ducts of two rats and at the same operation cannulae of a slightly greater bore were placed in the duodenum through a puncture wound in the anterior surface of the stomach near the pyloric antrum. This permitted the administration of oil without anaesthetising the animal.

The rats were given 0.5 N saline overnight and next morning 0.6 ml and 0.2 ml of castor oil was administered to the respective animals at a timed interval. After 45 minutes the animal given the larger dose of oil/

oil had diarrhoea. It was immediately killed by decapitation. The second animal was killed 45 minutes after the administration of oil. It did not have diarrhoea. The entire gut of each animal was removed, divided into large and small bowel and each part washed through thoroughly with water. Each portion of bowel was homogenised and extracted in acetone:ethanol (1:1).

The chyle collected during the 45 minute period after the administration of the oil was measured and extracted in chloroform: methanol (2:1). Total lipid estimation and GLC analysis of fatty acids were performed as already described.

Results

The results are given in Table XV.

The facts which emerge can be noted succintly. With the smaller dose of castor oil about 9 times the amount of ricinoleic acid is recovered from the chyle and the concentration of ricinoleic acid in the wall of the small intestine is double that of the purged animal. This plainly shows that purgation does not depend on the concentration of ricinoleic acid in the small bowel epithelium. It is of equal significance that in the purged animal there is no ricinoleic acid in the lipids of the large bowel either. This finding is at variance with the view that plasma borne ricinoleic acid causes irritation and increased peristalsis of the large bowel.

Comment

Purgation by castor oil is not caused by ricinoleic acid in the epithelium of the small and large bowel nor by circulating plasma acid/

Table XV Data from experiment of Section 4, Chapter 4, showing amounts of ricinoleic acid in chyle, small and large intestine after purgative and non-purgative doses of castor oil.

	Rat I	Rat II
Dose of castor oil	0.2 ml	0.6 ml
Purgation	None	After 45 min.
Volume of chyle	1.0 ml	0.9 ml
Total lipid in chyle	9.6 mg	6.2 mg
% ricinoleic acid in chyle	18.1	3.0
Total ricinoleic acid in chyle	1.57 mg	0.17 mg
Total lipid in small bowel	264.0 mg	342.0 mg
% ricinoleic acid	6.2	3.1
Total ricinoleic acid	16.1	10.6
Total lipid in large bowel	45.0 mg	41.5 mg
% ricinoleic acid	None	None
Total ricinoleic acid	None	None

acid. Purgation requires a certain minimum concentration of free ricinoleic acid in the lumen of the bowel. This not only causes purgation but also inhibits normal absorption. These two facts are almost certainly connected with each other, but whether causally or coincidentally cannot be stated at present. However, it seems likely that above a certain concentration of free ricinoleic acid in the gut lumen one or more step in the reactivation, esterification and absorption of fatty acid is interfered with. Bearing in mind the facts already demonstrated in the previous two sections, firstly that the efficiency of activation of a fatty acid falls off with increasing concentration and secondly that the phospholipid pathway of absorption is closed completely to ricinoleic acid, it is reasonable to suggest that the absorption mechanism is more readily overloaded where this fatty acid is concerned. While this may explain the poor absorption of ricinoleic acid it does not, of course, explain the mechanism of purgation.

SECTION 5

Rate and degree of absorption of castor oil compared with other triglycerides

Since it had been shown that castor oil was more readily hydrolysed than olive oil (section 1), and that ricinoleic acid was less readily activated than oleic (section 2), it seemed reasonable to investigate the practical implications of this during the actual digestion and absorption of these two oils.

Experiment/

Experiment 1

Methods

A polyethylene cannula was inserted into the thoracic duct of a 250 g rat and the animal was given saline but no food overnight. Next morning it was lightly anaesthetised with ether and 1 ml of a mixture of equal amounts of castor and olive oils was given by stomach tube. Purgation occurred 50 minutes later. Chyle was collected in three portions during the 9 hours following the administration of the oil - during the first hour, from the first to the third hour, and from the third to the ninth hour. Thereafter the animal was given food and saline overnight, and the experiment repeated next day. There was no purgation with the second dose.

The chyle was measured, extracted and analysed as in the last experiment.

Results

The results of the experiment are given in Table XVI, and a number of facts emerge.

It is again clear that castor oil is much better absorbed by the fed rat than by the fasting one. The total amount of ricinoleic acid in the chyle of the fasted rat recovered over the entire 24 hour period following the administration of the oil is 32.1 mg, compared with 85.9 mg for the 9 hour period following the administration of the same volume of oil to the same animal in the post-prandial state. This factor of 2.7 times is an underestimate of the ratio of efficiency, since the animal was killed before the chyle flow had stopped.

Secondly/

Table XVI Data from experiment 1 of Section 5, Chapter 4 showing volumes, lipid concentration, total lipid and fatty acid composition of chyle collected before and at intervals after the administration of a castor/olive oil mixture to a rat in the fasted and post-prandial state. The animal was fasted before the first dose of oil and fed before the second.

Hours after oil		Fast	1	3	9	24 Feed	1	3	9
Volume of chyle(ml)		113.0	0.8	2.0	13.0	51.0	0.4	4.6	12.6
Chyle lipid (mg)		169.0	2.1	15.0	74.2	362.0	1.0	66.3	356.0
Fatty acid	Oil mix.								
16	7.8	18.6	27.5	15.1	16.1	19.6	18.6	15.0	15.2
16:1	1.1	2.3	2.1	1.4	1.8	1.7	2.0	1.4	1.1
18	1.6	8.7	9.3	7.1	6.2	7.1	8.0	5.5	4.7
18:1	39.4	34.2	27.2	33.9	41.2	45.6	35.1	41.8	43.4
18:2	8.4	32.8	30.6	16.2	18.3	18.7	26.3	13.0	15.4
18:1:OH	41.7	None	None	22.8	13.8	5.1	7.7	22.0	20.1
20:4	None	3.3	3.3	3.4	2.6	2.0	2.3	1.1	None

Secondly, if we compare the two 9 hour periods following the giving of the oil, it can be seen that although the total volumes of chyle collected during these periods do not differ much (15.8 and 17.6 ml), the amount of lipid in the chyle of the fed animal is more than four times as much that in the fasted (91.3 to 423.3 mg.). While this probably reflects some of the difference between the amounts of lipid absorbed, some of the difference may be due to a reduction in contribution of endogenous lipid because of the purging.

Thirdly, if we compare the fatty acid composition of the control collection of chyle from the fasted animal (column 1) with that of the chyle collected during the first hour after the purgative dose of oil (column 2), there is a striking increase in the amount of palmitic acid. This increase is present in none of the other samples and since it is unlikely to be due to the selective absorption of this fatty acid at a time when purgation is taking place, it presumably is due to an increase in the endogenous contribution of palmitic acid to the chyle. The significance of this is not clear.

Fourthly, the volume of chyle recovered during each of the one hour periods after the giving of the oil, is very small. This is probably due to the inhibitory effect of the anaesthetic on gut function, and not due to purgation, since the volumes are low in the absence of purgation also.

Fifthly, it is plain that after purgation has occurred the animal can go on to absorb some (or perhaps all) of whatever ricinoleic acid remains in the gut.

However/

However, this experiment had two major disadvantages. Unfortunately the percentage amount of oleic acid in the oil mixture was too near that of the oleic acid in the control chyle (39.4 and 34.2). This made it impossible to interpret the subsequent analysis with reference to the absorption of oleic acid. Also the animal was anaesthetised for the administration of the oil and the poor flow of chyle during the immediate post-anaesthetic state blunts the point of an experiment such as this.

For these reasons another experiment was planned.

Experiment 2

Methods

Polyethylene cannulae were inserted into the thoracic duct and duodenum of a 250 g rat as described in Section 4 and the animal was given food and 0.5 N saline overnight. Next morning, when the chyle was seen to be flowing freely, 1 ml of a mixture of equal amounts of emulsions of castor oil and tristearin, containing about 80 mg of lipid, was administered through the duodenal tube, without anaesthetising the rat. Tristearin was quite soluble in hot ethanol and if the emulsion was prepared properly it was stable.

Chyle was collected in graduated centrifuge tubes at shorter intervals than in the previous experiment. The volume of each sample was measured, the lipids extracted, estimated and prepared for GLC analysis as before. Because there was no special interest in the oleic and linoleic acid components of the chyle lipids GLC analysis was carried out on the silicone gum column only. This column does not separate/

separate these two fatty acids and their aggregate percentage is given under 18:U in the Table.

Results

The volumes, lipid concentrations and total lipid of the chyle samples are given in Table XVII, and the fatty acid composition of the chyle samples in Table XVIII.

No purgation occurred in this animal and the volume of chyle collected during the first hour after giving the oil emulsion was more than five times that in the previous experiment (4.2 compared with 0.8 ml). During the subsequent 2 hours the volume was six times that during a comparable period in the last experiment (12.2 compared with 2.0 ml). Thereafter the volumes are similar. These facts testify to the value of the duodenal tube.

Once again there was a rise in the palmitic acid concentration before there was any trace of ricinoleic acid in the chyle or a definite change in the concentration of stearic acid. And again there is no obvious explanation for this, except the possibility that following the administration of the oil there is a brief period of inhibition of fat absorption and the increase in palmitic acid is due to an increase in the endogenous component of the chyle fatty acids.

The relative patterns of ricinoleic and stearic acid are illustrated in Figure 6. The concentration of ricinoleic acid rises and falls away more quickly than that of stearic. The increase in the concentration of stearic acid is more obvious in this experiment because/

Table XVII Volume, lipid concentration and total lipid of chyle samples collected before and at frequest intervals after giving 1 ml of a mixture of tristearin and castor oil to a fed rat. No purgation occurred.

Chyle sample	Volume (ml)	Lipid conc. (mg/ml)	Total lipid (mg)
Overnight	27.0	7.8	211.0
After oil			
15 mins	1.7	2.2	3.8
30	0.8	3.6	2.9
45	0.7	6.1	4.3
60	1.0	4.6	4.6
90	2.5	3.4	8.5
120	2.2	3.7	8.2
180	4.5	3.9	17.6
360	12.0	3.2	38.4
1080	28.0	9.0	252.0

Table XVIII Percentage fatty acid composition of the chyle samples of the previous table. The highest figure for fatty acids 14, 15, 17 and 17:1 was 1 per cent and they are not included in the table. Aggregate percentages do not therefore come to 100.

Fatty acid	Oil mix	Cont.	15	30	45	60	90 minutes	120	180	360	1080
16	4.9	24.4	29.2	19.5	19.3	21.4	22.9	23.8	22.6	24.1	24.0
16:1	-	2.0	2.5	1.4	1.4	1.6	1.8	2.1	1.7	1.5	1.8
18	49.7	10.4	11.3	19.2	23.4	21.5	19.5	16.9	12.9	12.0	10.5
18:U	6.6	51.5	42.3	33.7	30.0	36.1	40.8	44.9	50.0	51.1	51.5
18:1:OH	38.9	-	-	17.7	17.2	10.3	5.0	1.1	-	-	-
20	-	4.2	3.4	-	1.8	1.7	1.6	2.8	3.9	2.9	3.0
20:4	-	4.2	7.9	6.5	5.1	5.1	6.3	5.9	5.6	6.2	6.4

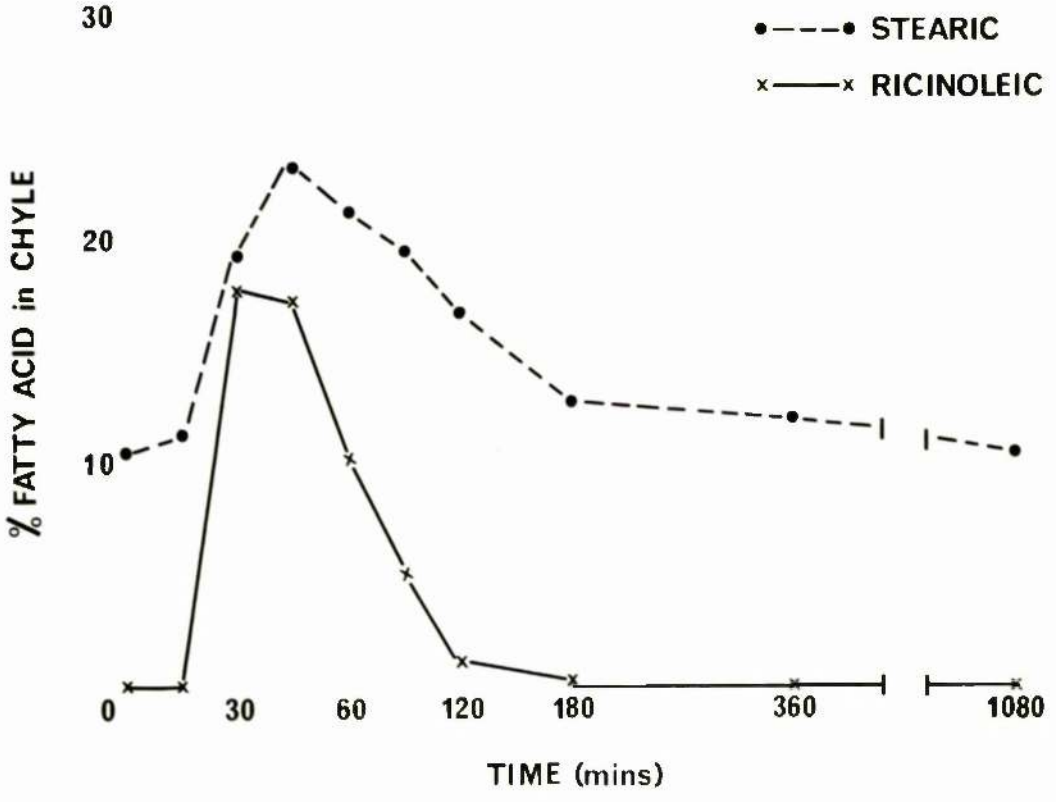


Figure 6. Graph illustrating the relative rates of absorption of stearic and ricinoleic acids in the rat as shown by the concentration of the fatty acids in thoracic duct chyle sampled at intervals.

because of its lower starting concentration. But even so, considering that there is about 10 per cent more stearic acid in the oil mixture and that the ricinoleic acid concentration rises from zero to 17.7 per cent, while the stearic only rises from 10.4 to 23.4 per cent, it does seem that there is a greater absolute absorption of ricinoleic acid. A firm opinion cannot be given about this in the absence of information about changes in the contribution of endogenous stearic acid. If this falls, for example, from 10 to 5 per cent then the contribution of the exogenous stearic acid is virtually the same as the ricinoleic.

The one thing which can be said without qualification is that in circumstances in which fat absorption is actually occurring, ricinoleic acid is at no disadvantage compared with another fatty acid of similar chain length and molecular weight.

GENERAL DISCUSSION

The work of this chapter has revealed much about the mechanism of absorption of castor oil. It is readily hydrolysed by pancreatic lipase, and the results tend to support the opinion of Hartwell (1938) that it is more readily hydrolysed than olive oil. On the other hand ricinoleic acid is less readily activated to its fatty acyl CoA than oleic acid, and this taken together with the inhibition of the activating system which occurs with increasing concentrations of free fatty acid suggests that there comes a point in the digestion of the oil when, if enough has been given the concentration of free ricinoleic acid is sufficient/

sufficient to inhibit completely further activation. This still leaves unanswered the question, ' How does this high concentration of free ricinoleic acid cause actual purgation?'

It seems certain that the purgative effect depends on the amount of ricinoleic acid in the lumen of the gut and not on the concentration within the epithelial cells. In the experiment of Section 4 purgation occurred in the rat given the larger dose of castor oil at a time when the mucosal concentration of ricinoleic acid was only half that in the animal given the smaller dose. As will be shown in Chapter 6, human balance studies corroborate that the most severe purgation and lowest absorption of ricinoleic acid are encountered with the largest doses of castor oil.

The nature of this intraluminal effect remains a matter for conjecture. It may depend on the amount of fatty acid soaps formed after the release of free ricinoleic acid, rather than on the amount of free acid. Pertinent to this speculation are the emesis which follows the ingestion of soap solution, the purgation which follows a soap suds enema, and the diarrhoea of sprue, where the calcium soaps of unabsorbed fatty acids are formed. Another possibility which the author hopes to be able to test in the future is that the free ricinoleic acid in the gut forms calcium soaps with endogenous calcium, and that the acute withdrawal of calcium from the bowel tissue causes an acute irritability of the bowel with greatly increased peristalsis. This hypothesis will be tested by measuring the amount of radioactive calcium, administered by intravenous injection, which appears in the stools/

stools in association with castor oil induced purgation, and comparing it with the normal loss.

A preliminary investigation into the effect of potassium ricinoleate on the behaviour of a rat intestinal loop preparation could not be followed up when it was realised that a large number of experimental variables, such as the concentration of potassium and calcium ions, required to be controlled.

It is clear from the experiments of Sections 4 and 5 that when the concentration of free ricinoleic acid is not such as to produce purgation, the absorption of the oil proceeds as rapidly and perhaps even more rapidly than either oleic acid or stearic acid.

Finally, the work of Sinclair and Stewart (1945) has been confirmed by different techniques. For some reason there is a bar to the incorporation of ricinoleic acid into phospholipid, whether of gut, liver or brain. This seems to be a unique property of the fatty acid, whatever its functional significance is shown to be. This appears to be the only point at which ricinoleic acid diverges fundamentally from the absorption pathway which has been demonstrated for all other fatty acids which participate in normal human lipid metabolism.

CHAPTER 5

THE METABOLISM OF RICINOLEIC ACID IN THE RAT

So far it has been shown that castor oil can be absorbed, and when absorbed deposited in adipose tissue to a maximum concentration of about 10 per cent. Having dealt with the problems of absorption and assimilation the next step seemed to be to study the fate of ricinoleic acid after its appearance in adipose tissue.

