TISSUE LIPIDES in Domestic Animals

with special reference to the Pregnant Ewe.

by

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C O N T E N T S.

INTRODUCTION.

Investigations into tissue lipides invariably lead to a consideration of the activities of the liver, for amongst the amazing variety of functions ascribed to that organ, not the least important is the role it plays in the metabolism of fat. An indication of the importance of the liver in lipide metabolism is given by the observation that in a great variety of conditions, neutral fat or choles-:terol esters may accumulate in it to a much greater extent than in any other organ. But here the border line between the normal and the abnormal is not well defined and such fatty changes have often been regarded as pathological rather than physio-:logical. And certainly, amongst the various conditions which result in an excess of fat in the liver, we find such pathological states of the body as diabetes mellitus, pernicious anaemia, and poisoning with phosphorus, chloroform or phloridzin. Various infective fevers such as typhoid, small-pox and diphtheria, or gross infestation with worm parasites as in ascariasis or ankylostomiasis may result in similar changes.

In/

In the conditions enumerated above there are usually some signs of damage to the liver cells, and this type of change is designated "fatty degeneration". In other cases, however, there is found an increase of fat in the tissue without any accompanying degeneration of the cell protoplasm, and such a condition is termed "fatty infiltration".

Fatty infiltration in the strict sense means merely accumulation in the cell of fat from the outside and might theoretically be due to increased transport of fat to the tissue or to diminished utilisation or to a combination of both. Thus there is an increase in the fat content of the liver within a few hours of the ingestion of a meal containing large amounts of fat, a phenomenon utilised on a commercial scale in the production of pâté de foie gras. And fatty infiltration is a frequent accompaniment of gross fatness whether the latter be purely nutritional or associated with endocrine disturbances. Such changes are probably mainly associated with increased assimilation. On the other hand, factors which interfere with normal oxidation and utilisation of fat, e.g. chronic alcoholism or pulmonary tuberculosis, have also an important effect in leading to accumulation of fat in the liver.

During/

During the last few years there has been a considerable amount of research on various factors associated with fatty infiltration of the liver. Thus, it has been reported to occur during fasting, and this has been ascribed to mobilisation of depot fat for energy requirements. Mottram (53) found that an increase in liver fat occurred in fasting rabbits whilst Best and Campbell (8) showed that fasting produced a similar condition in guinea pigs and mice. Rats, on the other hand, were found by Best and Ridout (14) to give rather variable results, the increase in liver fat apparently being dependent to a considerable extent on the calorie intake prior to fasting. That the fat which accumulates in the liver during fasting comes mainly, if not wholly, from the body depots has been shown by Barret and his co-workers (5) using deuterium as an indicator.

The production of fatty livers in rats by administration of a diet consisting of mixed grain and 40% beef fat was first demonstrated by Best, Hershey and Huntsman (11). Later it was shown by Best and Huntsman (12) that the inclusion of choline in the diet in the relatively small amount of about 1% prevented the appearance of this "fat" fatty liver. The phenomenon was further investigated by Best et al (9) who showed that it was the neutral fat faction of the liver which was increased in such experimentally produced/

produced fatty livers. And in addition to choline, various analogues such as homocholine, triethylcholine and tripropylcholine have been found by Channon,Platt and Smith (24) to be very efficient in preventing and curing such "fat" fatty livers of dietary origin.

It has also been found possible to produce fatty livers by feeding animals on diets containing choles-:terol. Thus Okey (56) showed that the dietary administration of 1% cholesterol to rats produced a large increase in the lipide content of the liver and, in particular, a remarkable rise in the amount of total cholesterol, far the greater part of this being present as cholesterol esters. Moreover, the inclusion of choline (9) and various homologues (24) will hinder the abnormal deposition of lipide in the liver by these high cholesterol diets.

Increasing the protein of the diet diminishes the deposition of fat in the liver. In 1935 Channon and Wilkinson (25) carried out experiments which indicated that the amount of glyceride in the "fat" fatty liver was inversely dependent on the amount of caseinogen in the diet. This work has since been extended, and it is now known that certain proteins such as caseino-:gen (10) and amino-acids such as methionine (23) exhibit a marked lipotropic action when added to diets which would otherwise produce fatty infiltration of the liver.

