pure ethyl dehydrocholate, m.p. 221–223°, and an impure but different ester, m.p. 157–166°. The infra-red spectra of these two substances in the range 1200–900 cm.$^{-1}$ showed an overall similarity but also definite differences.

The authors offer their thanks to the following, without whose generous help in collecting King penguin bile this work could not have been undertaken: Dr E. C. Appelby and the Royal Zoological Society of Scotland, and Bernard Stonehouse of the Falkland Islands Dependencies Survey and the Edward Grey Institute of Field Ornithology, Oxford.

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The Effect of Sheep-Rumen Contents on Unsaturated Fatty Acids

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(*Received* 19 *March* 1957)

Diets rich in unsaturated oils modify the depot fats of most animals to resemble the dietary fats, but ruminant depot fats are relatively unaffected (cf. Shorland, 1955). In addition, ruminant fats contain higher proportions of fully saturated glycerides than would be expected on the basis of the even or widest distribution rule of Hilditch (1956). Other peculiarities of ruminant fats include the presence of substantial amounts (5–10%) of *trans* unsaturated acids (Hartman, Shorland & McDonald, 1954, 1955) and of mono- and di-unsaturated acids which differ from oleic and linoleic acids in the position of their double bonds (cf. Shorland, 1956). Hilditch (1956) has suggested that the peculiarities of the glyceride structure of ruminant fats may be due to the hydrogenation *in situ* of preformed oleoglycerides. However, while this process may possibly occur, the most important factor is probably the hydrogenation of the dietary unsaturated fatty acids by the rumen micro-organisms, giving rise to less-unsaturated acids and stearic acid.

Perhaps the first observation of the effect of the rumen on the dietary fat was made by Reiser (1951). He reported that linolenic acid present in linseed oil was converted *in vitro* into linoleic acid by the action of sheep-rumen contents. Similar results were obtained by Hoflund, Holmberg & Sellman (1956), using fistulated sheep.

As a result of the observation of Willey, Riggs, Colby, Butler & Reiser (1952) that the depot fats of steers feed on cottonseed oil contained more stearic acid than the controls, R. Reiser (private communication) suggested that the high content of stearic acid in the depot fats of ruminants was due to the hydrogenation of dietary C_{18} unsaturated acids in the rumen. This suggestion was further supported

by Shorland, Weenink & Johns (1955), who showed that whereas linolenic acid was the main component of pasture lipids, in pasture-fed sheep the rumen contents contained stearic acid as the main lipid constituent. Furthermore, incubation of linolenic acid with sheep-rumen contents produced mainly stearic acid and not linoleic acid, as originally reported by Reiser (1951). Recently, Reiser & Reddy (1956) fed cottonseed oil and linseed oil to goats and found that the linoleic and linolenic acids present were decreased to a very low level in the rumen and increased amounts of saturated fatty acids were formed.

To provide more precise information about the hydrogenating action of the rumen micro-organisms, we now report the effect of sheep-rumen contents on oleic, linoleic and linolenic acids.

EXPERIMENTAL

Fatty acids used for incubation experiments

Oleic acid. The oleic acid, iodine value 90·1, was prepared from olive oil as described by Brown & Shinowara (1937).

Linoleic acid. This acid was prepared from cottonseed oil as described by Hilditch, Patel & Riley (1951). The iodine value was 173·7 (theoretical, 181·4). Analysis by the Brice, Swain, Herb, Nichols & Riemenschneider (1952) procedure showed the presence of 92·9% of non-conjugated dienoic acids, together with 0·2% of conjugated dienoic acids. Trienoic acids were not detected.

Linolenic acid. This was prepared from conophor oil, by the urea-complex procedure of Swern & Parker (1953). The linolenic acid concentrate was purified by fractional distillation of the methyl esters followed by adsorption chromatography as described by Reinbold & Dutton (1948). The acids finally obtained had an iodine value of 264·8 as compared with 274·1 required for pure linolenic acid. Analysis by the procedure of Brice *et al.* (1952) showed the presence of 91·3% of non-conjugated trienoic, 3·9% of non-conjugated dienoic, 1·9% of conjugated trienoic and 1·4% of conjugated dienoic acids.

By using the technique of Hartman *et al.* (1955) it was found that only negligible amounts of *trans* unsaturated compounds (less than 1% calculated as elaidic acid) were present in all of the above acids.