If C^{14}-ricinoleic acid had been available the work of this chapter would have been simpler and more direct. In the event indirect but otherwise valid methods were used.

SECTION 1

Disappearance of ricinoleic acid from adipose tissue

The first line of investigation was to see whether ricinoleic acid was turned over by the tissue lipid depots, or whether once deposited in adipose tissue it became a fixed component.

Methods

Six young rats were given the 20 per cent castor oil diet for 4 weeks. Five of them were used for the studies of this section and the sixth was used for the experiment of Section 2.

At the end of the four-week period of diet an epididymal fat pad was biopsied in each animal, which was then returned to normal feeding.

Further/

Further biopsies were made 7 and 14 days later. The lipid was extracted in the usual way with chloroform:methanol (2:1), fatty acid methyl esters prepared and analysed on the silicone gum column, under standard conditions.

The rat droppings were pooled on the 14th day of the castor oil diet and again 3 and 7 days after the return to normal feeding. These faecal samples were again extracted with chloroform:methanol and the fatty acid methyl esters analysed.

Results

The percentage amount of ricinoleic acid in each tissue sample is shown in Table XIX, and the GLC analysis of the faecal fatty acids in Table XX.

This experiment shows that ricinoleic acid disappears rapidly from adipose tissue when castor oil is withdrawn from the diet, the average ricinoleic acid concentration falling from 7.2 per cent to 2.1 per cent in 14 days. Within 48 hours of the return to normal feeding neither ricinoleic nor hydroxystearic acids could be detected in the faeces, though both of these fatty acids were present in faecal lipids while the animals were taking the castor oil diet. This suggests that the disappearance of the fatty acid from the adipose tissue is due to metabolic degradation rather than excretion. Indeed, one would anticipate that ricinoleic acid would follow the accepted pathway for fatty acid oxidation as indicated by the general reaction:-

$$CH_3(CH_2)_{2n}COOH + O_2 \longrightarrow CH_3(CH_2)_{2n-2}COOH + CH_3COOH$$

Comment/

Table XIX Percentage ricinoleic acid in adipose tissue of rats on the 28th day of the castor oil diet, and on the 7th and 14th days after returning to normal diet.

Rat No.	Castor oil	Normal diet	
	28th day	7th day	14th day
1	9.7	6.2	2.2
2	4.9	2.9	2.5
3	8.0	4.5	2.6
4	8.1	3.9	1.8
5	5.1	3.8	1.3
Mean	7.2	4.3	2.1

Table XX Fatty acid composition of rat faecal lipids during castor oil diet and after stopping it. GLC on silicone gum column only.

Fatty acid	Castor oil	Normal diet	
	14th day	3rd day	7th day
14	trace	1.5	2.0
15	1.5	3.5	6.5
16	25.2	29.0	30.0
16:1	trace	2.5	3.5
17	trace	2.0	1.0
18	12.5	17.5	20.0
18:U	30.6	44.0	37.0
18:OH	3.2	-	-
18:1:OH	27.4	-	-

Comment

It is now known that all even numbered fatty acids are oxidised as their acyl-CoA derivatives via a four-step spiral sequence involving four enzymes and requiring initial activation at the carboxyl group with ATP (Lynen, 1954). The first oxidation step is catalysed by a group of flavoprotein enzymes (Hauge, Crane and Beinert, 1956; Crane, Hii, Hauge, Green and Beinert, 1956; Crane and Beinert, 1956). The other three steps are fully and freely reversible under conditions consistent with the equilibria of the reactions involved. This beta-oxidation pathway was first suggested by Knoop in 1905.

However ricinoleic acid has a hydroxyl group to be accounted for and this might involve an early hydrogenation reaction mediated by diphosphopyridine nucleotide ($DPN^{+} \longrightarrow DPNH + H^{+}$) to produce oleic acid and one molecule of water. Alternatively a dehydration reaction might occur with the formation of linoleic acid. This possibility will be tested experimentally in Section 3 of this chapter. In so far as hydrogenation is a well established biochemical mechanism and dehydration is not, the former would be regarded as the likelier in this case.

Gensler (1957) discusses in detail the possible synthetic pathways for ricinoleic acid, but his suggestions apply only to the industrial chemistry of ricinoleic acid synthesis and do not consider the biosynthetic pathway.

SECTION 2

Release of ricinoleic acid from rat adipose tissue incubated in vitro

Gordon and Cherkes (1958) demonstrated that rat epididymal fat pads when incubated in vitro, under suitable experimental conditions, liberated fatty acids which could be recovered in their free state from the incubating medium. They showed that the fasting state and the addition of epinephrine to the medium accentuated the release of fatty acids, whereas the feeding of carbohydrate and the addition of insulin to the medium inhibited their release. The following experiment was carried out to discover whether ricinoleic acid would behave like other fatty acids in its pattern of release from rat adipose tissue.

Methods

The sixth rat of the experiment of the last section was used for this study. The animal was killed by decapitation and both epididymal fat pads removed. A small piece was taken from each of the pads for the analysis of the basal fatty acid composition of the adipose triglyceride, and thereafter each pad was dealt with separately. The pads were handled gently and immediately after weighing were placed in the incubation mixture.

The incubation mixture was Krob's bicarbonate buffer, with added epinephrine and bovine albumin, defatted by the method of Goodman (1957). The epinephrine is not essential but increases the yield of free fatty acids.

The bicarbonate buffer was made up as follows:-

sodium/

sodium chloride (0.154 M)	100 ml
potassium chloride (0.154 M)	4 ml
calcium chloride (0.11 M)	3 ml
potassium dihydrogen phosphate (0.154M)	1 ml
magnesium sulphate (0.154 M)	1 ml
sodium bicarbonate (0.154 M)	21 ml

To 100 ml of this mixture exactly 3 g of the defatted bovine albumin was added and stirred thoroughly. 3 ml of this mixture was pipetted into a small erlenmeyer flask and 0.05 ml of a solution of epinephrine containing 15 µg/ml was added. The fat pads were placed in the incubation mixture, the flask stoppered and incubated in a gently shaking water bath for at least 1 hour, at 37°C. For the first 5 minutes of this period a gaseous mixture of 5 per cent CO_2 and 95 per cent O_2 was blown over the contents of the flask, which was then sealed.

After incubation the fat pad was picked out of the mixture with forceps and drained of medium by touching it gently against the neck of the flask. The 3 ml of incubation mixture was transferred to a conical separating flask and extracted with 15 ml of Dole's reagent for 1 hour. The mixture was shaken vigorously for about 1 minute before being left to stand. At the end of the hour 6 ml of water and 9 ml of isooctane were added and the flask shaken vigorously for 2 minutes and again allowed to stand. Two phases formed with a sharp interface. The free fatty acids (FFA) are contained in the isooctane or upper phase.

As/

As much of the upper phase as possible was recovered without contaminating it by the lower and washed with an equal volume of 0.05 per cent sulphuric acid to remove any lactate. The lower phase was again discarded and the upper phase taken to dryness with gentle heat under nitrogen. Methyl esters of the fatty acids were prepared and analysed in both the silicone gum and ethylene glycol adipate columns to give a comprehensive fatty acid analysis.

Results

The results of this experiment are given in Table XXI.

This shows that ricinoleic acid is released from the adipose tissue triglyceride in proportion to the amount present. If this release occurs in in vitro conditions it is likely to occur in in vivo conditions. When castor oil feeding stops, ricinoleic acid disappears from the circulating plasma and there is a fatty acid concentration gradient for ricinoleic acid from adipose tissue to plasma. It is reasonable to assume that in these conditions ricinoleic acid will continue to be released from adipose triglyceride until it disappears.

SECTION 3

The metabolism of ricinoleic acid in essential fatty acid deficient rats

Although the oxidative degradation of ricinoleic acid seemed to be the likeliest explanation for its disappearance from adipose tissue, at least one alternative pathway was theoretically possible. In the introductory chapter the industrial dehydration of castor oil was mentioned as an important source of polyunsaturated fatty acids for the/

Table XXI Percentage fatty acid composition of epididymal fat pad triglyceride (TG) and the free fatty acids (FFA) released from it during incubation in Kreb's bicarbonate buffer at 37°C for 1 hour. The paired fat pads from rat No. 6 of the previous experiment were used.

Fatty acid	Fat pad 1		Fat pad 2	
	TG	FFA	TG	FFA
14	2.0	2.1	2.0	2.2
16	24.6	27.2	25.3	27.6
16:1	4.4	6.9	5.4	9.0
18	4.8	3.2	4.4	4.1
18:1	36.4	30.5	37.0	33.1
18:2	20.2	21.5	18.0	16.8
18:1:OH	6.8	7.2	7.0	6.5
20	0.4	0.0	0.3	0.0
20:4	0.5	1.3	0.5	0.7

the paint industry. It was important to see if biological dehydration might occur.

On theoretical grounds, to be discussed later, the metabolic dehydration of ricinoleic acid would be likely to produce linoleic or arachidonic acid. For this reason it was decided to investigate the effect of castor oil on essential fatty acid deficient rats. Developments from this work bearing on the morphology of the erythrocyte and the lipid structure of the red cell membrane have been published (Watson, 1963), but because the experimental methods did not directly involve the use of castor oil they have not been included in this thesis.

Methods

Following the recommendations of Holman and Peifer (1960) the first attempt to produce a group of essential fatty acid deficient rats was made with 15 day old animals whose average weight was about 30 g. Although each animal was given an injection of 15,000 units of penicillin when first introduced into the laboratory none survived. The second, and successful attempt was made with 25 day old rats which had been weaned at the 22nd day, and whose average weights ranged from 50 to 60 g. All of these survived.

The diet, based on that of Holman and Peifer, was made up as follows, the constituents being given as percentages:-

vitamin free casein (Nutritional Biochemicals Inc.)	18
sucrose	69
cellulose	4
Wesson salt mixture	4
hydrogenated/	

hydrogenated coconut oil	1
urea	3
cholesterol	1

Cholesterol was introduced by Holman and Peifer to accelerate the EFA deficient state. The basic vitamin needs of the animals were met by making up a triturate of the following vitamins in casein and adding them to the diet. The weight of each vitamin is the amount per kilogram of diet:-

vitamin A palmitate	5 mg
calciferol	100 µg
a-tocopherol	100 mg
ascorbic acid	500 mg
calcium pantothenate	70 mg
inositol	1,320 µg
2-methyl-1-4,naphthaquinone	6 mg
niacin	60 mg
p-aminobenzoic acid	600 mg
pyridoxine hydrochloride	30 mg
riboflavine	30 mg
thiamine hydrochloride	73 mg
choline hydrochloride	13 mg
folic acid	11 mg
biotin	500 µg
vitamin B_{12}	2 mg

The animals were restricted to 30 ml of water per day.

The/

The rats were weighed at regular intervals. By the end of 6 weeks it was apparent that they were thirsty, that their fur was deteriorating and that there was some scaliness of their feet and tails. They were not graded for severity according to the standards suggested by Holman and Ener (1954). They were not so frankly EFA deficient as the animals used in the study on the morphology and lipid composition of EFA deficient erythrocytes (Watson, 1963), but as will be seen in due course they were deficient enough for the purposes of this investigation.

Between the sixth and seventh week of the diet the lightest and two heaviest rats were killed, and their livers and one epididymal fat pad taken for lipid extraction and GLC of fatty acids. The livers of each of the animals were pale and the fat of the fat pads was of an unusual pale yellow colour.

When this analysis had shown that there was no linoleic or arachidonic acid in either the fat pad or liver lipids, the next part of the experiment was begun.

The 12 lightest animals had biopsies taken from an epididymal fat pad and were then divided into three groups of 4. Group 1 continued on the EFA deficient diet, Group 2 was given this diet containing 2 per cent castor oil by weight, and Group 3 the diet containing 20 per cent castor oil. It was necessary to give the castor oil in these two substantially different concentrations for the following reason. It will be remembered that the castor oil contained about 5 per cent of its fatty acids as linoleic acid. On the assumption that each animal/

animal was eating about 20 g of diet per day it meant that the animals in Group 2 were receiving about 320 mg of ricinoleic acid per day and about 15 mg of linoleic acid. This amount of linoleic acid is unlikely to correct the EFA deficient state. Turpeinen (1938) showed that 100 mg per day was the largest necessary dose for the maximum growth response of rats, that 50 mg was inadequate and that the benefit from 25 mg was marginal.

The animals in Group 3, on the other hand, were receiving about 160 mg linoleic acid per day and about 3.2 g of ricinoleic acid. This does not mean that the group was absorbing these amounts of the fatty acids, for the animals had chronic, mild diarrhoea and, as will be seen from the results, while they were absorbing some of the linoleic acid the amount was not sufficient to correct their biological deficiency.

The animals were weighed at weekly intervals and killed after 5 weeks. Samples of plasma, liver, adipose tissue and brain were obtained from 2 of the rats in each group and fatty acid methyl esters prepared for GLC on both the silicone gum and adipate columns.

Results

Both Group 1 and Group 2 rats continued to deteriorate as far as symptoms and signs of EFA deficiency were concerned. By the end of the 5 week period all of the animals in these groups had marked scaliness of the paws, dryness of the tail and skin and severe thirst. None of these features was so marked in the Group 3 rats. On the other hand these animals had gross congestion of the proximal part of the small/

small bowel, the naked eye appearance being that of frank inflammation. No histological examination of the bowel was made. It might have been instructive to study the microscopical appearances of the villi of the mucosal epithelium.

Clinically, it was plain that a daily dose of 320 mg of ricinoleic acid had not corrected the EFA deficiency of the Group 2 rats, whose further deterioration was as bad as that of the animals continuing on the original, unaugmented diet. The slight improvement, or perhaps absence of further deterioration, on the Group 3 rats was probably due to the larger amount of linoleic acid in their quota of oil rather than to any effect that might be attributed to the much larger amount of ricinoleic acid.

The average weights of the animals in each group are recorded in Table XXII. The data of this table may show a slight advantage in favour of the Group 2 rats, for whereas they started lighter than their Group 1 litter-mates, their final mean weight of 233 g is substantially higher than the mean weights of 215 and 209 g in Groups 1 and 3 respectively. Not too much will be made of these figures in view of the more specific data obtained from the GLC analysis of the various tissues.

The fatty acid analyses of the fat pad biopsies from each of the 12 EFA deficient rats before they were allocated to their dietary groups are shown in Table XIII, which also contains for comparison the fatty acid pattern of two animals from the same litter which were reared normally. The significant features are the complete absence

of/

Table XXII Weights of EFA deficient rats on the day of the fat pad biopsy and at weekly intervals thereafter during the 5 weeks on the modified diets. A = EFA deficient diet; B = A + 2% castor oil; C = A + 20% castor oil. Weights in grammes.

Diet	Rat No.	Week					
		0	1	2	3	4	5
A	1	150	178	200	208	207	216
	2	175	188	220	228	215	227
	3	130	128	177	180	201	203
	4	152	175	193	200	215	226
	Mean	152	167	197	204	209	208
B	5	145	170	195	192	231	247
	6	125	137	162	153	184	205
	7	155	181	195	187	229	246
	8	138	162	178	174	213	233
	Mean	141	163	183	177	209	233
C	9	142	157	180	184	203	217
	10	150	153	175	183	169	176
	11	120	127	164	174	192	205
	12	145	158	198	212	230	239
	Mean	139	149	179	188	199	209

Table XXIII Fatty acid patterns of the fat pad biopsies from the 12 rats of Table XXII, taken after 7 weeks on the EFA deficient diet. The mean figures for 2 animals from the same litter and reared normally are given for comparison at the end of the table. The percentages do not aggregate 100 since minor components have been omitted

Rat No.	Fatty Acid (%)						
	14	16	16:1	18	18:1	18:2	20:4
1	1.8	31.7	13.3	4.1	48.9	-	-
2	1.9	34.0	10.4	5.4	48.2	-	-
3	2.1	33.7	9.6	5.2	48.7	-	-
4	2.0	32.7	12.7	4.3	48.0	-	-
5	2.0	31.0	14.9	3.4	48.3	-	-
6	2.1	30.7	12.2	4.9	50.4	-	-
7	1.7	32.5	14.3	3.8	47.5	-	-
8	2.1	31.1	12.8	4.2	49.5	-	-
9	1.9	31.3	10.1	4.7	51.6	-	-
10	2.1	29.4	11.2	4.2	52.5	-	-
11	1.6	32.6	12.0	4.1	49.3	-	-
12	1.7	32.0	12.1	4.6	49.6	-	-
Mean	1.9	31.9	12.1	4.3	49.3	-	-
Normal	2.2	27.3	6.1	4.6	37.3	20.6	0.9

of linoleic acid and arachidonic acids from the deficient rats and the substantial increase in the amount of palmitoleic acid.

The GLC analyses of the plasma, liver, adipose and brain fatty acids are recorded in Tables XXIV, XXV, XXVI and XXVII respectively and considered separately in the following paragraphs.

Plasma fatty acids (Table XXIV)

The main facts from these results are illustrated in Fig. 7. The EFA deficient animal has an excessive amount of palmitoleic and eicosatrienoic (20:3) acids. The former reduces in amount as linoleic acid begins to appear and the latter finally vanishes when arachidonic acid is present in substantial concentrations.

Although there is a very small amount of linoleic acid in group 2 plasma there is no arachidonic and this will explain why these rats remained clinically deficient. Ricinoleic acid does not appear until the bigger dose of castor oil is given.