Another/

Another dietary factor concerned with fat metabolism in the liver is the vitamin B complex. According to McHenry(48 & 49) vitamin B_1 was found markedly to increase the amount of fat in the liver of rats maintained on a low choline diet. This was confirmed by Best and Ridout (14) who showed that the addition of crystalline vitamin B_1 to diets low in choline or to sucrose diets increased the deposi-:tion of fat in the livers of normal rats.

Endocrine factors have not been neglected, and in particular it has been shown that injections of anterior pituitary extracts rapidly produce an increase in liver lipides. In 1936, Best and Campbell (7) studied the action of a ketogenic ex-:tract of the anterior pituitary which produced an intense infiltration of fat and a rapid increase in the size of the livers of fasting rats; and subse-:quent observations by Mackay and Barnes (48) con-:firmed their results. Further work by Best and Campbell (8) showed that in fasting animals the anterior pituitary preparation used caused a much greater increase in the liver fat in guinea pigs and mice than in rats.

Other internal secretions may also be of importance in this respect. Some workers claim to have demonstrated in pancreatic extracts a lipotropic factor/

factor other than choline and the factors associated with protein (22), though this claim has been disputed (15). And the suggestion that the adrenals may also be concerned in the deposition of liver fat has been put forward by Issekutz and Verzar (44).

While there has thus been intensive research work during recent years on several dietetic and hor-:monal factors associated with fatty changes in the livers of various species, relatively little attention has been paid to the effect of pregnancy on liver lipides.

According to some authors, e.g. Gaiger and Davies (35), a fatty infiltration of the liver is a normal accompaniment of pregnancy in various species. But a search of the rather scanty literature on this subject reveals considerable divergence of views. In 1900, Miotti (52) recorded an abnormal amount of fat in the livers of pregnant rabbits, and this was confirmed by Coope and Mottram (26) who found a decided increase in the liver fat of cats and rabbits during pregnancy. On the other hand, MacLean [quoted by Best and Ridout (13)] failed to demonstrate a constant increase in the amount of liver fat in pregnant rabbits even when these animals received a diet rich in fat; and Snook (63) found no evidence of fatty infiltration of the liver near term in pregnant/

pregnant rats, rabbits, guinea pigs and sheep.

Accumulation of fat in the liver is the most striking post-mortem feature in ewes affected with a condition known as "pregnancy toxaemia", and this is often used to confirm the diagnosis made on the history and clinical symptoms. It has been suggested, however, by various authors (27, 38, and 69) that fatty infiltration of the liver might be characteristic of the pregnant ewe near term. Such suggestions were presumably based on post-mortem experience, but no data are quoted in support of the contention.

While there are numerous studies of the liver lipides in small experimental animals such as rats and rabbits, little analytical work has been done on the liver lipides of sheep. Turner (68) analysed five specimens of liver from non-pregnant sheep and found that the total fatty acids averaged 4% of the moist tissues, with an iodine value about 120 and a mean molecular weight of about 300. Hilditch and Shorland (42) made a more comprehensive study of the livers of 22 sheep, also non-pregnant, and fractionated the fatty acids obtained. They found that on an average the livers contained 5.4% of ether-soluble material, about half of which was phosphatide and the remainder glyceride plus unsaponifiable material. Snook (63) analysed three liver samples from non-pregnant sheep, and found the average chloroform extract to amount to about/

about 5% of the wet liver tissue.

Only two workers give figures for the fat content of the livers of healthy pregnant ewes. Roderick, Harshfield and Merchant (59) reported that in 6 normal pregnant ewes the fat present in the liver amounted on an average to 7% of the dry tissue (about 2% of the fresh liver) as compared with 60% of the dry tissue in eight cases of pregnancy toxaemia. Snook (loc.cit) found that in twelve healthy pregnant ewes, the chloroform extract amounted to about 5.5% of the wet liver. Of this, ether-soluble phosphorus formed about 2% of the total extract, and unsaponifi-:able material about 8.5%, these figures being very similar to the findings in non-pregnant sheep. Snook therefore concluded that fatty infiltration of the liver is not a necessary concomitant of pregnancy in the ewe.