Incubation of the fatty acids

Fistulated sheep maintained on a ryegrass–white clover pasture were used as a source of rumen contents. Approximately 1 l. of contents was removed from each sheep after the animals had been withheld from pasture for 6 hr. to produce reasonably fluid contents. The samples were mixed under anaerobic conditions. The rumen contents were not strained, as it was considered that the fermentation of the residual plant material would be necessary for the production of hydrogen for the hydrogenation reaction. The combined rumen samples were divided into portions of 700 ml. Of these, one was used for the control experiment, and to the remainder 5 g. samples of oleic, linoleic and linolenic acids were added. They were incubated in 1 l. Erlenmeyer flasks, fitted with rubber bungs through which passed tubing for gassing and sampling, as well as a valve to allow the escape of fermentation gases. The flasks were gassed with carbon dioxide and incubated at 37° for 2 days, being shaken at hourly intervals by hand during the day. Samples were withdrawn via the sampling tube at approximately 3 hr. intervals on the first day and once on the second day for determination of pH.

The pH values of the contents of the flask were kept within the range 6·5–5·8 by the addition, when necessary, of solid sodium bicarbonate to the flask. This was done by quickly removing the rubber bung, adding the bicarbonate, replacing the bung, shaking and regassing with carbon dioxide. The pH range was chosen as that found in normal rumen contents of pasture-fed sheep at the Plant Chemistry Laboratory.

The reactions were stopped by adding an equal volume of ethanol and heating to boiling point. The aqueous ethanol was evaporated *in vacuo* on the water bath and the residue extracted first with acetone and then continuously with ethyl ether. The extracts were evaporated, dissolved in ethyl ether and washed with water, and the total lipids recovered by evaporation of the ether on the water bath. The last traces of solvent and water were removed *in vacuo* at 100°.

Analysis of lipid fractions

The total lipid fractions of the control and experimental samples were saponified and the unsaponifiable matter was extracted as outlined in the standard method of the Society of Public Analysts (1933). The aqueous solutions were acidified and the fatty acids recovered by extraction with ethyl ether. Under these conditions of saponification the amounts of conjugated acids formed from linolenic acid are less than 1%. The methyl esters of the fatty acids, prepared as described by Hilditch (1956), were separated into 'solids' and 'liquids' by crystallization from 15 vol. of acetone three times at −35° (Shorland & De la Mare, 1945). The methyl esters of the solids and liquids were fractionated by using column E, described by Shorland (1952), and the proportions of C_{18} constituents determined from the saponification equivalents and iodine values of the fractions. The proportions of diene and triene constituents were determined by the technique of Brice *et al.* (1952). By means of the infrared method described by Hartman *et al.* (1955) the contents of *trans* double bond expressed as elaidic acid were measured from the graphs of 5% (w/v) solutions of the methyl esters in carbon disulphide, compensated with a similar concentration of methyl palmitate in the same solvent. Fractions particularly rich in methyl esters of *trans* acids (greater than 30%) were measured as above but at a lower concentration, namely 1% (w/v).

The results of the incubation experiments shown in Table 1 have been expressed in terms of the differences between the weights of the various C_{18} fatty acid constituents present in the control sample and in the samples to which the fatty acids have been added. The amount of added C_{18} unsaturated acids was about four times that present in the control experiment.

In view of the substantial amounts of dienoic acids, both conjugated and non-conjugated (see Table 1), after the incubation of linoleic acid the infrared absorption spectra were re-examined for

Table 1. *The component fatty acids formed during incubation of unsaturated fatty acids with sheep-rumen contents*

The figures in parentheses denote the amounts formed, expressed as a percentage of the wt. of the added acid. The composition of the fatty acids in the control sample (no acid added) was as follows (wt. %): saturated C_{16}, 14·1; C_{18}, 50·5; C_{20} and above, 3·2. Unsaturated C_{14}, 1·4; C_{16}, 1·6; C_{18} monoene, 28·4; C_{18} non-conjugated diene 0·1, triene 0·4; C_{18} conjugated diene 0·3, triene trace.

Fatty acid added		Fatty acids formed (g.)						
			C_{18} unsaturated					
				Non-conjugated†		Conjugated		
Nature	Wt. (g.)*	Stearic	Monoene	Diene	Triene	Diene	Triene	*trans* (as elaidic)
Oleic	5·00	1·13 (22·6)	2·95 (59·0)	0·03 (0·6)	0·05 (1·0)	0·07 (1·4)	0·04 (0·8)	0·86 (17·2)
Linoleic	20·00	3·13 (15·6)	8·94 (44·7)	3·77 (18·9)	0·03 (0·2)	3·00 (15·0)	0·05 (0·3)	9·58 (47·9)
Linolenic	20·00	3·37 (16·8)	14·45 (72·2)	Trace —	0·14 (0·7)	0·29 (1·4)	Trace Trace	13·47 (67·3)

* Uncorrected for impurities.