The values for linoleic and ricinoleic acids in the second and third groups could be explained in three ways. Firstly, ricinoleic acid may have been altered to linoleic acid; secondly, it may have been less readily absorbed; thirdly, it may have been more rapidly metabolised. One or all of these explanations could account for the fact that although linoleic acid is present in castor oil in only about one twentieth the concentration of ricinoleic acid, it is present in plasma in much greater concentrations. Against these possibilities, however, must be laid the data of Table XXVI, which show that ricinoleic acid is present in adipose tissue in greater or equal concentrations

than/

Table XXIV Percentage fatty acid composition of the plasma lipids from 2 of the rats in each of the dietary groups of Table XXII, after 5 weeks on the diet.

Fatty acid	Diet A		Diet B		Diet C	
	Rat 2	Rat 3	Rat 5	Rat 8	Rat 9	Rat 12
14	1.7	1.2	0.9	0.9	trace	trace
16	28.3	27.0	26.3	28.1	24.8	21.4
16:1	17.2	17.3	10.0	12.4	5.8	5.4
18	6.1	5.5	11.4	7.6	8.4	9.3
18:1	39.1	41.2	38.6	39.0	37.5	36.8
18:2	-	-	1.3	2.1	9.6	9.0
18:1:OH	-	-	-	-	2.8	4.3
20:3	7.6	7.8	11.6	9.9	-	-
20:4	-	-	-	-	10.8	13.7

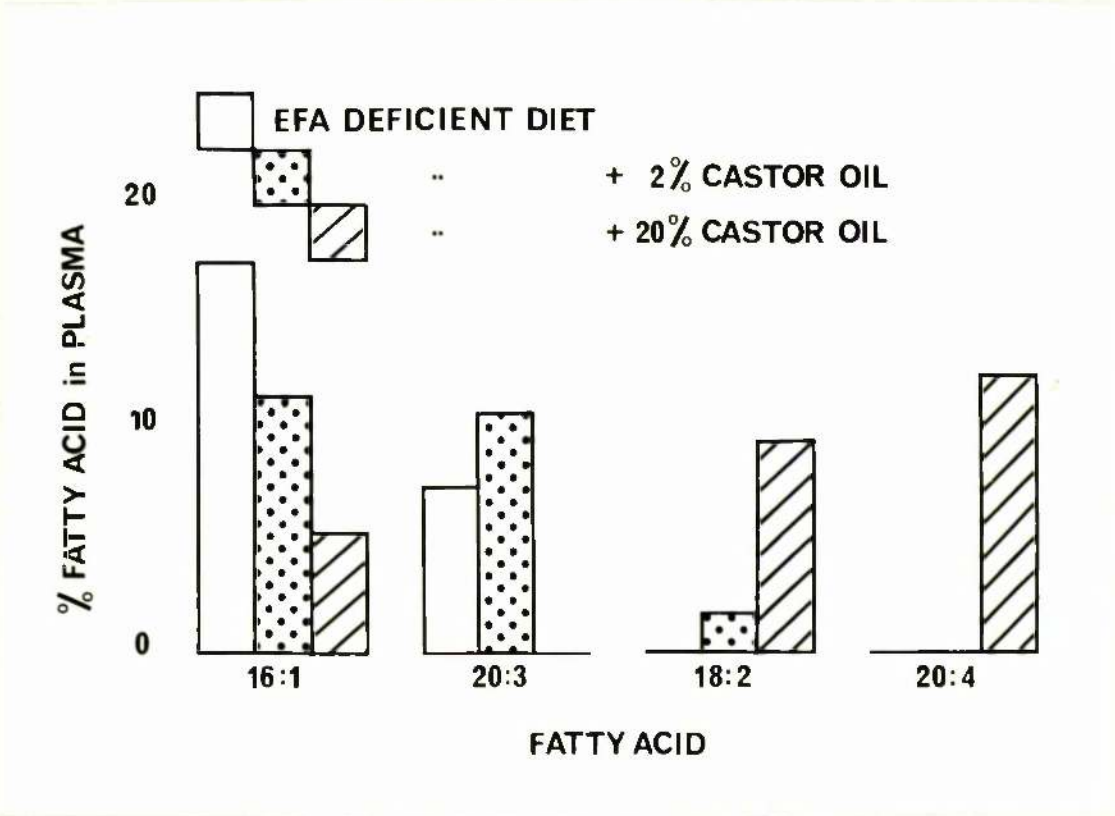


Figure 7. Figure illustrating the fall in the concentration of palmitic (16:1) and eicosatrienoic (20:3) acids and the rise in linoleic (18:2) and arachidonic (20:4) acids in the plasma lipids of EFA deficient rats having increasing amounts of castor oil added to their EFA deficient diets.

than linoleic acid, and its lower concentration in plasma may simply be due to more selective deposition in adipose tissue.

Liver fatty acids (Table XXV)

The results for the liver fatty acids are similar to those for plasma, except that there is a small amount of arachidonic acid in group 2, and eicosatrienoic acid has not completely disappeared from group 3. There are no experimental data which explain this, but it may be due to the slower turnover of fatty acids in liver phospholipid. Fig. 8 illustrates once more that palmitoleic and eicosatrienoic acids are reciprocally reduced as linoleic and arachidonic acids increase.

Adipose tissue fatty acids (Table XXVI)

Apart from the presence of small amounts of linoleic and ricinoleic acid in the group 3 rats there has been no significant change in the fatty acid patterns of all 3 groups.

Brain fatty acids (Table XXVII)

Little can be said about the brain lipids. There is no linoleic in any of the samples, and arachidonic is present even in the group 1 rats, although its concentration increases as that of eicosatrienoic falls and finally disappears. The 24 carbon fatty acid is probably tetracosenoic acid (24:1, nervonic acid), which Fulco and Mead (1961) suggest is derived from oleic acid by the addition of three acetate molecules. As in the initial feeding experiments ricinoleic acid is again absent from the brain lipids.

Table XXV Percentage fatty acid composition of the liver lipids from the same rats as in Table XXIV.

Fatty acid	Diet A		Diet B		Diet C	
	Rat 2	Rat 3	Rat 5	Rat 8	Rat 9	Rat 12
14	0.8	1.0	1.0	0.9	0.6	0.6
16	29.9	26.7	26.1	32.7	27.6	23.1
16:1	16.4	16.6	10.5	11.6	6.2	5.4
18	6.2	5.8	11.2	8.0	8.7	10.3
18:1	38.6	44.8	42.0	39.0	41.4	43.0
18:2	-	-	2.5	2.0	6.1	6.5
18:1:OH	-	-	-	-	2.5	2.5
20:3	7.1	5.6	5.1	4.3	1.3	1.5
20:4	-	-	1.7	0.8	5.5	7.2

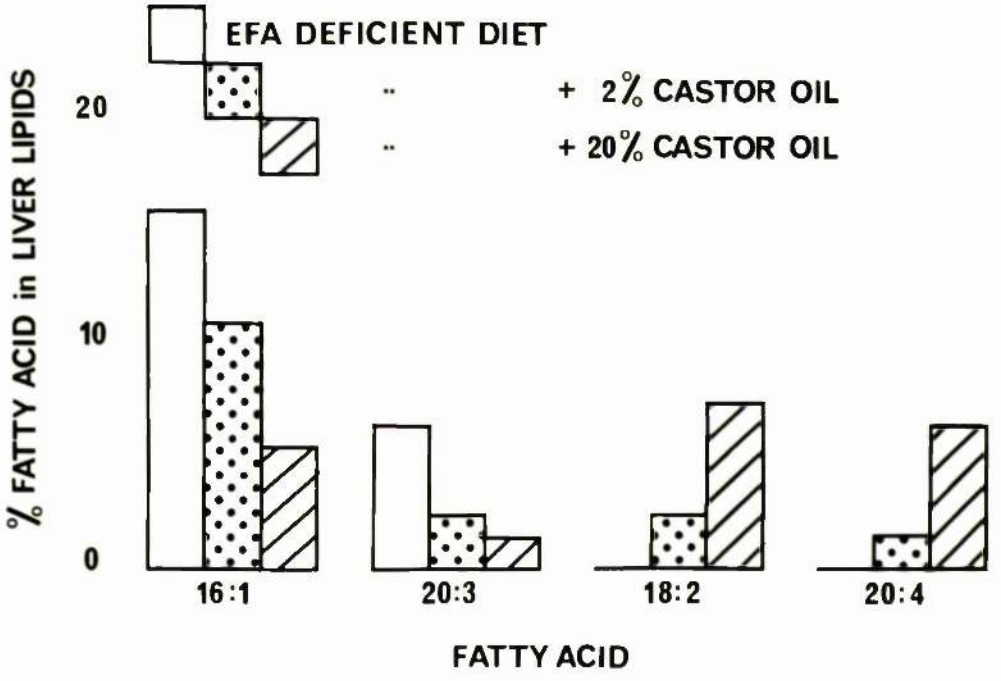


Figure 8. Figure illustrating fatty acid changes similar to those of Fig. 7 in the liver lipids of E.F.A. deficient rats on castor oil supplemented EFA deficient diets.

Table XXVI Percentage fatty acid composition of the fat pad lipids from the same rats as in Table XXIV. There is no eicosatrienoic (20:3) or arachidonic (20:4) in any of them.

Fatty acid	Diet A		Diet B		Diet C	
	Rat 2	Rat 3	Rat 5	Rat 8	Rat 9	Rat 12
14	2.3	1.7	1.5	1.9	1.8	1.7
16	28.0	29.8	32.1	30.9	32.2	31.0
16:1	13.4	17.4	15.0	16.3	13.5	14.0
18	3.9	3.3	3.8	3.5	4.1	3.9
18:1	52.0	48.0	47.6	47.5	42.3	46.2
18:2	-	-	-	-	2.4	1.6
18:1:OH	-	-	-	-	3.6	1.6

Table XXVII Percentage fatty acid composition of the brain lipids from the same rats as in Table XXIV. There is no ricinoleic acid or linoleic acid in any of the samples.

	Diet A		Diet B		Diet C	
Fatty acid	Rat 2	Rat 3	Rat 5	Rat 8	Rat 9	Rat 12
16	20.0	22.1	21.3	22.1	25.5	21.6
16:1	-	1.1	1.1	1.4	1.1	1.2
17	-	2.2	2.7	2.8	2.7	2.4
18	20.0	20.8	22.1	22.8	18.8	20.8
18:1	23.9	26.9	23.5	23.9	22.0	24.2
20	1.2	-	1.9	2.0	-	1.8
20:3	3.1	3.5	1.8	1.9	-	-
20:4	6.3	7.2	7.5	7.6	10.7	11.6
24:1	25.4	13.0	14.8	12.3	16.2	13.5

Comment

In an admirable series of studies published between the years 1954 and 1960, Mead and his colleagues demonstrated the biosynthetic pathway for arachidonic acid in the normal rat and for eicosatrienoic in the essential fatty acid deficient rat (Mead, Steinberg and Howton, 1953; Steinberg, Slaton, Howton and Mead, 1956; Mead and Howton, 1957; Fulco and Mead, 1959; Fulco and Mead, 1960, Howton and Mead, 1960). They showed that the starting point in the synthesis was linoleic acid, the essential fatty acid which mammals cannot synthesise and which, therefore, must be supplied in the diet.

Linoleic acid is first converted to gamma-linolenic acid by dehydrogenation. This in turn is converted to 8,11,14-eicosatrienoic acid by chain lengthening through the addition of one molecule of acetate and the final step to arachidonic acid involves a further dehydrogenation in the 5 position. The reaction proceeds so rapidly through its intermediate stages that only trace amounts of gamma-linolenic and 8,11, 14-eicosatrienoic acids can be isolated from the animal body, even after their ingestion (Mead, 1957). This fact is certainly fully confirmed by the many GLC analyses performed throughout this study, in none of which were recognisable amounts of these two fatty acids found in normal tissues.

In the EFA deficient rat on the other hand, eicosatrienoic acids do appear. The 5, 8, 11-eicosatrienoic acid is derived from oleic acid, while the 7, 10, 13-eicosatrienoic acid probably comes from palmitoleic acid/

acid. These two trienoic acids are indistinguishable by the analytical methods used here and it is not possible to say which begins to disappear first as the rats return to their normal metabolic state. There is probably some significance in the fact that the tissue concentration of palmitoleic acid shows a quicker fall to more normal levels than does oleic acid.

The positions of the double bonds within the different unsaturated fatty acids are critical in determining the synthetic development of the fatty acid. Linoleic acid is 9, 12-octadecadienoic acid, and this is an obligatory starting point if the 8, 11, 14-eicosatrienoic acid, which is the immediate precursor of arachidonic acid, is to be derived. The dehydrogenation of oleic acid, which is 9-octadecenoic acid, results in 6, 9-octadecadienoic acid, and the subsequent production of the 5, 8, 11-eicosatrienoic acid.

Now ricinoleic acid is 12-hydroxy-9-octadecenoic acid, and if a simple enzymatic dehydration of the fatty acid were possible it is likely that it would occur at the hydroxy position, giving 9, 12-octadecadienoic acid, the correct isomeric form of the dienoic acid for further conversion to arachidonic acid. That this does not occur seems likely from the work of this section. The amount of ricinoleic acid given to the second group of rats was sufficient to cure their EFA deficient state had it been wholly or mostly converted to linoleic acid. There is neither clinical not analytical proof that this occurred. It seems likely, therefore, that the metabolic fate of ricinoleic acid is/

is that of straightforward oxidation. If this is so, then this fatty acid, like other fatty acids, is an energy source.

GENERAL DISCUSSION

If C^{14}-ricinoleic acid had been available it might have been possible to demonstrate the oxidation of the fatty acid by the recovery of expired $C^{14}O_2$. But even without this convenient tool it has been possible to study some aspects of its metabolism both directly and indirectly, and to show that ricinoleic acid behaves in a manner similar to other long chain fatty acids.

It is mobilised from adipose tissue in vivo, and released from it to the same degree as other fatty acids in vitro. During the period of disappearance from adipose tissue neither it nor its hydrogenation product, hydroxystearic acid, can be detected in the faecal lipids, so that it is unlikely that it is being excreted either in the bile or from the gut wall.

The clinical and analytical data of Section 3 make it unlikely that ricinoleic acid is dehydrated, either in the course of absorption, assimilation or mobilisation. It seems improbable that ricinoleic acid is so biochemically unconventional as to require a completely new metabolic pathway for its degradation, and therefore, reverting to the theoretical considerations of page 71, it is suggested that this pathway is the established one of oxidation with at one point a single hydrogenation reaction mediated by diphosphopyridine nucleotide to split off the hydroxyl group with the production of one molecule of water.

CHAPTER 6

THE ABSORPTION OF CASTOR OIL IN MAN

The previous chapter concludes the experimental work carried out on the rat. The next three chapters give an account of clinical studies which demonstrate points of similarity between the fate of castor oil in rat and in man, and show how the oil may be used as a tool in clinical investigation and research.

SECTION 1

Hans Meyer demonstrated in 1890 that the purgative action of castor oil was due to ricinoleic acid. Despite the later work of Valette and Salvanet (1936) and Stewart and Sinclair (1945), which demonstrated the absorption of ricinoleic acid in man and in animals, the belief persisted that castor oil was not absorbed. This belief partly underlies its use as a safe and non-toxic purgative.

This is an account of a series of balance experiments with castor oil in man which show that the absorption is inversely related to the size of the dose. Secondly, it is a description of a new technique for faecal fat balance, utilising the gas-liquid chromatography of ricinoleic acid on the silicone gum column, and incorporating an improved technique for the collection, preparation and extraction of faecal lipid.

Methods/

Methods

Eighteen of the subjects were under investigation for hypertension, but were otherwise metabolically normal. One of these patients participated twice. Three others were young, normal, male volunteers.

Castor oil of medicinal purity and containing a trace amount of oil labelled with radioactive iodine^{131}, prepared by the method of Rutenberg, Seligman and Fine (1949), was administered in a range of doses from 4-60 g containing about 6 μc of radioactivity. The dose of castor oil administered was determined by weight. A paper cup was weighed, and the desired dose of castor oil, plus an average of about 5 g was placed in it. The full cup was weighed, and the cup plus residual oil was finally weighed again after the patient had drunk as much oil as would easily flow out. Thus, the quantity of oil consumed could be determined, and a known amount remained in the cup, to be used as a standard for comparison with excreta in the subsequent analytical procedures.

A 120 mg dose of potassium iodide was given each subject to block thyroid uptake of radioactive iodine. The hypertensive patients were given castor oil at 5 p.m. on the day before a scheduled intravenous pyelogram, and allowed only a light meal of coffee and toast thereafter. Stool collections, uncontaminated by urine, were made in two parts; for the first 24 hours after the dose and during the subsequent 72 hours. The normal volunteers were given small doses of oil and allowed free diet. Their stools were collected in 3 samples of 2, 2 and/

and 3 days after the oil.

Stools were collected in 1 gallon paint cans and mixed as suggested by Gordon (1959). In the course of the investigation the method was modified to give a more homogeneous and stable emulsion as follows.

To each can were added approximately 50 g coarse granular silica (4 mesh obtained from Fisher Scientific Company), water to bring the wet weight of faeces to 970 g, 100 ml of 0.4 per cent cellulose gum solution (carboxymethylcellulose sodium salt, type 70 High, Hercules Powder Company), and 1,000 ml 95 per cent ethanol. The can was then tightly closed and agitated for 15 minutes in a commercial paint shaking machine (Red Devil Paint Conditioner in U.S., Holmes Speedy Agitator in U.K.). This produces a homogeneous emulsion of faeces in a volume of 2 litres. The emulsion is moderately stable, but if the can is left standing for more than 15 minutes it is advisable to shake it once or twice by hand.

The paper cup containing the weighed residue of castor oil was treated in the same way as the stools. Radioactivity was measured by placing the paint can on a scintillation counter, taking care to match the position of each can.