On the other hand, many veterinary practitioners with considerable experience in post-mortem examination of ewes in the field and in the abattoir hold that fatty changes in the liver are quite common in healthy pregnant ewes. In view of this diversity of opinion and of the diagnostic importance often attached to the post-mortem finding of fatty infiltration in the livers of ewes suspected of pregnancy toxaemia, it seemed desirable to make a comprehensive survey of the lipide content of the liver in healthy pregnant and non-pregnant ewes.

This/

This was rendered possible by the kind co-operation of Sir Joseph Barcroft, who was making a study of the development of the foetal nervous system in the sheep, necessitating the killing of in-lamb ewes at various stages of pregnancy. Arrangements were made to obtain specimens of liver from these animals, and in addition a number of random samples from pregnant and non-pregnant ewes were obtained from other sources.

It has also been stated [Gilruth (37) and Dryerre (31)] that in pregnancy toxaemia the kidneys are also frequently pale and fatty. As there are no recorded observations on the effect of pregnancy on kidney lipides, occasional specimens of kidney were also analysed from healthy pregnant and non-pregnant ewes to see if the lipide content differed in the two groups.

METHODS.

Scheme of Analysis.

Before commencing the analysis of liver and kidney specimens, a comprehensive survey was made of the various methods recommended for studying tissue lipides. The details are discussed in subsequent pages, but the following outline gives an indication of the general scheme of analysis finally adopted.

A small portion of tissue($\frac{1}{2}$ - 1 gm.) was taken for determination of moisture content and occasional specimens submitted to histological examination. Some 20 gm. of tissue sampled from different parts of the organ were accurately weighed, ground up with anhydrous sodium sulphate and then extracted with alcohol and ether, the extracted lipides being finally taken up in 250 c.c. of ether. This was then analysed as follows:-

(1) 25 c.c. were evaporated down, dried in vacuo and weighed to give a measure of the total lipides present.

(2) 100 c.c. were saponified and divided into (a) unsaponifiable material and (b) total fatty acids, each of these fractions being taken up separately in 200 c.c. of petroleum ether:-

(a) 50 c.c. were taken for the estimation of total cholesterol whilst the remainder

evaporated/

evaporated down and weighed gave the total unsaponifiable material.

(b) 100 c.c. evaporated down and weighed gave the total fatty acids. These were then redissolved in alcohol and titrated with N/10 NaOH to get their mean molecular weight.

The remaining 100 c.c. of petroleum ether extract were evaporated down and the iodine value of the fatty acids obtained.

(3) Free cholesterol was estimated on 25 c.c. of the original extract.

(4) Lipoid phosphorus was estimated on 5 c.c. portions of the original extract. (In occasional specimens the phospholipides in the remaining ether extract were precipitated by acetone plus magnesium chloride and weighed as a check on the lipoid P. estimation).

From the data obtained, the following were calculated:-

(a) Combined cholesterol

(b) Fatty acids combined with cholesterol

(c) Phospholipides

(d) Phospholipide fatty acids

(e) Neutral fat fatty acids

(f) Neutral fat.

Extraction of Tissue Lipides.

Complete extraction of lipoid material from tissues is no simple matter. Thus Leathes and Raper (47) found that after four successive extractions with alcohol followed by two with ether there remained unextracted in the tissue more than 3% of the total fatty substance of the liver. In the last decade many modifications of Leathes and Raper's procedure have been suggested (16, 67, and 45) with a view to increasing the efficiency of the extraction, but the essence of all the methods has been dehydration with alcohol followed by a varying number of extractions with boiling alcohol and ether, the extracts being finally evaporated to dryness and the residue taken up in ether or chloroform. The most exhaustive series of extractions is that used by Best, Channon and Ridout (9) who subjected the minced tissue to three extractions with hot alcohol followed by four with ether, the combined alcohol-ether extracts being evaporated to dryness in vacuo and the residue re-extracted with warm ether several times and filtered.