† As customarily designated but preferably termed conjugatable under experimental conditions used.

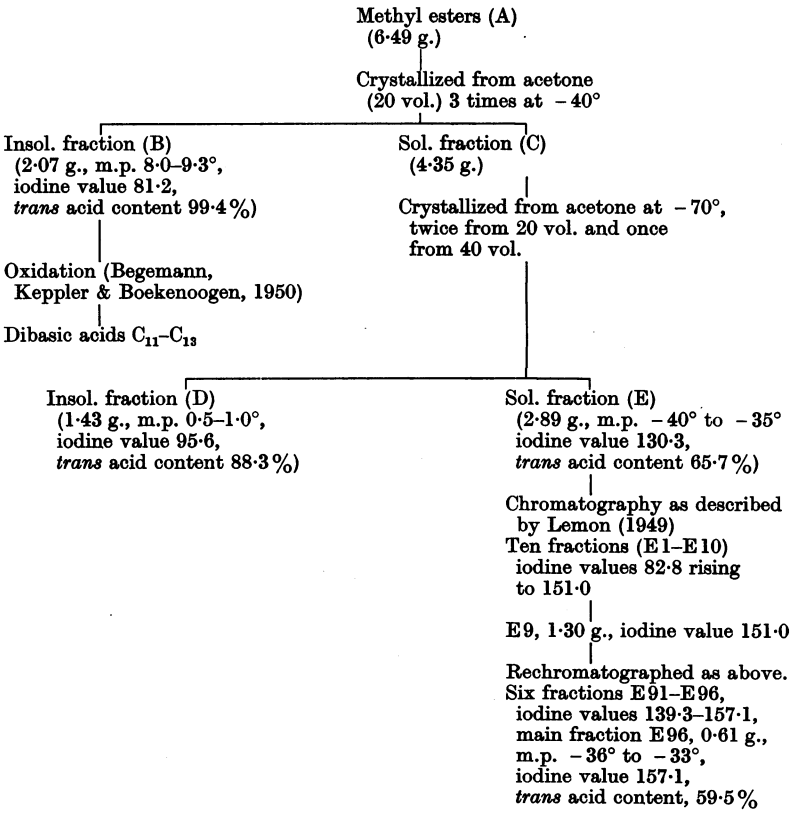

Fig. 1. Separation of a C_{18} non-conjugatable dienoic acid concentrate after the incubation of linolenic acid with sheep-rumen contents.

the presence of conjugated *trans* acids. From the extinction-coefficient and peak-frequency data of Ahlers, Brett & McTaggart (1953) it was calculated that 5·3 % of *trans–trans* conjugated and 2·0 % of *cis–trans* conjugated dienoic acids were present.

Carotene was determined according to method B of the Grass Driers' Association (1941).

The non-conjugatable dienoic acids from the linolenic acid incubation experiment

The Wijs iodine values and the calculated iodine values (Brice *et al.* 1952) of the C_{18} unsaturated fraction from the oleic and linoleic experiments agreed, but there was a wide discrepancy in the linolenic acid experiment. Here the Wijs iodine value was 107·4 and the calculated value 89·5. This difference, if calculated as dienoic acid, represents about 21 % of the fraction.

The methyl esters of C_{18} unsaturated acids from the linolenic acid experiment (denoted A) were therefore separated as shown in Fig. 1 for further examination.

From the results given in Fig. 1 the main concentrate of mono-unsaturated acids appears to consist of a mixture of *trans*-Δ^{11}-, Δ^{12}- and Δ^{13}-octadecenoic acids. Fraction E 96 (0·61 g.) appeared from the iodine value to contain considerable proportions of dienoic acid, and was therefore selected for further investigation. Analyses by the Brice *et al.* (1952) procedure showed 2·8 % of conjugatable trienoic acids together with traces of conjugated dienoic and trienoic acids. However, assuming the presence of mono- and di-enoic constituents only, it may be calculated from the iodine value of 157·1 that the content of methyl octadecadienoate was 82 %.