At first, total fatty acids were estimated by the method of van de Kamer, Ten Bokkel Huinink and Weyers (1949), but early in the study it was suspected that the results were too low. This led to a reinvestigation of the method for extraction of faecal fatty acids, and this/

this work was carried out by a colleague in the laboratory and published elsewhere (Jover and Gordon, 1962). The writer has made extensive use of the method and established the range of values for faecal fatty acids in normal patients and those with steatorrhoea (Watson and Dickson, 1964). The principal details of the method are now given briefly.

Extraction and Titration of Faecal Fatty Acids

10 ml of the faecal suspension prepared above, is placed in a 125 ml flask and 5 ml of 66 per cent KOH(W/V), and 40 ml of 95 per cent ethanol (neutral to thymol blue) are added. The flask is fitted with a reflux condenser, and the contents boiled for 20 minutes. After the flask has been well cooled in ice, 17 ml of a 1:4 (V/V) dilution of reagent grade, concentrated HCl, is cautiously added. The flask is cooled again, and 50 ml of toluene added.. The flask is stoppered, shaken vigorously for one minute, and then allowed to stand for 5 to 10 minutes, to allow effective separation of the two phases.

25 ml of the toluene phase is then transferred to a 60 ml Erlenmeyer flask and evaporated over a steam bath. The residue is redissolved in 10 ml of 95 per cent ethanol, 2 drops of thymol blue solution (2 per cent in 50 per cent ethanol) are added, and the acids are titrated with NaOH solution of known normality. Standard alkali of about 0.1 N may be used conveniently with a microburette. A reagent blank should be run in parallel with each set of faecal fatty acid determinations.

The patient's output of fatty acids, in mEq per day, is calculated as/

as follows:

$$\text{Output} = \frac{50 \times A \times B \times 2000 \times 1.15}{25 \times 10 \times C} = \frac{460 \times A \times B}{C}, \text{ where}$$

A = known normality of the standard NaOH solution,

B = volume of NaOH solution (minus the blank) in ml,

C = number of days' output collected

The factors 50, 2000, 25 and 10 are the volumes of the various aliquots used; they may be changed if special circumstances make it advisable to do so. The factor 1.15 is an empirically determined correction factor, which compensates for the fact that the volume of toluene is increased by 8 per cent due to solution of ethanol in it, and for the fact that the extraction of fatty acids from alcohol - water by toluene in one step is only 93 per cent complete. The completeness of extraction is virtually the same for all of the higher fatty acids tested, with the exception of ricinoleic and 12-hydroxystearic acids, for which extraction was 90 per cent. On this account a correction factor of 1.18 was employed in recovery experiments using castor oil. In unusual circumstances it would be advisable to check extraction in individual cases by extracting the acid-alcohol-water mixture with a second aliquot of toluene.

The original paper should be consulted for an account of the various procedures which were used to demonstrate the accuracy of the method for different kinds of fats and oils and for the uniformity of the results obtained no matter whether the faecal contents are sampled from the top, middle or bottom of the can.

The/

The procedure for the determination of faecal fatty acids, originally published by van de Kamer et al (1949) was modified primarily to provide for the satisfactory recovery of hydroxy acids. Hydroxy acids, and lipids containing them, do not dissolve in petroleum ether, but are freely soluble in the slightly more polar toluene. This point was found to be of critical importance in connection with the human castor oil balance studies.

Total lipid for the preparation of methyl esters was extracted from the faecal homogenate by the method of Bragdon (1960). Methyl esters were prepared and GLC carried out by the standard methods.

Calculation

I. Faecal ricinoleic acid = Total titratable faecal fatty acid x per cent ricinoleic acid obtained by GLC (mEq)

II. Ricinoleic acid administered =

$$\text{dose of castor oil (in g)} \times \frac{9}{10} \times \frac{896}{988} \times \frac{1000}{298} \text{ (mEq)}$$

$$= \text{dose} \times 2.7 \text{ (mEq)}, \text{ where}$$

$\frac{9}{10}$ is a factor accounting for the fact that ricinoleic acid is 90 per cent of castor oil fatty acids,

$\frac{896}{988}$ is the factor accounting for the glycerol component of castor oil

298 is the molecular weight of ricinoleic acid.

$$\text{The recovery of ricinoleic acid} = \frac{I}{II} \times 100 \text{ (per cent)}$$

Results

The fatty acid composition of the castor oil is given in Table III. Allowing for the presence of the doubly unsaturated linoleic acid (4.7 per cent) and the mono-unsaturated oleic acid (3.3 per cent) one might expect that at least 13 per cent of the radioactivity in the I^{131}-labelled oil would be due to radioactivity incorporated in these fatty acids.

The results of the 22 I^{131}-castor oil balance studies are shown in Table XXVIII. Clearly I^{131}-labelled castor oil can be absorbed, and the degree of absorption is approximately inversely related to the dose (Fig. 9). With the smallest doses of oil absorption is 99 per cent, whereas with the large purgative doses faecal excretion approaches 90 per cent. Thus, even in the presence of gross purgation there probably is some absorption of the oil. This could, however, be due entirely to absorption of label in the oleic and linoleic acids.

Four grammes is not the maximum amount of oil that may be absorbed since 50 per cent of 20 to 30 g doses was absorbed in these studies.

In 17 of the 18 patients who received doses of 10 g or more, almost all of the radioactivity recovered was present in the first 24 hour faecal collection, and each of these individuals had either frank purgation or mild laxation. In one subject a dose of 43.9 g was not noticeably effective, and of the 61.5 per cent recovery of radioactivity 31.5 per cent was present in the first 24 hour collection and the remainder in the second collection. In a general way the degree of recovery correlated with the purgative effect. The volunteers receiving/

Table XXVIII Faecal recovery of I^{131}-ricinoleic acid following the oral administration of various doses of castor oil. Results of 22 experiments on 21 subjects - 18 hypertensive patients and 3 normal volunteers.

	Dose of Castor Oil (g)	% Dose of I^{131} in stools
Normal volunteers	3.8	0.5
	3.9	0.3
	3.9	0.9
Hypertensives	10.0	11.4
	18.3	42.9
	32.2	31.1
	33.4	60.7
	37.4	53.5
	42.2	72.7
	42.9	71.5
	43.6	75.4
	43.9	61.5
	44.4	86.0
	46.3	72.2
	46.4	59.6
	46.4	64.9
	47.9	81.0
	49.3	54.6
	50.2	64.5
	53.7	84.0
	57.5	82.5
	60.6	89.7

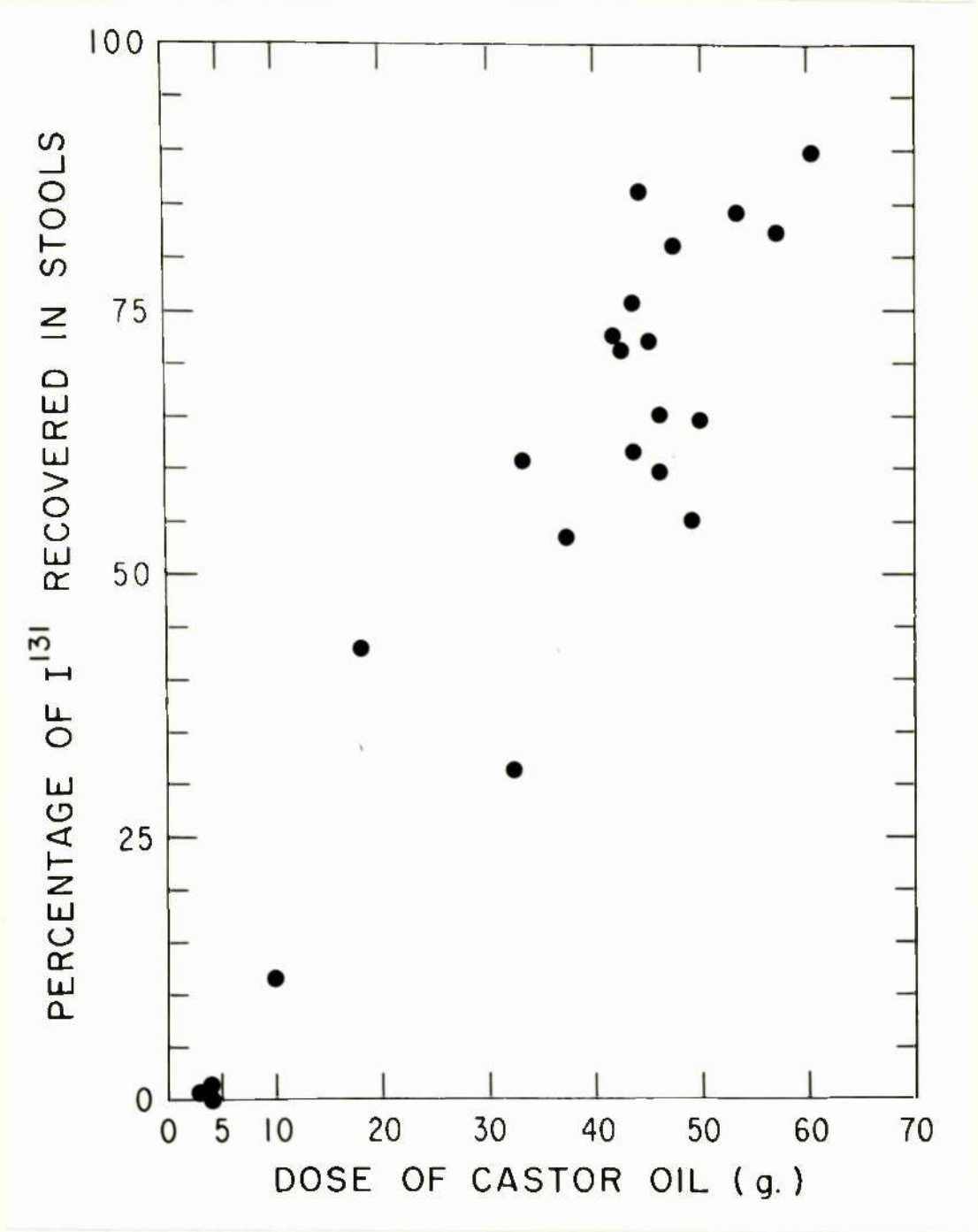


Figure 9. Graph illustrating the relationship between the dose of I^{131}-castor oil and its excretion. This graph is drawn from the data of Table XXVIII.

receiving a 4 g dose had no alteration of normal bowel function.

During the initial phase of this study poor correlation between the results obtained by the I^{131} tracer and chemical techniques led to a critical scrutiny of the latter and the relevant modifications incorporated in the technique have been described in the methods section. Results obtained by the revised procedure are compared in Table XXIX with radioisotopic data obtained simultaneously in 5 patients. The comparison shows substantial agreement between the techniques in 4 cases. In subject No. 2 however, there is a difference between the results which is greater than should be accountable on the basis of analytic errors alone.

Comment

The appearance of ricinoleic acid in faeces uniquely follows the administration of castor oil. James, Webb and Kellock (1961) have reported the presence of hydroxystearic acid, but not ricinoleic acid, in the faeces of individuals on normal diet. It is this exclusive occurrence of ricinoleic acid and its rapid resolution by gas-liquid chromatography on a silicone gum column which make possible the technique for accurate and specific fat balance reported here.

The labelling of unsaturated fatty acids by radioactive iodine has two main disadvantages. Firstly, the exact fate of the I^{131} label in the gut is not known. Cox (1961) has discussed at length the reasons for the unreliability of I^{131}-triolein, mentioning in particular the evidence for the instability of the I^{131} label in the gut. Dissociation of the label from the oil will lead to absorption and lower faecal recovery/

Table XXIX Comparison of faecal recovery of castor oil in 5 subjects using I^{131}-castor oil and the chemical balance technique.

Dose of Castor Oil (g)	% Recovery from Faeces	
	Chemical	I^{131}
10.0	12.7	11.4
18.3	55.0	42.9
33.4	61.9	60.7
37.4	57.1	53.5
44.4	90.0	86.0

recovery. A chemical method avoids this fallacy. Secondly, iodine labelling alters the chemistry of a fatty acid. The pharmacology of ricinoleic acid may be different from that of hydroxyiodochlorostearic acid, the product of its iodination by the method of Rutenberg and his colleagues, where iodine monochloride is the iodinating agent. In this respect it is reassuring that in the cases studied by the improved chemical technique and by iodine labelling there is a close relationship between the results.

However, differences do exist and they are substantial in case 2. While this is probably due to the imperfections of the isotope method there are two possible sources of error in the chemical technique. Firstly, if absorbed ricinoleic acid is re-excreted into the gut this will raise the faecal recovery of ricinoleic acid and lead to under-estimation of the real degree of absorption. There is no evidence about this for man, but the animal studies of section 1 of the previous chapter show that the intestinal excretion of unaltered ricinoleic acid is not a mechanism in its overall metabolism in the rat, and this is also likely to be the case in man.

Secondly, and of more practical importance, the result will be affected by any chemical modification of ricinoleic acid in its passage through the gut. This is not a problem when castor oil is administered in purgative doses. But in 2 of the 3 normal subjects given the small non-purgative doses of castor oil, and in rats on chronic castor oil feeding, hydroxystearic acid appeared in the faeces, although/

although it had not been detectable in pre-castor oil faecal collections. This finding is consistent with the belief that intestinal hydrogenation of fatty acids occurs, and is studied further in Chapter 8. It also indicates that difficulties will arise in the interpretation of balance studies made with non-purgative doses of castor oil, unless it can be shown that hydroxystearic acid is not present in faeces collected before the administration of castor oil. Absence of hydroxystearic acid was demonstrated in those faecal samples listed in Table XXIX. In such a case, if hydroxystearic acid does appear in the post-castor oil faeces it can be assumed to be derived from ricinoleic acid and its amount taken into account in calculating the unabsorbed ricinoleic acid.

SECTION 2

Fatty acid analysis of human intestinal succeal lipids after the ingestion of castor oil.

The evidence of the previous section for the absorption of castor oil in man, though suggestive, is not absolute. It depends on the assumption that what is not excreted is absorbed. It is indirect. It seemed better to show by direct means, if possible, that ricinoleic was actually absorbed and simply just not excreted. The demonstration of plasma radioactivity after the ingestion of the I^{131}-labelled castor oil would also have been indirect unless it could have been shown that the circulating radioactivity was actually bound to ricinoleic acid.

To/

To show this was simply to demonstrate ricinoleic acid in the plasma fatty acids, but this was attempted and failed because the concentration of ricinoleic acid in the plasma was never great enough at any one moment of time.

The following direct approach was tried, and although it also failed in its objective the attempt is described because it introduces a technique which the author has used successfully in other fields of research into alimentary disease (Hazan and Watson, 1963).

Methods

Two patients under investigation for the malabsorption syndrome were studied. As part of their routine clinical investigation a Crosby capsule was passed into the duodenum by the peroral route, for biopsy of the intestinal epithelium. One hour before the capsule was fired each patient was given a 5 g dose of castor oil. After the capsule had been fired the piece of tissue, measuring about 8 mm in diameter, was divided by a sharp razor blade and one half fixed in 10 per cent formalin, for routine histology. The other half was homogenised in 1 ml water and extracted in 24 ml chloroform:methanol (2:1). The small amount of succus entericus which was sucked into the capsule at the time of firing was also extracted and the lipids of both tissue and juice were esterified and analysed on both the silicone gum and ethylene glycol adipate columns.

Results

The amount of lipid from both sources was very small, but as

Figure/

Figure 10 shows, there was enough to give a fatty acid pattern by GLC, although virtually the entire sample had to be used to obtain this. However, no ricinoleic acid was present in any of the samples and no conclusions can be drawn about the absorption of the fatty acid.

The juice sucked into the capsule at the time of firing is a sample of the intestinal contents in direct contact with the fragment of intestinal mucous membrane and is therefore most likely to contain the mixture of fatty acids presented to it for absorption. Since there was no ricinoleic acid in the sample of juice, it is not surprising that there was none in the tissue.

It is possible that if this technique had been explored further experimental conditions, more likely to give a positive result, would have been defined. But since absolute proof of ricinoleic acid absorption was to be obtained otherwise, this more detailed exploration was not carried through. The initial attempt was worthwhile, nevertheless, and showed that tissue obtained by the Crosby capsule method was large enough for a fatty acid analysis of the lipids of the intestinal villi. There are other areas of gastrointestinal research within which this knowledge can usefully be applied.