As a preliminary, it was decided to compare the completeness of extraction by various methods, those chosen being that of Bloor (16), which was the most comprehensive/

comprehensive of the earlier series, and that of Best et al (9). The principle used was to take in each case some 20 - 30 gm. of tissue and extract the lipides in the manner recommended. Then the total lipides extracted and the residual tissue were saponified separately by the method of Lieberman (47) and the total fatty acids plus unsaponifiable material estimated in each case. It was thus possible to get a measure of the completeness of the extraction by the percentage of fatty acids plus unsaponifiable material left in the extracted tissue. As a result of a number of determinations it was found that the method of Best et al was slightly more thorough than that of Bloor, the percentage of lipoid material remaining unextracted being usually about 2% with the former as compared with slightly over 3% with the latter. Experience with these methods, however, suggested that certain alterations might be an improvement. Thus, the use of alcohol as a drying agent was found to introduce complications later when evaporating down the various extracts, as considerable frothing occurred in the presence of water. It was decided therefore to adopt the technique of Best et al. with minor modifications, viz.,

(a) the use of anhydrous sodium sulphate to remove water and facilitate the grinding of the tissue,

(b)/

(b) to discard the second grinding of the tissue which proved tedious and was unnecessary in view of the thorough trituration obtained with (a),

(c) to keep the alcohol and ether extracts separate so as to facilitate recovery of solvents,

(d) to substitute extraction in a soxhlet apparatus for shaking with hot solvent.

Having adopted these modifications, it remained to see how many extractions were necessary to get as nearly complete removal of lipides as possible. In order to do this some 20 gm. of tissue were extracted and the various fractions kept separate, evaporated down and weighed. It was found that the use of a soxhlet in place of shaking with hot solvent enabled one to extract all but 1 - 2% of the lipoid material from the tissues whilst omitting several of the extractions used by Best and his colleagues. Thus, extracting the dried powdered tissue overnight with cold alcohol was found to remove some 60% of the total lipides whilst subsequent shaking with warm alcohol for a few hours removed another 20%. Treating the tissue with alcohol in a soxhlet apparatus then succeeded in removing another 10% of lipides, whilst subsequent extractions with boiling alcohol proved relatively ineffective. But two further/

further extractions of the tissue with ether in a soxhlet for 2-3 hours each time removed respectively some 6% and 2% of the total lipides, whilst further extractions with ether removed only very small traces. The method finally adopted, therefore, was as follows:-

About 20 gm. of liver were taken, being sampled from various parts of the organ, avoiding fat and connective tissue as far as possible. This was weighed accurately, transferred to a glazed mortar and thoroughly pulped. Then about 25 gm. of anhydrous sodium sulphate were added and the whole ground thoroughly into a paste. After about 30 minutes the resulting dry mass was thoroughly powdered and transferred completely to a conical flask with 100-150 c.c. of absolute alcohol. After being shaken, this was stoppered and set aside for several hours, often overnight. Then the alcohol was decanted through a fat-free filter paper into a 500 c.c. conical flask. About 100 c.c. hot alcohol were added to the residue and set aside for a few hours with occasional thorough shaking. The alcohol was then decanted off as before and the tissue transferred to a soxhlet extractor and treated with 100 c.c. of boiling alcohol for about three hours. The alcoholic extract was then filtered off as before and the tissue extracted twice with 100 - 150 c.c. of ether, for about three hours each time.

The/

TABLE I.

Liver Lipides in Domestic Animals.

Species of Animal	Number of Specimens	Total Lipides (gm. per cent. moist tissue) Mean S.D.
Horse	4	4.62 ± 0.62
Ox	6	6.05 ± 0.8
Sheep	6	6.6 ± 1.2
Pig	4	4.83 ± 0.9
Dog	5	3.8 ± 1.0

The alcohol and ether extracts were then evaporated to dryness under reduced pressure, the temperature not being allowed to rise above 40° C. The residue was extracted five times with warm ether, filtered and made up to 250 c.c. in a standard flask, then transferred to a tightly stoppered container and suitable aliquots taken for analysis.

In repeated experiments with this method it was found possible to extract practically 99% of the lipoid material from the tissues.

Estimation of Total Lipides.

The method used was to pipette 25 c.c. of ether extract into a weighed extraction flask and evaporate down on a water bath in a stream of CO_2 until all the ether had been removed. Then the CO_2 was shut off and a vacuum applied for 10 - 15 minutes. The flask was then cooled for several hours in a vacuum dessicater and weighed. Using 20 - 25 gm. of tissue for the original extraction and making the ether extract up to 250 c.c. it was found that 50 c.c. aliquots gave results within half a per cent. of each other, whilst 25 c.c. portions gave a variation of one to two per cent. at the most.