Ozonolysis of methyl linoleate (m.p. of tetrabromide of acids 114°) by the procedure of Klenk & Bongard (1952) followed by chromatography of dibasic acids as described by Marvel & Rands (1950) gave malonic acid only. Under similar conditions E 96 gave the following molar percentages of dibasic acids, based on the weight of ester taken: malonic, 3·6; succinic, 17·0; glutaric, 2·0; adipic, 1·2. These results show that the main dienoic constituent present in fraction E 96 contains two methylenic groups between the double bonds. The presence of some malonic acid is to be expected from the residual conjugatable trienoic acid.

In determining the monobasic acids the oxidation procedure of Allen & Kiess (1955) was used. The resulting acids were determined by the method of James & Martin (1952). A linolenic acid concentrate containing 69·8 % of linolenic acid, 18·8 % of linoleic acid and 11·4 % of oleic acid gave the following molar percentages of monobasic acids: propionic acid, 70·0; hexanoic acid, 19·5; nonanoic acid and higher fatty acids, 10·5. In a sample of fraction E 96 there were found the following molar percentages: propionic acid, 64; butyric acid, 20; hexanoic acid, 16.

DISCUSSION

The object of the fermentation *in vitro* was to examine qualitatively the conversion of unsaturated acids by rumen organisms without the necessity of preparing the larger amounts of materials that are necessary to dose directly into the animal. The difficulty with a fermentation *in vitro* technique in this case is that a relatively large amount of the acids is necessary for the methods of analysis employed. The most satisfactory method of incubating rumen contents *in vitro* appears to be the use of a semipermeable sac in which rumen liquor is dialysed against a mineral solution (Warner, 1956). However, it was thought that the number and size of the 'artificial rumens' necessary to carry out the experiment with the amount of substrate involved made their use impracticable. Hence recourse was made to the all-glass impermeable system. As the pasture on which the sheep were feeding contains the acids as substrates in the present experiments, the bacteria necessary for their hydrogenation should be present in the rumen ingesta.

As shown in Table 1, 15·6–22·6 % of stearic acid was formed when oleic, linoleic or linolenic acids were incubated with rumen contents. The change in composition of the added fatty acid was least marked with oleic acid, 59·0 % remaining unhydrogenated as compared with 18·9 % for linoleic acid and 0·7 % for linolenic acid. These changes were paralleled by *trans* acid formation, which amounted to 17·2, 47·9 and 67·3 % from oleic acid, linoleic acid and linolenic acid respectively (see Table 1). In the linoleic acid experiment, of the total (33·9 %) dienoic acid remaining, 15·0 %, or nearly half, had been converted into the conjugated form. If these results are compared with those reported by Reiser & Reddy (1956) for steers, it appears that in our experiments the degree of hydrogenation of linoleic acid was similar, but that linolenic acid was more completely hydrogenated, with not more than traces of di- and tri-enoic acids remaining as determined by alkali-isomerization technique. However, hydrogenation of linolenic acid led to the formation of non-conjugatable dienoic acids.

The formation of *trans* and positional isomers of unsaturated fatty acids is known to occur during the industrial hydrogenation of natural fats (cf. Markley, 1947). The mechanism of the reactions involved is not completely understood, but in the simplest case, the hydrogenation of methyl oleate, Allen & Kiess (1955) have shown that the double bond migrates equally in either direction along the

carbon chain with the formation of positional and geometrical isomers. In the hydrogenation of methyl linoleate Allen & Kiess (1956) consider that the methylene-interrupted system shifts to a considerable extent to a conjugated system before hydrogenation takes place. On the other hand, there is evidence that in the hydrogenation of linolenic acid the central double bond is attacked preferentially, leading to the formation of dienoic acids with the double bond separated by more than one methylenic group (cf. Rebello & Daubert, 1951). It could be expected therefore that, if the hydrogenation by the rumen micro-organisms parallels catalytic hydrogenation, the dietary unsaturated acids consisting mainly of linolenic acid could be expected to produce a wide variety of dienoic and monoenoic acids in addition to the oleic and linoleic acids normally found in natural fats.

This expectation appears to be realized; *trans* acids were formed in all cases, conjugated dienoic acids resistant to hydrogenation were present only in the linoleic acid experiment and non-conjugatable dienoic acids were produced by the incubation of linolenic acid (see Table 1). In the latter experiment several isomers with isolated double bonds were present. As propionic and succinic acids were the main products isolated after ozonolysis, octadeca-11:15-dienoic acid would appear to predominate. The presence of octadecadienoic acids with separated double bonds has been inferred by other workers (Garton, 1954), and such acids have been isolated from lamb caul fat (Weenink, 1956). Though analogy between catalytic hydrogenation and hydrogenation by micro-organisms has been demonstrated in this work, there are differences, suggesting that the hydrogenases present in the rumen micro-organisms are to some extent specific. The more complete hydrogenation with accompanying isomerization of linolenic acid as compared with linoleic acid indicates that the octadecadienoic acids (mainly non-conjugatable) formed by the hydrogenation of linolenic acid are more readily hydrogenated under the conditions prevailing in the rumen than is linoleic acid itself. No evidence has been found for the formation of linoleic acid from linolenic acid by rumen micro-organisms.