SECTION 3

Demonstration of ricinoleic acid in human chyle

In the normal course of events it is not possible to recover human chyle under experimental conditions. The opportunity to do so presented/

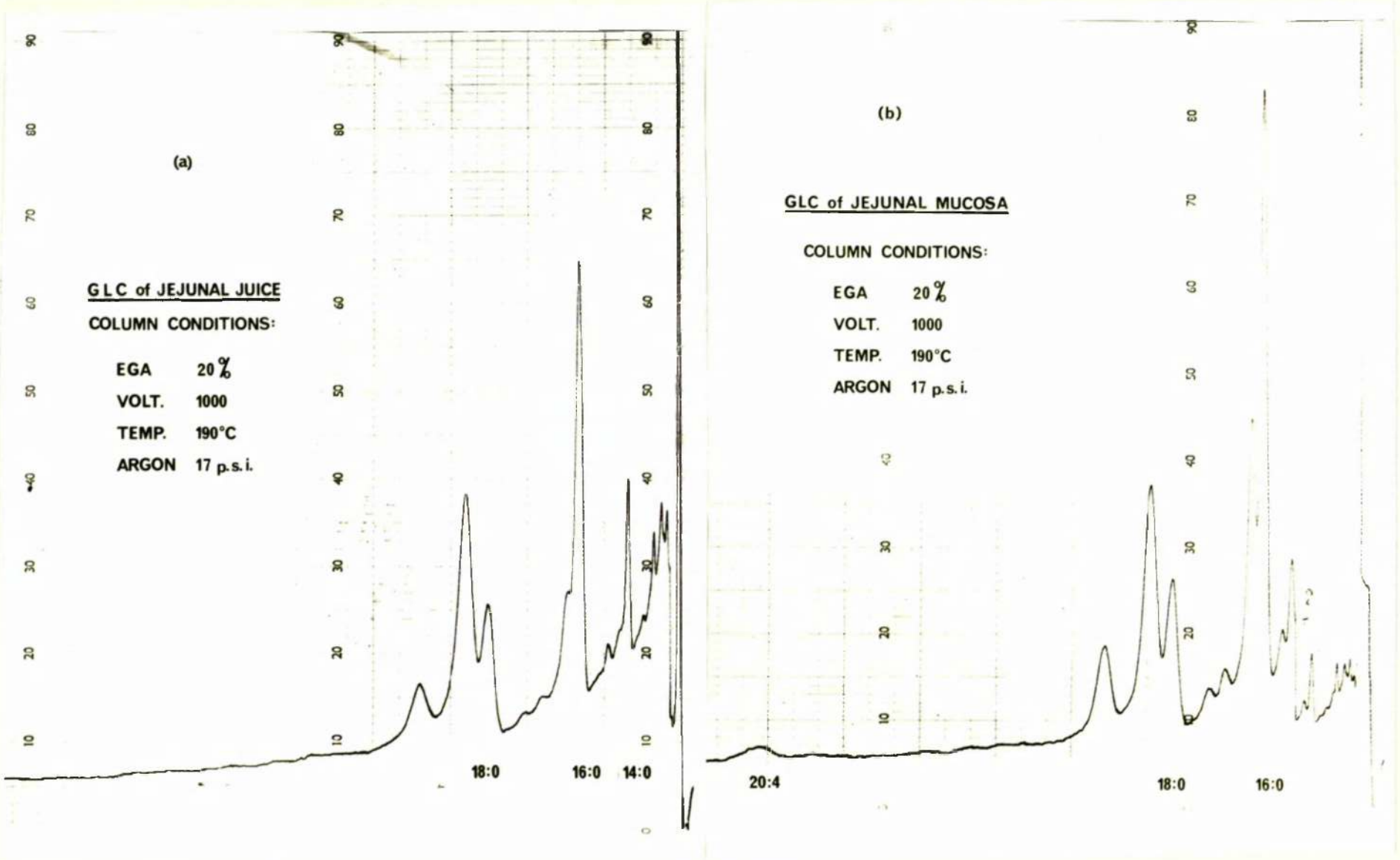


Figure 10. Fatty acid patterns of the lipids of jejunal juice and jejunal mucosa obtained at intestinal biopsy using the Crosby capsule. The patterns are grossly similar apart from the presence of arachidonic acid in the mucosal lipids.

presented quite unexpectedly, and was used in the manner about to be described to prove beyond doubt the absorption of ricinoleic acid by the human subject.

Methods

Two male patients were referred to the writer for the investigation of chylothorax. In both cases this was shown to be due to lymphosarcoma involving lymph glands in general and the mesenteric lymphatic system in particular. In the first patient (J.W.) the chylous effusion involved the right side of the chest, while in the other (J.D.) it was bilateral, though greater on the right side. Ultimately both patients had surgical ligation of the thoracic duct as a palliative procedure, and the diagnosis was confirmed both by inspection and biopsy at operation, carried out by Mr. J.D. Thomson.

During the preoperative management of the patients, pleural paracentesis had to be carried out frequently for the relief of dyspnoea. With the cooperation of the patients and without any extra discomfort to them, the experimental investigation was carried out as follows.

The pleural cavity was drained of chylous material as completely as possible and this sample was kept for later analysis. Thereafter, each patient took 5 g of castor oil three times a day, immediately after meals, for four days. This dose of oil caused neither diarrhoea nor nausea, and indeed the patients did not particularly object to it. After the 5 day period further paracentesis was carried out and this sample and the one obtained previously were analysed simultaneously.

200/

200 ml of each sample of pleural fluid was spun at 20,000 x g for 1 hour in a Spinco Model L ultracentrifuge. The chylomicron layer was recovered, extracted with chloroform:methanol (2:1) and the fatty acid methyl esters prepared and analysed on the silicone gum column.

Results

The results were similar in both subjects and are illustrated in Figure 11, which shows the fatty acid patterns of the chyle obtained from patient J.D. before and after the ingestion of the small doses of castor oil. The GLC tracings show that whereas there was no ricinoleic acid present in the pre-castor oil chyle it is clearly present in the subsequent sample. This proves beyond dispute that ricinoleic acid can be absorbed by the human intestine.

GENERAL DISCUSSION

The work of this chapter has shown that in man, as in the rat, castor oil can be digested and absorbed. With small doses of the oil absorption is virtually complete. But even with purgative doses a certain amount of absorption still occurs. The apparently normal absorption of the small doses of the oil was followed up in a clinical investigation which will be described in the next chapter.

The techniques which have been described and tested in this chapter, taken together with the biological uniqueness of ricinoleic acid, as far as its absence from normal animal tissue is concerned, offer/

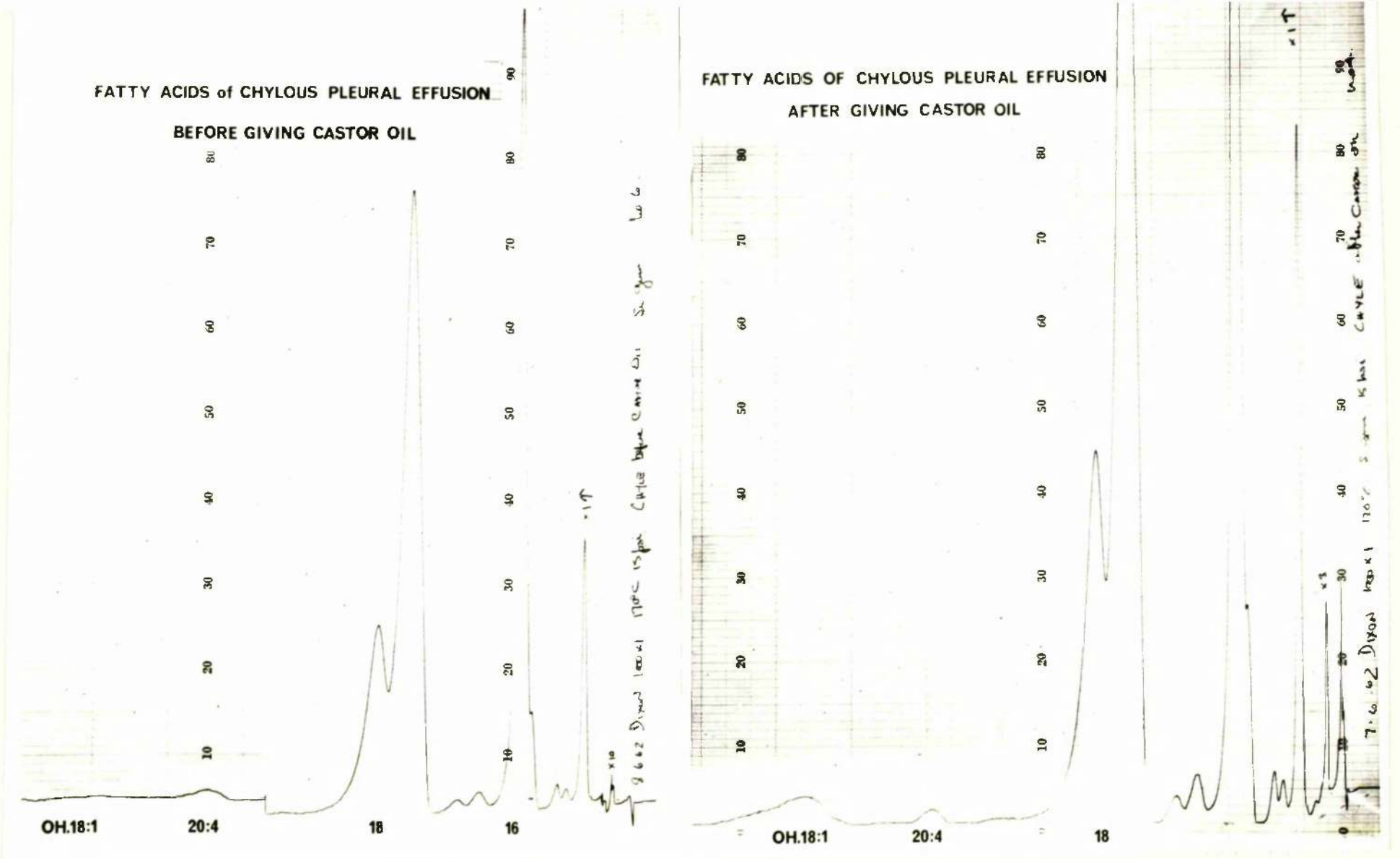


Figure 11. Fatty acid patterns of chyle obtained before and after the administration of small doses of castor oil to a patient with a chylous pleural effusion. Ricinoleic acid appears after the giving of castor oil.

offer some new approaches to certain aspects of lipid metabolism. The easy recognition of ricinoleic acid by GLC suggests uses for it as a nonradioactive tracer material in studies on lipid metabolism in humans. In this respect, chapter 8 will give an account of the use of castor oil to demonstrate the role of intestinal bacteria in the hydrogenation of dietary fatty acids. Finally, tissue obtained by the Crosby capsule has been used for autoradiographic studies of iron absorption and the absorption of I^{131}-labelled fats in humans.

CHAPTER 7

CASTOR OIL IN THE DETECTION OF LATENT STEATORRHOEA

In the diagnosis of the malabsorption syndrome, steatorrhoea or the excessive excretion of fat in the stools, is an important feature. However, it is not an invariable finding and at an early stage in the writer's interest in this group of diseases, he encountered three patients who, by certain clinical and laboratory criteria, suffered from adult coeliac disease, and yet none of whom had steatorrhoea. In each case the diagnosis was confirmed by biopsy evidence of subtotal atrophy of the jejunal mucosa in patients whose clinical presentation was a megaloblastic anaemia with free acid in the gastric juice, impaired xylose absorption and a haematological response to folic acid.

It seemed possible that these patients might have latent steatorrhoea in the sense that they would either develop frank steatorrhoea as their condition advanced, or that their present mechanism for fat absorption, although normal, was barely coping with the demands being made upon it and might fail if the normal demands were increased or altered in some way. From these speculations arose the idea of a provocative test to unmask potential or latent steatorrhoea.

It had been shown by this time that small doses of castor oil were absorbed completely, or at least as well as other dietary oils, and/

and yet at the same time it was obvious that the absorption of the oil challenged the efficiency of the absorbing mechanism in a way that the others did not. For with increasing doses of the oil its absorption deteriorated until the percentage recovery of the oil from the faeces represented castor oil steatorrhoea.

Therefore, the idea that a small dose of castor oil might be useful as a provocative test for latent steatorrhoea seemed worth investigating.

Methods

I^{131}-labelled castor oil was prepared as previously described. It was administered to patients in the manner explained in the preceding chapter, so that approximately 4 g was given to each. It was given at 11a.m.,approximately 3 hours after a normal breakfast. This was an important precaution, because even the small dose had been noted to cause diarrhoea on one occasion, when it was given to a fasting subject.

A 4 day faecal collection was made and the faecal fat and radioactivity were calculated as described in Chapter 6.

78 patients were studied and comprise 3 groups. Group 1, the normal or control group, consisted of 54 patients under investigation for peptic ulcer, chronic bronchitis, rheumatoid arthritis, cerebrovascular disease and rheumatic heart disease. Group 2 was made up of 12 patients suffering from various forms of the malabsorption syndrome. The diagnosis for each patient is given in Table XXXI. Group 3, the group of special interest to the purpose of this chapter, also consisted of 12 patients and is called the "latent steatorrhoea/

steatorrhoea group". None of the patients in it had steatorrhoea as determined by the chemical method of fatty acid titration, but all of them had definite malabsorption of the small dose of castor oil.

It is necessary to give more than a bare diagnosis for a full and relevant assessment of these patients against their fat balance results. These can only be interpreted in the light of the complete clinical presentation, as far as this is known. Therefore, a short summary of the clinical details of each patient will now be given. The numbers and initials of the patients correspond to those in Tables XXXII and XXXIII.

Clinical Protocols

No. 1 S.M. Aet 60, Female

Admitted for the investigation of anaemia. Haemoglobin 52 per cent, M.C.H.C. 22 with a blood film showing the hypochromic microcytosis characteristic of a severe iron deficiency anaemia. Barium meal showed a large reducible hiatus hernia and barium enema showed diverticulitis of the distal descending colon. Occult blood was detected in the faeces on three occasions and the anaemia was regarded as being due to chronic haemorrhage from the lesions in the alimentary tract.

Free hydrochloric acid was found in the gastric juice after the full body dose of histamine, a 25 g D-xylose absorption test gave a 5 hour excretion of 3.1 g in 430 ml of urine and this represents

definite/

definite malabsorption of xylose, in the presence of a normal blood urea (32 mg per cent) and a normal creatinine clearance.

Serum calcium, phosphorus and alkaline phosphatase were normal, and there was no radiological evidence of osteoporosis or osteomalacia. The anaemia responded rapidly to oral iron.

Final diagnosis: Severe iron deficiency anaemia secondary to alimentary blood loss from hiatus hernia and diverticulosis.

No. 2 H.M. Aet.66, Female

Admitted for investigation of possible gastric neoplasm. There was a history of gradual weight loss, poor appetite, dyspepsia and slight dysphagia. Haemoglobin 45 per cent, M.C.H.C. 23 with a blood film of severe iron deficiency. Radiology of the alimentary tract revealed no lesion. The 25 g xylose test gave a 5 hour excretion of 5.4 g in 550 ml of urine. This is normal. A glucose tolerance curve showed a mild diabetic pattern. There was electrocardiographic evidence of myocardial ischaemia.

Tests for occult blood in the faeces were negative on 3 occasions. Detailed dietary history suggested a significant lack of iron containing foods. The anaemia responded rapidly to oral iron.

Final diagnosis: Severe iron deficiency anaemia, probably due to poor diet.

No. 3 J.W. Aet. 59. Female

Admitted for investigation of anaemia. Haemoglobin 40 per cent.

Full/

Full haematological investigation showed the presence of both pernicious anaemia and iron deficiency. Evidence for the former was a megaloblastic marrow, malabsorption of Cobalt^{58}-labelled vitamin B_{12} (the Schilling test) corrected by the administration of oral intrinsic factor, a histamine fast achlorhydria, a reticulocytosis of 13 per cent after the administration of vitamin B_{12} (cytamen), and a serum vitamin B_{12} level of 55 µµg/ml.

The iron deficiency state was suggested by the appearance of the peripheral blood film, by a M.C.H.C. of 27 and serum iron level of 40 µg/100 ml, the iron saturation being 12 per cent of the total iron binding capacity of the serum. These iron values are particularly indicative of an iron deficiency state in the presence of pernicious anaemia, where, because of non-utilisation of iron, the serum iron levels are high and the percentage saturation is often greatly elevated.

A 25 g xylose test gave a 5 hour excretion of 1.7 g in 300 ml urine. This result is quite abnormal. In addition to the signs and symptoms directly attributable to the anaemia the patient complained of paraesthesia and there were objective signs of peripheral neuritis which cleared quickly after the administration of cytamen. There were no long tract signs of subacute combined degeneration of the cord.

There was no evidence of blood loss from the alimentary tract and alimentary radiology was normal. The dietary history was poor. She never ate meat, eggs or pulses. This was regarded as the main reason for the iron deficiency.

Final/

Final diagnosis: Pernicious anaemia and iron deficiency with peripheral neuritis. Satisfactory response to cytamen and oral iron.

No. 4 F.H. Aet 70. Female

Admitted for the investigation of anaemia. Haemoglobin 65 per cent, M.C.H.C. 31, blood film slightly hypochromic. The sternal marrow showed normoblastic erythropoiesis and free hydrochloric acid was present in the gastric juice without histamine stimulation. The serum iron was only 7 µg/100 ml, the iron saturation being 1.7 per cent of the total iron binding capacity, a remarkably low figure.

A 25 g xylose test gave a 5 hour excretion of 3.8 g in 130 ml of urine. This could not be regarded as signifying malabsorption of xylose, since the blood urea was 56 mg per cent and the creatinine clearance was 54 ml per minute.

Radiology of the alimentary tract showed normal oesophagus, stomach and duodenum, while the follow through examination showed the pattern of "small bowel dyspepsia", often found in the malabsorption syndrome, though not pathognomonic of it. The barium enema showed diverticulosis and diverticulitis in the pelvic colon only. Tests for faecal occult blood were negative on three occasions, but once more the dietary history revealed a poor intake of iron-containing foods.

There was a good response to parenteral iron, which was given after a poor response to oral ferrous sulphate, and in view of the very low serum iron level. The haemoglobin ultimately rose to 90 per cent.

Final/

Final diagnosis: Severe iron deficiency anaemia, probably due to poor diet and intermittent bleeding from colonic diverticulae.

No. 5 J.S. Aet 40, Female

Admitted for the investigation of anaemia. Haemoglobin 51 per cent, M.C.H.C. 28, the blood film being that of severe iron deficiency anaemia. The sternal marrow showed hyperplastic, normoblastic erythropoiesis. Free acid was present in the gastric juice after histamine. Serum iron 38 µg/100 ml, with 9.4 per cent saturation of the total iron binding capacity.

Serum calcium, phosphorus and alkaline phosphatase were normal. Xylose excretion after the 25 g dose was 5 g in 480 ml urine. Radiology of the alimentary tract showed no lesion.

The iron deficiency was regarded as being due to the iron demand of 16 pregnancies, aggravated by poor diet. There was a good response to oral and parenteral iron therapy.

Final diagnosis: Severe iron deficiency anaemia.