As very few data were available on the lipide content of the liver in domesticated animals a few analyses were made to find the range of variation which/

which might normally be encountered. The results are given in Table I.

Separation of Tissue Lipides.

For a study of the effect of pregnancy on liver lipides, more detailed information was thought desirable and the following methods were adopted.

Estimation of Total Fatty Acids and Unsaponifiable Material.

Two methods for this were tried out, viz., Lieberman's method (47) and that of Channon and El Saby (21). The first was found to be less tedious and more thorough than the second and was therefore adopted with a further slight simplification. 100 c.c. of the original ether extract were evaporated down and 20 c.c. of absolute alcohol plus 5 c.c. of 2.5% NaOH added. The solution was heated on a water bath for one hour, then transferred to a separating funnel with 50% alcohol and extracted five times with 20 - 30 c.c. portions of petroleum ether to remove unsaponifiable material.

(a) The combined petroleum ether extracts were shaken twice with 50% alcohol, transferred to a standard flask and made up to 200 c.c. A portion of this was then set aside for total cholesterol estimation, and the remainder transferred to a weighed flask and evaporated down as for total lipides. The increase in weight gave a measure of the total unsaponifiable/

unsaponifiable material present.

(b) The combined alcoholic solutions were acidified with 10% H_2SO_4 until acid to phenolphthalein. Then the fatty acids were extracted five times with 20 - 30 c.c. portions of petroleum ether, the combined extracts washed twice with distilled water and then made up to 200 c.c. in a standard flask.

100 c.c. were taken for iodine value determination and the remainder evaporated down and weighed, giving the total fatty acids.

Mean Molecular Weight of Fatty Acids. (47)

The total fatty acids weighed in the previous estimation were dissolved in 25 c.c. of alcohol and a drop of 1% phenolphthalein added. They were then titrated with N/10 NaOH, as also a blank containing only alcohol plus indicator. The difference gave the amount of alkali required to neutralise the fatty acids and the mean molecular weight =

$$\frac{\text{wt. of fatty acid (mgm)}}{\text{c.c. N/10 NaOH}} \times 10$$

Iodine Value of Fatty Acids. (Wij's method). (47).

100 c.c. of the petroleum ether extract of fatty acids were evaporated down as for total lipides but instead of being cooled and weighed were immediately taken up in 10 c.c. of pure carbon tetrachloride and 25 of Wij's iodine solution added. At the same time a control containing solvent and iodine/

iodine solution only was also prepared. The flasks were stoppered and set aside in the dark over night, The next morning the contents of the flask were poured into a 150 c.c. Erlenmeyer flask and 10 c.c. of 10% KI run into the former to dissolve traces of iodine left in it. The contents were then washed quantitatively into the Erlenmeyer flask with water and the volume of fluid made up to 300 c.c. and titrated with N/10 Sodium thiosulphate using starch solution as an indicator. The difference between the two titrations (✱ c.c.) gave the amount of iodine absorbed by the fatty acids and the iodine value =

$$\frac{12.2 \times}{10 \times \text{wt. of fatty acids}}$$

With the quantities normally available this method was found to give results reproducible within 5%.

Estimation of Phospholipides.

Phospholipides may be precipitated from alcoholic solution by means of acetone containing traces of magnesium chloride (62). This method is useful where a detailed analysis of the phospholipide is required, but as a method of estimation it is somewhat unsatisfactory, as it is by no means certain that precipitation is always complete. A common method of estimation therefore is to determine the amount of ether-soluble phosphorus by a method such as that of Fiske and Subbarow (32) or its modifications and to calculate the amount of phospholipide on the assumption/

assumption that it is present as lecithin.

The method of lipoid P. estimation adopted in the present investigation was that of Stewart and Hendry (65) which had been found simple and very satisfactory for blood lipides. The method was modified slightly by adapting it to estimate somewhat larger amounts of phosphorus, thus avoiding any necessity for further dilution of the ether extract.