A further example of the apparent resistance of conjugated systems to hydrogenation by rumen micro-organisms is indicated by the fact that considerable proportions of carotene remain in the rumen contents after prolonged incubation. Our earlier unpublished results showed that the carotene content of ingested pasture is 8·0 mg./g. of unsaponifiable matter as compared with 2·1 mg./g. of unsaponifiable matter in the sheep-rumen contents. In the present work, in each experiment, as well as in the control, the carotene content remained constant at about 1·8 mg./g. of unsaponifiable matter.

The results in Table 1 support the conclusion that the di- and tri-unsaturated acids of the diet are considerably hydrogenated, so that the smallness of the proportions (2 % or less of the fat) of di- and poly-enoic acids in the depot fats of ruminants can be explained.

The amount of *trans* double bonds in the linolenic acid experiment when based on the unsaturated acid content was approximately 91 %. This is higher than was found in the equilibrium mixture (66 %) when methyl oleate was catalytically hydrogenated (Feuge, Pepper, O'Connor & Field, 1951) but comparable with that obtained (89 %) for methyl linoleate (Feuge, Cousins, Fore, Dupre & O'Connor, 1953) in similar circumstances.

Shorland *et al.* (1955) found that in the rumen of fistulated sheep 50 % of the pasture linolenic acid was converted into stearic acid. In the present work we have found that only 16·8 % of the linolenic acid was converted into stearic acid. It appears therefore that *in vivo* the hydrogenation is more complete. However, the present results give an indication of the differences in the facility with which different unsaturated fatty acids are hydrogenated and add to our knowledge of the many different types of unsaturated acids occurring in ruminant fats but not found in non-ruminant fats or vegetable oils.

SUMMARY

The incubation of oleic, linoleic or linolenic acid with rumen contents of sheep in a carbon dioxide atmosphere at 37° resulted in the production of stearic acid as well as *trans* and positional isomers of the unsaturated acids. These results further support the view that many of the peculiarities of ruminant fats arise from the hydrogenation of the dietary unsaturated acids, particularly linolenic acid.

The authors are indebted to Mr C. N. Hooker of the Fats Research Laboratory for the estimation of diene and triene acids as well as for the determination of carotene.

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The Ninhydrin Reaction and its Analytical Applications

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(*Received* 25 *January* 1957)

The colorimetric or spectrophotometric measurement of the absorption produced by the purple reaction product (ANP) between α-amino acids and ninhydrin at 570 mμ has become one of the most widely used methods for the quantitative determination of small quantities of α-amino acids. Its analytical application is, however, sometimes adversely affected by one or more of the following factors. Since the ANP has not been prepared in the solid state, its extinction coefficients could not be determined; in consequence it has been necessary to express analytical results in terms of 'colour yield' in relation to that given by known quantities of a reference standard reacting under defined conditions (Moore & Stein, 1948). It has often been observed that the ANP yield was not constant owing to the occurrence of oxidative side reactions decomposing it to colourless products. Moore & Stein (1948) sought to overcome this effect by the addition of reducing agents like stannous chloride or hydrindantin to the reaction systems. The absorption maximum of the ANP was found to vary within the 500–580 mμ range, thus making impossible absorption measurements at a fixed wavelength. A method devised to eliminate this effect, by treating with alkali spots of ANP on chromatograms, was described by Kay, Harris & Entenman (1956).

The sensitivity of the reaction, i.e. the colour yield by a given amino compound produced under defined conditions, is significantly influenced by the composition of the solvent in which the reaction takes place. It is highest in organic solvents and decreases in solvent mixtures containing water (Yanari, 1956).

The experiments reported in this paper were designed to investigate the properties and behaviour of ANP, especially its absorption spectrum and its changes under different conditions and to correlate the changes in ninhydrin reactivity with measurable properties of this compound and, in turn, with structural changes undergone by the ninhydrin molecule in different solvents.

MATERIALS AND METHODS

All chemicals used were of analytical grade. The disodium salt of ethylenediaminetetra-acetic acid (EDTA) was recrystallized from water before use. Hydrindantin was