No. 6 M.F. Aet 64, Female

Admitted for the investigation of anaemia. This was a very complex clinical problem. Haemoglobin 45 per cent, M.C.H.C. 26, the blood film being that of a typical iron deficiency anaemia.

The clinical findings in this patient were extremely important. There were symptoms of glossitis, paraesthesia chiefly involving the hands, intermittent diarrhoea with stools that were sometimes pale,

and/

and weight loss of 7 pounds. The chief signs were pallor, koilonychia, a smooth red tongue, angular stomatitis, thickening of the skin of the hands with associated dermatitis, enlargement of the spleen and liver, tender calves but normal vibration sense and light touch.

The initial clinical impression was of a woman with malabsorption syndrome and multiple vitamin deficiencies. At first she was given nicotinic acid only and there was some response to this in that her tongue became less painful and the hands became softer and pinker.

By this time a number of investigations had been completed and there was no evidence of steatorrhoea. Tests for occult blood in the stools were negative on three occasions. Radiology of the alimentary tract showed no lesion. Xylose excretion was 7.9 g in 730 ml of urine. There was a histamine fast achlorhydria but the serum vitamin B_{12} level was 600 $\mu\mu$g/ml and the sternal marrow showed normoblastic erythropoiesis. Liver function tests, serum calcium, phosphorus and alkaline phosphatase were normal and the bromsulphalein clearance test was also normal.

By now a Crosby capsule had become available. Jejunal biopsy was performed on this patient and showed normal mucosa with tall columnar surface epithelium with well developed brush borders and no undue cellular infiltration in the lamina propria.

There was, however, some evidence of impaired absorption of vitamin B_{12} by the Schilling test. The 24 hour urine excretion of orally administered Co^{58} - vitamin B_{12} was 8.5 per cent of the dose (normal/

(normal > 12 per cent). This degree of malabsorption of vitamin B_{12} is not as severe as that seen in patients with pernicious anaemia, where the excretion is usually less than 5 per cent. The excretion rose to 12 per cent when the test was repeated with the administration of 30 mg of intrinsic factor. This is in keeping with some degree of intrinsic factor deficiency, although it is just as likely that the difference in readings is within the range of experimental error and spontaneous variation of response.

After the course of nicotinic acid further treatment consisted of oral iron and a parenteral multivite preparation. The haemoglobin rose satisfactorily, the liver and spleen reduced in size and the paraesthesia disappeared.

Final diagnosis: Iron deficiency anaemia with multiple vitamin deficiencies, not apparently due to inadequate diet.

No. 7 I.R. Aet 52. Female

Admitted for the investigation of anaemia. Haemoglobin 43 per cent, M.C.H.C. 24, blood film that of severe iron deficiency anaemia. Serum iron 35 µg/100 ml with 5 per cent saturation of the total iron binding capacity. The reason for the iron deficiency in this case was obvious. There had been a partial gastrectomy for a bleeding duodenal ulcer in June 1962, and during the present investigation the stools were constantly and grossly positive for occult blood.

Sternal marrow showed normoblastic erythropoiesis and the serum vitamin/

vitamin B_{12} level was 155 µµg/ml. Barium meal showed the Polya gastrectomy without evidence of stomal ulceration. Liver function tests were normal. Xylose excretion was 4 g in a urine volume of 920 ml.

There was a good response to oral and parenteral iron. The stools gradually became negative for blood and to date the management of this patient has remained medical.

Final diagnosis: Severe postgastrectomy iron deficiency anaemia.

No. 8 M.McB. Act 66. Female

This is another complex case. The patient was first admitted and investigated for anaemia in 1957. Her haemoglobin then was 32 per cent and the peripheral film showed predominantly macrocytic red cells. The sternal marrow showed frankly megaloblastic erythropoiesis and there was a histamine fast achlorhydria. However the response to cytamen was incomplete, the reticulocytosis being no greater than 10 per cent and the haemoglobin rising no further than 65 per cent. When folic acid was given there was no further rise in the haemoglobin. There was no evidence of iron deficiency.

Thyrotoxicosis was suspected and proved by radioiodine studies. She was treated with radioactive iodine and ultimately became euthyroid.

Fat balance studies done at this time were normal, duplicate tests showing 95 and 96 per cent absorption of fat. But because the haematological picture was unusual a gluten free diet was started in case/

case she might have a mild or early form of gluten enteropathy. Peroral jejunal biopsy was not available at this time. Cytamen and folic acid were also continued and she was maintained on this regime until she was reinvestigated in 1962.

On this occasion the haemoglobin was 103 per cent, there was no history of diarrhoea and the xylose absorption was probably normal. The urine excretion of xylose was 4.5 g in 860 ml of urine, a borderline figure, but the blood urea was 45 mg/100 ml and the creatinine clearance was only 35 ml/minute.

Jejunal biopsy showed a normal mucosa with well developed villi and tall columnar surface epithelium with normal brush border. There was no undue cellularity of the lamina propria. In the light of the completely normal histology the gluten free diet was discontinued. There was no subsequent deterioration in the alimentary or haematological condition.

However at this time she developed painful lumps on both shins which disappeared and later recurred intermittently on both legs and arms. There was an associated arthralgia affecting the ankles, knees and fingers and the E.S.R. remained moderately elevated at levels between 20 and 40 mm in 1 hour. The haemoglobin remained satisfactory around the 100 per cent level.

The skin lesion was diagnosed both clinically and histologically as an allergic vasculitis. No treatment was given for this initially, but later, because of disabling arthralgia steroids were prescribed and/

and the arthralgia then rapidly improved. This patient is still on steroids at the time of writing and remains in reasonable health.

Final diagnosis: Atypical megaloblastic anaemia, probably pernicious anaemia, thyrotoxicosis and allergic vasculitis. Possible multiple autoimmune disease.

No. 9 J.J. Aet 23, Female

Admitted for the investigation of anaemia. Haemoglobin 43 per cent, M.C.H.C. 20, blood film that of severe iron deficiency anaemia. Serum iron 20 µg/100 ml with 2.9 per cent saturation of the total iron binding capacity. The reason for the anaemia seemed to be severe menorrhagia and there was a good haematological response to oral iron.

There was a history of intermittent diarrhoea, but xylose excretion was 9.1 g in 840 ml of urine and jejunal biopsy showed normal histology.

Final diagnosis: Severe iron deficiency anaemia caused by menorrhagia.

No. 10 B.D. Aet 63. Male

Admitted for the investigation of anaemia. Haemoglobin 49 per cent, M.C.H.C. 26, blood film that of moderate iron deficiency anaemia. Serum iron 75 µg/100 ml with 15 per cent saturation of the total iron binding capacity. Sternal marrow showed normoblastic erythropoiesis. There was a histamine fast achlorhydria. Xylose excretion was 4.2 g in 1060 ml of urine, but the blood urea was 40 mg per cent and the urea/

urea clearance was 56 per cent of normal.

There were moderately severe haemorrhoids for which the patient refused surgical treatment, but in addition there was an interesting history of an emergency laparotomy in 1960 when a strangulated loop of jejunum was discovered, but since it was unobstructed it was left in situ. His postoperative recovery at this time was uneventful and later radiology of the alimentary tract was normal, as were liver function tests.

There was a good response to oral iron, the haemoglobin rising to 103 per cent.

Final diagnosis: Iron deficiency anaemia due to bleeding from haemorrhoids.

In these 10 cases anaemia was the central clinical problem. In the remaining 2 cases the clinical presentation was different.

No. 11 A.H. Aet 59. Male

Admitted with acute myocardial infarction, proved by the standard clinical criteria. Haemoglobin 93 per cent, normal blood film. The castor oil test was carried out during the 3rd week of the illness. By this time the patient was being allowed out of bed. There was no history suggesting malabsorption or diarrhoea. He was not given anticoagulants because of past symptoms indicating peptic ulcer dyspepsia.

Final/

Final diagnosis: Uncomplicated myocardial infarction.

No. 12 T.S. Aet 65, Female

Admitted with acute myocardial infarction, proved by the standard clinical criteria. Haemoglobin 84 per cent, blood film normal. The castor oil test was carried out during the 3rd week of the illness. There was no history suggesting malabsorption or diarrhoea. She was not given anticoagulants because of a peptic ulcer history.

Final diagnosis: Uncomplicated myocardial infarction.

Results

The results of the faecal fat analysis and the I^{131}-castor oil balance studies for the three groups are summarised in Table XXX.

The average daily faecal fatty acid excretion of 9.6 mEq for the normal group of 54, with a range whose upper level is 21.3 mEq, corresponds closely to the figures of a larger series of 128 normals, of which the present group was a part (Watson and Dickson, 1964).

The percentage recovery of the I^{131}-castor oil, namely 2.2 $\pm$ 2.4 per cent of the dose, compares closely with the figures for the I^{131}-triolein balance studies of Grossman and Jordan (1958), 2.7 $\pm$ 1.2 per cent of the dose, Pimparkar, Tulsky, Kaiser and Bockus (1960), 3.0 $\pm$ 1.9 per cent of the dose, and Cox (1961), 2.3 $\pm$ 1.3 per cent of the dose.

In/

Table XXX Summarised data for 78 patients studied by the small dose I^{131}-castor oil test. The mean, standard deviation and range for the daily faecal fatty acid (FFA) excretion and percentage recovery of radioactivity is given for each group.

Group	No.	FFA(mEq/day)			% Recovery I^{131}		
		Mean	S.D.	Range	Mean	S.D.	Range
1. Normal	54	9.6	4.6	3.0-21.3	2.4	2.2	0.2- 9.7
2. Malabsorption syndrome	12	34.9	19.0	17.1-75.0	6.2	4.9	0.8-13.8
3. Latent steatorrhoea	12	10.9	4.3	4.1-16.9	27.8	17.5	10.6-77.0

In Cox's 67 normal cases without steatorrhoea, the range was 0.6 - 8.7 per cent of the dose. In the present group of 54 normals without chemical steatorrhoea the range was 0.2 - 9.7 per cent of the dose. These comparisons show that in normal people without steatorrhoea the small dose castor oil test gave virtually identical results with 3 other series which used the radioactive triolein technique. Further, the results extend the experience of Chapter 6 in showing that in small doses castor oil is utilised like more normal dietary oils and that its efficient absorption can be expected in the normal subject. The important corollary to this, from the point of view of this study, is that significant deviations from the normal range of values must indicate some significant abnormality in either the digestion or absorption of the small dose of castor oil.

It was also important to assess the small dose castor oil balance test in patients with frank steatorrhoea. The summarised results for this group in Table XXX, are given in detail in Table XXXI, where the clinical diagnoses of the 12 patients are also listed.

If we regard a recovery of 5 per cent of the dose as the upper limit of normal, as did Cox (1961), then only 5 of the 12 patients with chemical steatorrhoea has "isotopic" steatorrhoea. This again almost exactly repeats the findings of Cox for radioactive triolein, for although the range of recovery results for his 33 patients was greater than for this smaller group of 12, only 15 of his subjects had recoveries which were in excess of the 5 per cent upper limit, and 18 had/

TAble XXXI Results of small dose I^{131}-castor oil test in 12 patients with the malabsorption syndrome and definite steatorrhoea, that is faecal fatty acid excretion > 17 mEq/day.(Group 2).

Diagnosis	Faecal fatty acid (mEq/day)	% Recovery I^{131}
Adult coeliac disease	17.1	4.2
"	20.5	6.8
"	22.0	3.6
"	59.6	10.4
"	75.0	13.6
Chronic pancreatitis	18.3	13.8
Carcinoma of pancreas	56.3	11.8
Carcinoma of duodenum	39.5	2.8
Mesenteric lymphosarcoma	27.4	0.8
Postgastrectomy diarrhoea	26.2	1.6
Mesenteric tuberculosis	21.3	3.9
Addison's disease	35.5	1.3
Mean ± S.D.	34.9 ± 19.0	6.2 ± 4.9
Range	17.1 - 75.0	0.8 - 13.8

had less than this. The results of the small dose castor oil test for this group show, therefore, that in the presence of steatorrhoea the excretion of this small amount of castor oil does not rise to an exaggerated degree when compared with a test using its nearest equivalent, triolein, and it seems to reflect the same incidence of "isotopic" steatorrhoea as the radioactive triolein test.

If we consider again the 54 normals, the excretion of the small dose of castor oil was greater than 5 per cent in 5 of them. This shows that whereas the test may err considerably in giving a 60 per cent incidence of false negative results in the presence of chemically demonstrated steatorrhoea, it only gives 9 per cent false positives if we regard 5 per cent as the upper limit of normal, and none at all if we take 10 per cent as the upper limit. These figures make it clear that the 4 g radioactive castor oil test is not biased towards giving abnormally high results. The 9 per cent incidence of false positives is exactly that found by Cox in his 67 normals if he took 4 per cent excretion as the upper limit of normal.

In other words, the small dose castor oil test, as far as the results of the first two groups are concerned, gives representative normal and abnormal recovery results when compared with the radioactive triolein test.

In the light of this information about the test we turn to the data for the third group with interest and with confidence that whatever the test shows it is reasonable to regard the results as being valid, whatever/

whatever their significance may be.

Table XXXII gives the daily faecal fatty acid excretion and the percentage castor oil recovery of the 12 patients who did not have chemical steatorrhoea but who, on the basis of castor oil recovery of more than 10 per cent, had isotopic steatorrhoea. The clinical details of these patients have already been presented. Nos. 1 - 10 were under investigation primarily because of anaemia. Nine of the ten were anaemic at the time of the test, and the average haemoglobin level for this group was 48 per cent. In these 9, iron deficiency of moderate to severe degree was present. Five of the ten had a urinary xylose excretion of 4.2 g or less, which is definitely abnormal, although in two of the cases the results have to be interpreted in the light of some impairment of renal function. Two of the ten had pernicious anaemia. One had features suggesting multiple vitamin deficiency, and her response to nicotinic acid suggested that there was an element of pellagra in the clinical syndrome.

Cases 11 and 12 were in their third week following a myocardial infarction. Neither was anaemic and neither complained of diarrhoea. Neither was on anticoagulants, so that their isotopic steatorrhoea cannot be attributed to phenindione (dindevan) which occasionally causes diarrhoea and steatorrhoea.

Discussion/

Table XXXII Results of the small dose I^{131}-castor oil test in 12 patients with definite malabsorption of the labelled oil and normal faecal fat excretion by chemical analysis (Latent steatorrhoea, Group 3). The test was repeated in Case 1.

Case	Faecal fatty acid (mEq/day)	% Recovery I^{131}
1 S.M.	8.4	25.3
"	7.2	51.3
2 H.M.	14.0	21.9
3 J.W.	4.6	77.0
4 F.M.	14.7	36.8
5 J.S.	4.9	12.6
6 M.F.	11.5	25.0
7 I.R.	16.9	18.6
8 M.McB.	10.2	22.7
9 J.J.	10.9	10.6
10 B.D.	11.9	40.1
11 A.H.	11.5	11.9
12 T.S.	4.1	23.1
Mean ± S.D.	10.9 ± 4.3	31.4 ± 17.5
Range	4.1 - 16.9	10.6 - 77.0

Discussion

Steatorrhoea is not always found in the malabsorption syndrome. The author now has records of 4 patients with severe villous atrophy, malabsorption of xylose, megaloblastic anaemia responsive to folic acid and associated with free acid in the gastric juice, and yet without steatorrhoea at the time of presentation. These people certainly have deranged function of the small bowel, which is either just coping with fat absorption or has selective enzyme deficiencies not involving those concerned with fat absorption.

Provocative tests are not uncommon in medical diagnosis. The Master "two-step test" was introduced to provoke recordable ischaemia in the myocardium. Trousseau's sign is the demonstration of latent tetany by occlusion of the blood supply to a limb. Hess's test reveals the purpuric tendency associated with thrombocytopenia. Frequent blinking of the eyelids may expose the barely concealed fatigusability of the neurochemical mechanism which is at fault in myasthenia gravis. The current widespread interest in prediabetes has been stimulated to some extent by the development of tests based on the deterioration in glucose tolerance which may follow the administration of steroids.

Such tests are eminently reasonable, for while many diseases involve a sudden change from the completely normal to the frankly abnormal, there are many others which come on gradually. If a disease process involves a number of functions, some may be affected earlier

than/

than others.

The novel approach to steatorrhoea which has been explored in this study is quite different from that of Parker and Ross (1960), who were concerned not with the problem of latent steatorrhoea but with that of asymptomatic steatorrhoea. They followed up the suggestion of Cooke, Elkes, Fraser, Parkes, Peeney, Sammons and Thomas (1946) that minor and potentially important anomalies of fat absorption were more common than was generally supposed. Using the serum carotene level as a screening test, they investigated 230 convalescent patients who had no alimentary symptoms and found that of 11 of these with low carotene levels, 5 had definite steatorrhoea, an incidence of 2.3 per cent.

Parker and Ross claimed on the basis of their work that asymptomatic steatorrhoea was a more frequent occurrence than had been suspected. None of their 5 patients had impaired xylose absorption, if 4.2 g is taken as the lower limit of urine excretion in 5 hours. They do not give haemoglobin levels for these subjects.

The group of 12 patients described here is quite different. Nine were severely anaemic and one had an unusual anaemia. Two were recovering from a myocardial infarction. None had steatorrhoea by chemical analysis. All, however, on the basis of the I^{131}-castor oil small dose test, clearly shown in the preliminary studies not to overestimate fat loss from the bowel, had definite steatorrhoea of the isotopically labelled fat.

The/

The contention of this chapter is that the small dose castor oil test as described here is capable of unmasking a marginally competent fat absorbing mechanism. Anaemia, with its associated tissue hypoxia, interferes with cellular and enzymatic activity. Xylose absorption is impaired in pernicious anaemia (Helmer and Fouts, 1937; Bezman, Kinnear and Zamcheck, 1959; Fowler and Cooke, 1960). Iron deficiency of a chronic and severe degree has long been known to be associated with gastric atrophy (Witts, 1930) and various degrees of chronic gastritis and gastric atrophy have been demonstrated in about 74 per cent of patients with iron deficiency anaemia (Davidson and Markson, 1955). It is unlikely that achlorhydria itself is the cause of the iron deficiency, since patients with uncomplicated pernicious anaemia are seldom iron deficient, and indeed the tissues of such patients are often replete with iron in spite of the complete failure of gastric secretion and the gastric atrophy, often present for many years. And it is equally unlikely that iron deficiency is the cause of the atrophy, since iron medication does not improve the gastric mucosa, judging by serial biopsy samples, and in fact deterioration may take place (Lees and Rosenthal, 1958).

The mechanism for fat digestion and absorption is complex, and for castor oil it is normally less efficient than in the case of other dietary fats. Increasing complexity often means increasing vulnerability, and this may be why castor oil absorption deteriorates at a stage of failing function which is still adequate for other fats. It is also possible/

possible, of course, that it is some single step in the absorption pathway, peculiar to castor oil, which fails, and the test is detecting a precise defect in fat absorption of no importance to other fats. It is difficult to see what this might be. The only point at which it has been shown that ricinoleic acid differs fundamentally from other fatty acids in the absorption route, is in not participating with intestinal phospholipid. On the other hand, no suggestion has been offered regarding the special difficulties which the OH group in the ricinoleic acid molecule seems to present, and it may be in this area of activity, indeed it is likely to be in this area, that the critical deterioration in the absorption mechanism occurs.

The proposition being tested in this study has therefore some prior grounds for consideration and the results obtained give it qualified support.

The castor oil steatorrhoea found in the 2 patients after a myocardial infarction is also of interest. Steatorrhoea is frequently found in patients with the clinical picture of mesenteric ischaemia, and the author has personal experience of two such cases, one proved by lateral aortography (Mr. J.K. Watt), the other at laparotomy, and one of them cured by arterial anastomosis. Mesenteric ischaemia most often occurs in the presence of generalised arterial degenerative disease, and one of these two patients had already suffered a myocardial infarction.

In view of the findings in the two infarct patients a systematic survey of faecal fat excretion during the third week following an acute/

acute myocardial infarction has been undertaken, and so far approximately 15 per cent of the patients have been shown to have steatorrhoea by the standard chemical method of analysis.

It seems possible therefore, both on theoretical and factual grounds, that the small dose castor oil test is a valid provocative test for latent steatorrhoea. It will be of interest to see what developments in the understanding and diagnosis of malabsorption ensue from the clinical application of the procedure.

CHAPTER 8

THE INTESTINAL HYDROGENATION OF DIETARY FATTY ACIDS

In none of the many tissue lipid analyses carried out hitherto has hydroxystearic acid been present, either by itself or in conjunction with ricinoleic acid. This applies to rat intestine mucosal glycerides, rat and human chyle, and rat adipose tissue and liver lipids. Thus, whatever the metabolic fate of ricinoleic acid may be during its transport to, deposition in or mobilisation from these tissues or tissue fluids, it is not hydrogenated to hydroxystearic acid, at least not in detectable amounts. Furthermore, it is unlikely that it is hydrogenated in the lumen of the small bowel, for any hydroxystearic acid so produced would be likely to be absorbed to some extent and thus be demonstrable in the mucosal lipids.

On the other hand hydroxystearic acid has been encountered in faecal lipids. Figure 12 shows it to be present in the faecal lipids of a rat on the 20 per cent castor oil diet, although this animal had no hydroxystearic acid in its faeces before the diet was started. Figure 13 shows its presence in the faecal lipids of one of the subjects of chapter 6. This patient had no hydroxystearic acid in the control specimen of stool and none in the first sample collected while the castor oil was exerting its purgative effect. But in the sample of stool collected/

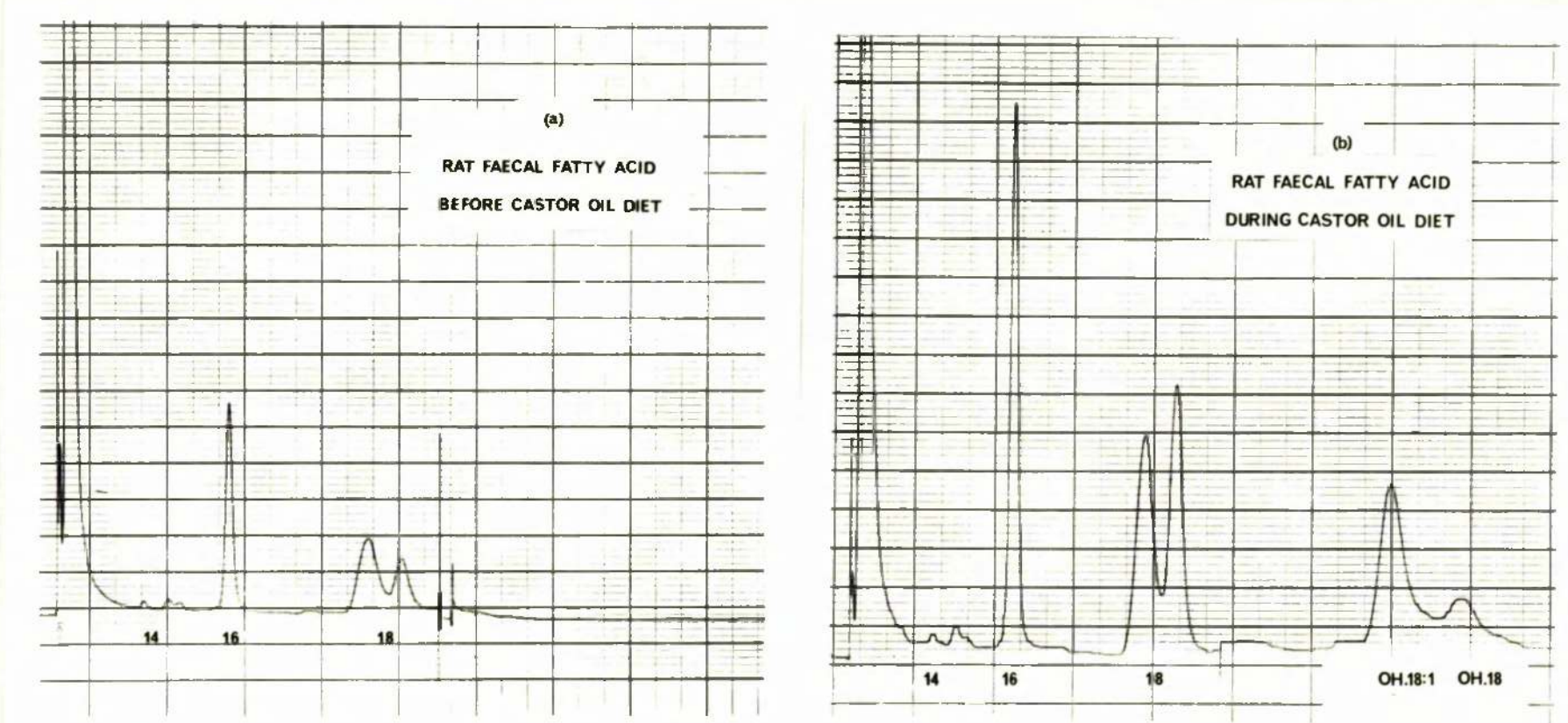
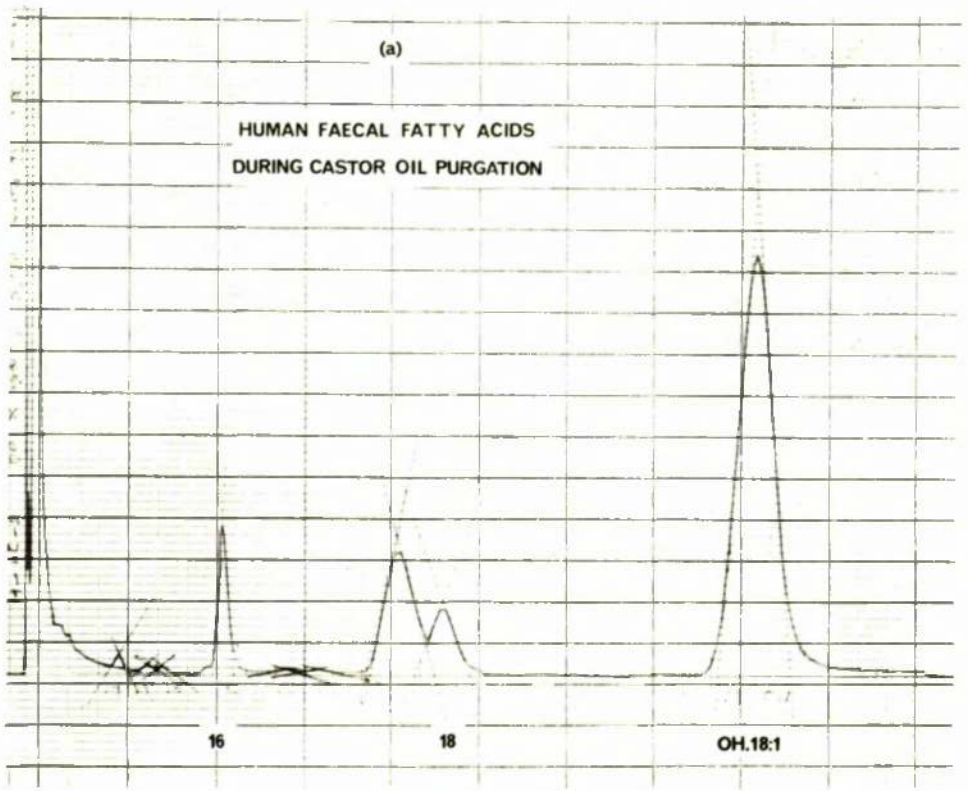


Figure 12. Fatty acid patterns of rat faecal lipids before and during the feeding of a castor oil supplemented diet. Ricinoleic and hydroxystearic acids are both present in the faecal lipid of the castor oil fed rat.



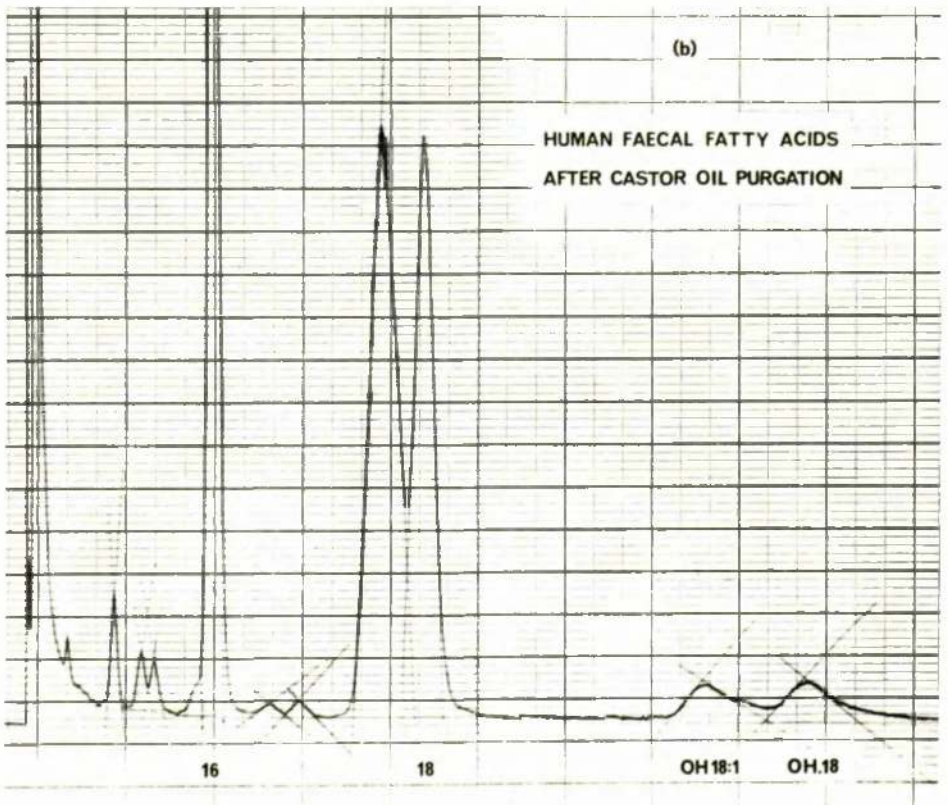


Figure 13. Fatty acid patterns of human faecal lipids from a patient during and immediately after castor oil purgation. Hydroxystearic and ricinoleic acids are present in equal concentration in the second sample.

collected after the purgative action of the castor oil had ceased the fatty acid was present to a significant degree. This sequence of analytical events was encountered, to a varying extent, in most of the patients studied.

These facts suggested that the hydroxystearic acid was derived from ricinoleic acid. Theoretically the likeliest mechanism for this chemical transformation was hydrogenation and the likeliest agents the bacteria of the large bowel. James, Webb and Kellock (1961), who described the occurrence of unusual fatty acids in the faeces of humans with normal and abnormal fat absorption, encountered varying amounts of hydroxystearic acid, and suggested that it might arise from intestinal hydrogenation. However, they did not encounter ricinoleic acid in the faecal lipids of any of the people investigated, and thus the source of the hydroxystearic acid was uncertain.

Fernandes, van de Kamer and Weijers (1962) also suggested the possibility of the intestinal hydrogenation of fatty acids when they noticed the presence of behenic acid (22:0) in the faecal lipids of a patient with steatorrhoea who had been fed experimentally with erucic acid (22:1).

The experiments of this chapter were designed to corroborate that hydrogenation of fatty acids did occur in the gut and that the mechanism was likely to involve bacterial activity in the large bowel.

Methods

Two female patients agreed to take regulary small doses of castor oil/

oil for a number of days. The dose, which was not critical, was about 4 or 5 g three times a day after meals. Three stool collections were made in each case. The first was a 2-day collection immediately before the administration of castor oil begun, and while the patient was on a normal diet. The castor oil was then started and after the patient had been taking it for two days a 4-day collection was made. The delay period gave time for ricinoleic acid to appear in the stools. On completion of this collection neomycin was given orally in a dose of 1 g four times a day. After 24 hours of this a further 2-day collection of faeces was begun. Castor oil was given continuously until the completion of this third stool collection.

Thus, there were three stool samples for analysis. One collected while the patient was on normal diet, one while on diet and castor oil and a third while on diet, castor oil and neomycin. The faecal lipids were extracted as before and the fatty acid methyl esters prepared. These were analysed on both the silicone gum and ethylene glycol adipate columns.

Results

Neither of the patients had diarrhoea while on castor oil alone. Patient M.N., however, developed diarrhoea while taking neomycin. This result of neomycin was anticipated.

The faecal fatty acid analyses are recorded in Table XXXIII. The figures combine the data from the two GLC columns. The results from the two studies are rather different, probably due to the fact that/

Table XXXIII Percentage fatty acid composition of normal human faeces (A), during the administration of non-purgative doses of castor oil (B) and during the administration of castor oil and neomycin (C). The results combine data from GLC on silicone gum and ethylene glycol adipate columns.

Fatty Acid	Patient M.M.			Patient L.M.		
	A	B	C	A	B	C
14	2.2	1.7	1.2	2.5	3.9	4.4
16	33.4	26.4	22.9	37.9	29.3	29.0
18	45.6	39.4	22.9	36.4	41.9	24.3
18:1	5.9	12.3	14.2	17.6	8.8	28.0
18:2	8.6	2.8	5.1	3.8	1.3	7.0
20:	4.3	-	-	1.8	-	-
18:OH	-	9.5	-	-	9.6	1.7
18:1:OH	-	7.9	40.5	-	5.2	5.6

that one of the patients had diarrhoea and the other did not. They are therefore considered separately.

In case M.M., hydroxystearic acid appears in the stools (9.5 per cent) following the administration of castor oil, but when neomycin is added to the regime it disappears again. At the same time the relative amounts of stearic and oleic acids are altered considerably, the oleic:stearic ratio rising from 0.13 before castor oil administration, to 0.31 before neomycin and finally to 0.62 after neomycin. The large fraction of ricinoleic acid in the post-neomycin stool depresses the percentages of the other fatty acid fractions, but does not conceal the change in the balance of the non-hydroxy fatty acids with respect to one another.

In case L.M., hydroxystearic acid again appears in the stools following the administration of castor oil. On this occasion it does not disappear when neomycin is given, but its percentage concentration falls from 9.6 to 1.7, although the amount of ricinoleic acid remains virtually the same. Once again there is a change in the oleic:stearic ratio, more striking even than in the previous case. It rises from 0.21 before neomycin to 1.15 after it. In both subjects the other major unsaturated fatty acid, linoleic, is affected in a similar way, though to a less pronounced degree.

Discussion

It is well established that the unsaturated C-18 fatty acids are partially hydrogenated to stearic acid in the presence of a flourishing/

flourishing microflora in the rumen of sheep (Shorland, Weenink, Johns and McDonald, 1957). Partial evidence for the intestinal hydrogenation of unsaturated fatty acids in man has been presented by two groups of authors in the past three years, from studies on very small numbers of patients. Fernandes et al (1962) observed the appearance of behenic acid in the faecal lipids of their patient who had been given erucic acid. Gompertz and Sammons (1963) followed up this observation in greater detail, but in only 1 of 6 people studied (3 normal volunteers and 3 patients with steatorrhoea) was there evidence of hydrogenation of the diet-incorporated erucic acid to behenic acid.

Erucic acid (22:1) is, however, a very long chain fatty acid and its fate in the gut may be quite unrepresentative of the more normal 18 carbon fatty acids. For this reason the data obtained from the use of ricinoleic acid are worth adding to that obtained from the feeding of erucic acid.

The two experiments reported here, together with the relevant observations from earlier chapters, offer good evidence that hydrogenation of dietary fatty acids occurs in the intestinal tract. This process is undoubtedly modified by the oral administration of neomycin, and the most reasonable and obvious explanation to offer for this action by the antibiotic is that it completely or partially sterilises the gut and inhibits the hydrogenating activity of intestinal bacteria. There is no other obvious explanation for its action in producing a greater degree of unsaturation of the faecal fatty acids. Since the small bowel

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is normally free of bacterial contamination the sterilising effect of neomycin must be principally on the large bowel. The corollary to this is that it is large intestine bacteria which are responsible for the hydrogenation mechanism.

There is, of course, another reason why the ratio of oleic acid to stearic acid is much lower in faecal lipids than in dietary lipids. The recent work by Fernandes et al (1962) on children with steatorrhoea has shown that there is selective absorption of individual fatty acids, absorption decreasing with increase in chain length and being more complete for the unsaturated fatty acids. This view is partly confirmed by fatty acid analyses on the faecal discharge from an ileostomy in one patient (Sammons, 1961) which showed that even before the faeces entered the large bowel and were exposed to its bacterial flora, the oleic:stearic ratio was much lower than in the diet and was already similar to that in the faecal lipids from one normal control patient. But the ileostomy bowel cannot be considered sterile, and Sammons does not consider the possible role of small bowel bacteria in contributing to his fatty acid data.

The composition of faecal fatty acids is therefore likely to be due to a combination of these two factors, the differential absorption of fatty acids in the small bowel, and bacterial hydrogenation in the large.

It is not clear why neomycin causes diarrhoea. The explanation is simple enough in case M.N., where the concentration of ricinoleic acid in/

in the diarrhoea stool was 40 per cent. But the diarrhoea-producing effect of neomycin is frequently encountered in patients who are not receiving castor oil and at present there is considerable speculation about the possible toxic suppression of intestinal enzymes by this antibiotic. The author is currently engaged in a research programme covering the general field of the alimentary metabolism of lipids. The effect of neomycin on the fate of dietary lipids is one of the specific problems awaiting study.

CHAPTER 9

GENERAL DISCUSSION AND THEORETICAL CONSIDERATIONS ON CASTOR OIL AND RICINOLEIC ACID

Each experimental section of the thesis has been accompanied by relevant discussion and it is unnecessary to repeat points which have already been made. Here we shall summarise what we know of the biochemistry of castor oil and ricinoleic acid, indicate further fields of investigation and finally discuss some of the theoretical concepts which may underly the unique biological properties of these substances.

The experimental work has shown conclusively that ricinoleic acid, the principal fatty acid of castor oil, is absorbed by both the rat and human intestine and subsequently appears in body tissues. Since ricinoleic acid can be demonstrated in rat and human chyle it means that it follows the main pathway of absorption for long chain fatty acids through the intestinal mucous membrane into the mucosal lymphatics and through these to the thoracic duct and the bloodstream. Its presence in chyle also means that it has been exposed to the biochemical processes which esterify a fatty acid, coat it with a protein shell and so transform it into a very low density lipoprotein or chylomicron. It is also certain that this process can occur without the participation of phospholipid. This seems to be an absolute fact for ricinoleic acid/

for ricinoleic acid, and it seems to show that phospholipid is not essential for the absorption of a fatty acid, even such a 'difficult' one as ricinoleic.

In the process of digestion and absorption castor oil participates in the normal process of lipolysis, and the free ricinoleic acid can take part in the initial step of fatty acid activation, though to a lesser extent than oleic acid. The experiments with stearic and hydrostearic acids suggest that this lesser capacity for activation is common to the hydroxy fatty acids and attributable to their OH group.

Ricinoleic acid is readily deposited in adipose tissue and readily mobilised, to be oxidised and utilised as an energy source. The experiments with essential fatty acid deficient rats show that there is unlikely to be a mechanism for biological dehydration of a fatty acid. The transformation of ricinoleic acid by this process to an unconjugated Delta $^{9,10;\ 12,13}$ isomer of linoleic acid, would have corrected the clinical effects of the EFA deficient state in the rats, while the transformation to a conjugated isomer, the Delta ^{9,10,11,12} form, although it would not have improved the clinical conditions, would have been detectable by gas-liquid chromatography. Thus, while the dehydration of castor oil ricinoleic acid is an important industrial process, it has no biological counterpart.

It was fortunate in so much of this work that ricinoleic acid could be identified so easily by gas-liquid chromatography and that it is normally absent from animal tissue. Dr. R.S. Gordon attempted a number of syntheses of C^{14}-ricinoleic acid, without success. We are unaware of

any/

any other or more successful approach to the problem. The use of I^{131}-ricinoleic acid was valuable in permitting a large series of absorption studies with castor oil in humans. A parallel group of studies using the newly devised GLC technique for the quantitative recovery of ricinoleic acid in faeces showed that, with minor reservations, I^{131}-castor oil was acceptable as a tracer material.

The I^{131} label is not suitable, however, for studying the tissue metabolism of a fatty acid. Without a C^{14}-labelled ricinoleic acid it was not possible to obtain that sensitivity and flexibility of detection with small amounts of a fatty acid which were necessary for the study of its absorption through the portal venous system to the liver and its fate in that organ. This remains a possible pathway of absorption and esterification for ricinoleic acid on which we have neither information nor opinions.

The author has been growing castor oil plants and attempting to label the naturally developing oil both by watering the plants with tritiated water and by injecting C^{14}-acetate into the bean-bearing stems. As yet there are no results from this method, but one anticipates that the yield and specific activity of the labelled oil will be low.

The studies of Chapter 8 establish that fatty acid hydrogenation occurs in the rat and human intestine and that the likely agents for this transformation are large bowel bacteria. The biological significance of this is not clear and in any case is probably of no importance.

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The implications of the work of Chapter 7 have already been discussed in detail. It appears that there is something about the absorption of ricinoleic acid which is so unique as to test critically some link in the normal chain of fatty acid absorption, or a link which is peculiar to ricinoleic acid. From the nature of the patients studied it is possible that it is either anaemia as such, or iron deficiency anaemia in particular, or perhaps tissue iron deficiency which affects a critical absorptive link. Among various possibilities there may be the effect of anaemia on the potency or viability of the thiokinase fatty acid activating enzymes, or the effect of iron lack on certain cytochrome oxidases. Whatever the implications, there is in the small dose castor oil test something which, in a special clinical situation, reveals a special deficiency in the absorption of the fatty acid.

Mechanism of Purgation

When this work was begun there was the hope that from it would emerge an explanation for the purgative action of castor oil. Unfortunately this has not been realised. But the problem may not be too far from solution and it might be of value to indicate, on theoretical grounds, those areas in which the solution may be found.

In considering the possible mechanisms for the purgative action of castor oil it seems important to pay particular attention to some aspects of the physical and chemical theory of monolayers and to the problems of surface phenomena in general. Two books which are particularly valuable for the novice in this field are the monograph on the "Physical Chemistry of Surface Films" by Harkins (1952),

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a prolific writer and investigator of this subject, and the symposium on "Surface Phenomena in Chemistry and Biology", edited by Danielli, Pankhurst and Riddiford (1957). An attempt will now be made to relate what is know about ricinoleic acid to the facts and theories of monolayers and their physical and chemical effects.

There seems to be little doubt that the purgative action of castor oil depends on the presence of free ricinoleic acid in sufficient concentration within the lumen of the small bowel. That free ricinoleic acid is necessary has been known for 70 years (Meyer, 1890) and the present study has shown that purgation is associated with the minimum absorption of ricinoleic acid and the smallest concentrations of the fatty acid within the intestinal mucosa. The questions which therefore seem to require answering are, why does ricinoleic acid cease to be absorbed and how does increasing concentration of the free acid in the intestinal lumen cause purgation?

As far as cessation of absorption is concerned Kornberg and Pricer (1953) have shown, and our own results confirm, that with increasing concentrations of free fatty acid in an experimental system, fatty acid activating enzymes act less efficiently and above certain concentrations are likely to be almost completely inhibited. This fall-off in efficiency will probably occur at concentrations of ricinoleic acid which are lower than those of other fatty acids.

Again, according to Frazer, Schulman and Stewart (1944), the optimum emulsifying system for fat absorption is one comprising fatty acid, bile salts and monoglyceride. The monoglyceride contribution is/

is critical and is usually provided by the normal hydrolysis of the oil. There is no information about the production of monoricinolein during the lipolysis of castor oil, and this is another aspect of the subject which might repay study. As yet there is no evidence that castor oil can be absorbed as lipid micelles by the process of pinocytosis suggested for other fats by Palay and Karlin (1959).

There is one final theoretical consideration which might explain the poor absorption of ricinoleic acid. There are circumstances, perhaps in the presence of the phospholipid phosphate radical, in which the molecule might bend on itself, forming a bond between its OH^+ and COO^- groups. In this form it would be inaccessible to the normal processes of fatty acid activation and esterification. Or, of course, molecules of ricinoleic acid might bond to each other through the $OH^+ - COO^-$ linkage.

It seems that the purgative effect of ricinoleic acid is due to an irritant action on the surface of the intestinal membrane. The older writers freely use the word 'irritant' for the action of ricinoleic acid without defining or explaining what this irritant action is, or is due to.

It is unlikely that castor oil acts by a bulk effect, although there is evidence, not for castor oil and ricinoleic acid, that alterations in the ratio of free fatty acid and triglyceride affect the temperature and the area of the mixture. Dervichian (1958) has shown that as the ratio of myristic acid to trimyristin rises the mean molecular area per/

per chain may increase from about 22 Å to 45 Å. It is conceivable that this happens with the release of ricinoleic acid and its admixture with the castor oil.

If some kind of surface activity caused by castor oil is the likelier mechanism for purgation, how may this be mediated? One of the principal considerations in monolayer theory is the orientation of molecules at surfaces. Normal fatty acids, which have the terminal COO^- as their only polar group, take up a vertical position at aqueous or polar interfaces, with the polar group facing into the liquid and the non-polar paraffin chain projecting into the vapour phase. This arrangement allows the maximum number of molecules per surface area.

This kind or orientation was first demonstrated by Harkins, Davies and Clark (1917), but at this time they also considered the possibility that the distribution of other polar groups at suitable points along the chain of a long chain paraffin would give a monolayer in which the molecules would lie flat. To test their hypothesis they obtained a series of long-chain linear ω-hydroxydecanoic acid polymers with chain lengths from 60 to 1970 Å. They found with these polymers, where there was an OH group at every tenth carbon atom, that the molecules lay flat, giving a tightly packed monolayer, about 4.5 Å thick and with about 4.5 Å between the centres of the chains (Harkins, Carman and Ries, 1935).

The importance of the two types of orientation is that they produce monolayers with different properties. For example, with the long axis of the chain vertical, the molecular area is independent of/

of the length of the chain, whereas with the long axis horizontal the molecular area increases with the chain length, or conversely fewer molecules take up more space at the surface. The surfaces also show different responses to pressures, the vertical monolayer withstanding high pressures and the horizontal one collapsing at low pressures. There are also significant differences in the surface potentials and compressibilities of such monolayers.

It seems likely, therefore, that ricinoleic acid, which has an OH group at the 12 carbon position, will form a horizontal monolayer with the intestinal cell membrane, rather than a vertical one, and will cover a larger area of the intestinal surface than a similar amount of oleic acid, which forms vertical monolayers. This, however, depends on the assumption that cell membranes will participate in surface chemistry in the same way as other substances of simpler molecular construction.

Gorter and Grendel (1925) found that the membranes of red cells contained enough lipid to make a membrane about two lipid molecules thick. This led them to suggest that the membrane was simply a bimolecular leaflet of lipid molecules, the molecules being orientated perpendicular to the membrane-water interface, with their polar groups in these interfaces. Later work showed that the cell surface was not solely lipid, but consisted of lipid upon which was adsorbed a monolayer of protein. Such a structure was compatible with all other observations available at that time, and qualitatively at least could account for many permeability properties of the cell (Danielli and Harvey, 1934).

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The protein of such membranes is usually orientated so that its carbonyl group (>C = O) is facing outwards.

It seems, then, that one has to think of the molecules of free ricinoleic acid lying horizontally in the lumen of the gut, and with their polar OH groups directed towards the membrane. Since the outer surface of this membrane is protein, with free carbonyl groups facing into the lumen, the possibility of hydrogen bonding has to be considered. It is unlikely that this kind of physico-chemical relationship exists between the non-hydroxy fatty acids such as oleic, stearic and palmitic, and the intestinal membrane.

If the surfaces of protein or fatty acid monolayers are studied in media of constant pH and ionic strength, and containing varying ratios of sodium to calcium, it is found that the amount of calcium associated with the surface is greatly in excess of that which would be found with an uncharged surface (Danielli, 1944; Webb and Danielli, 1940). The excess is partly due to the electrostatic effect of the surface charge, which pulls an excess of calcium into the electrical double layer, but is mainly due to the formation of a complex with carboxyl groups and other anions. In the case of carboxyl groups the stoicheiometry is that of the reaction:

$$Ca^{++} + -COO^{-} \rightleftharpoons -COOCa^{+}$$

It is reasonable to suppose that analogous reactions occur between the anions present at the surfaces of cells and cations in the environment/

environment. When the data for cell surfaces were examined from this point of view, it was found that when the surfaces were in equilibrium with media containing a sodium to calcium ratio of 100 to 1, the ratio in the surface phase was about 1 to 1. This represents a remarkable concentration of calcium at such interfaces. In clinical steatorrhoea the unabsorbed fatty acid combines with dietary calcium which is lost and this may ultimately lead to a low serum calcium, osteomalacia and even tetany. It is possible that in a situation in which ricinoleic acid is forming an extensive monolayer with the intestinal membrane, and where dietary calcium is not present, that there may be a rapid and sizeable loss of endogenous calcium, which in turn might produce a state of "irritability" of the intestinal membrane leading to increased motility and purgation.

It should be possible to test this hypothesis by experiments in which intravenously administered isotopic calcium is sought in the faeces, following the administration of castor oil. A great excess of the isotope compared with its normal excretion under control conditions would favour the hypothesis. It is hoped to carry out such a study at some time in the future.

There are other effects, however, besides that of "intestinal tetany" (the author's phrase), which the above mechanism might produce. The remarkable effects of sodium hydroxide, sodium chloride and calcium chloride upon an interfacial film of oleic acid were discovered by Clowes (1916) and were found to be even more striking when investigated quantitatively by Harkins and Zollman (1926).

The/

The interfacial tension between an aqueous solution and a 0.001 molar solution of oleic acid in olive oil was found to be only 0.002 dyne cm^{-1} when the aqueous phase contained 0.001 molar NaOH, and 0.0015 molar NaCl. This is the lowest interfacial tension between water and an oil thus far measured. However, if this same aqueous solution was made 0.0015 molar with respect of $CaCl_2$, the interfacial tension rose by a factor of about 3,500 to 6.88 dyne cm^{-1}, an extraordinary increase.

There is considerable speculation about the effect of calcium at interfaces. Calcium ions do not seem to pack fatty acid chains more tightly. At pressures below 25.5 dyne cm^{-1} they transform the less condensed liquid phase into a more condensed solid phase. According to one view advanced by Harkins, a fatty acid-calcium complex such as calcium stearate is insoluble, and forms a solid monomolecular sheet in which two stearate ions are combined with one calcium ion. Monolayers of this type exhibit considerable rigidity and may be classed as truly solid. Ricinoleic acid may form this kind of complex with calcium, but since it is much more likely to be a horizontal monolayer than a vertical one, its physical properties may be quite different from those of a calcium stearate complex, and in any case it is impossible to speculate on what their biological effects might be.

Studies on permeation kinetics and active transport mechanisms have shown that there is a whole range of specialised processes of permeation, some requiring energy and some not, which are not to be explained in terms of a simple lipoprotein structure for the membrane. There/

There is much evidence to indicate that these specialised permeations are restricted to a small part of the cell surface area and that there are many resemblances between these specialised regions and enzymes.

Goldacre (1952) postulated the presence of contractile proteins at the cell surface which could effect the transfer of molecules. His argument was to a large degree based on analogy with the contractile protein system of muscle cells. In muscle, a complex structure exists based mainly on two proteins, actin and myosin. Myosin has enzymic activity, being able to hydrolyze an orthophosphate ion from adenosine triphosphate, with the liberation of about 12,000 calories. The actin-myosin system is able to utilise part of the energy liberated in this hydrolysis to perform mechanical work in a reversible contraction-relaxation cycle.

It is certain that there are contractile elements in or adjacent to the plasma membrane which are responsible for many of the movements of the cell surface. But evidence bearing directly on Goldacre's hypothesis is scanty. Cytochemical studies have shown that the surfaces of cells concerned in active transport frequently, possibly invariably, have associated with them a hydrolytic enzyme, alkaline phosphatase. It has been suggested (Danielli, 1952) that this enzyme is the enzymic contractile protein postulated by Goldacre. Much work remains to be done before this can be accepted. For example it needs to be shown that these phosphatases are indeed contractile, and also that they are situated in the membrane in such a manner as to be able to transport molecules by contraction.

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It is possible that such contractile enzymes, in a special orientation with ricinoleic acid, might contract horizontally rather than vertically, thus promoting peristalsis.

In conclusion, it has seemed pertinent to cast the net of hypothesis widely and in unusual biochemical waters, for it is extraordinary that a fatty acid, which differs from oleic acid by only one OH group, should differ so substantially from it in its biological behaviour. Whatever the explanation for this profound difference, it seems certain that it will be attributable to this simple OH group, which confers extra polarity upon the fatty acid molecule, at a biochemically strategic site on the straight chain.

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