5 c.c. portions of the original ether extract were pipetted into two 6 x 1 in. pyrex boiling tubes and a glass bead added to each. The ether was then evaporated off on a water-bath and 5 c.c. of 10 N H_2SO_4 added. Next each tube was heated over a microburner until complete charring occurred. After cooling, a few drops of 30% H_2O_2 (perhydrol) were added and the tube again heated. If the solution did not clear completely, two more drops of perhydrol were added and the heating repeated. This process of adding perhydrol, one or two drops at a time, and heating after each addition, was continued until the liquid was clear. Then the solution was boiled up again for a few minutes, when further charring usually took place. This was cleared as before by the addition of a few drops of perhydrol, and then the H_2O_2 was boiled off. After the solution had cooled, it was transferred with distilled water to a 100 c.c. standard/

standard flask and a further 5 c.c. of 10 N H_2SO_4 added. In another flask were placed 10 c.c. of 10 N H_2SO_4 and 5 c.c. of standard phosphorus solution (1 c.c. = 0.1 mgm. P.). To each flask was added 10 c.c. of 2.5% ammonium molybdate and 5 c.c. of an 0.25% solution of 1-2-4 amino-naphthol-sulphonic acid. The contents of the flasks were then made up to the mark and thoroughly mixed. A portion of the contents of each flask was transferred to a separate boiling tube and the tubes placed in a boiling water-bath for ten minutes. After cooling in running water, the solutions were compared in a colorimeter, with the standard set at 20.

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times 0.5 = \text{mgm P. present in 5 c.c. ether extract.}$$

Repeated experiments with this technique gave results reproducible within 2 - 3%.

Precipitation of phospholipides.

As a check on the reliability of the above method for giving a measure of the phospholipides present, a number of experiments were carried out, where, in addition to estimation of ether-soluble phosphorus, the phospholipides present were precipitated and estimated both gravimetrically and by colorimetric estimation of phosphorus in the precipitate. Thus, in one sample where estimation of/

of ether-soluble phosphorus gave a calculated phospholipide value of 0.730 gm. present in the 250 c.c. of ether extract, direct estimation by precipitation and weighing gave a value of 0.712 gm. i.e. within 2 - 3%. Colorimetric estimation of lipoid phosphorus in the precipitate gave a calculated value of 0.745 gm. showing that the factor used in the calculation was a satisfactory approximation to the true value. It would appear, therefore, from this and other check experiments that estimation of ether-soluble phosphorus gave a reliable estimate of the amount of phospholipides actually present in the tissues.

Estimation of Cholesterol.

The small quantities of cholesterol normally present in tissue extracts are rather difficult to estimate accurately, especially in the presence of large quantities of other lipides. The methods employed depend on digitonide precipitation and the presence of large amounts of lipides renders the filtration and washing of the digitonide tedious and uncertain. Various techniques were tried out, and the most satisfactory method of separation was found in Yasuda's modification of Okey's method (71). For the actual estimation Yasuda employed Bloor's oxidative technique but this was found to give very variable results, so a gravimetric method of estimation

was/

was sought. The most satisfactory proved to be the method of Szent-Gyorgi as modified by Jowett and Lawson (45). The method finally adopted, therefore, consisted of a combination of these two techniques.

(a) Total Cholesterol.

Aliquots were taken from the petroleum ether extract of unsaponifiable material, the solvent being evaporated off on a water-bath and the lipides taken up in 5 c.c. of absolute alcohol. 5 c.c. of an 0.5% solution of digitonin in alcohol were added, the mixture heated almost to dryness on a hot plate and the last traces of alcohol removed in a stream of CO_2. Then 10 c.c. of distilled water were added and the mixture heated with constant shaking till it began to boil. After cooling, 20 c.c. of acetone were added and the mixture thoroughly shaken. It was then filtered through a weighed sintered glass filter (G_3) using gentle suction. The inside of the flask and the filter were washed twice with acetone and three times with ether, sucking dry each time. After drying in a hot-air oven at 112° C. for 1 hour, the filter was cooled in a dessicator and weighed.

Wt. of cholesterol = 0.2431 x wt. of cholesterol digitonide.

(b)/

(b) Free Cholesterol.

25 c.c. portions of the original ether extract were evaporated down on a water-bath and the lipides taken up in alcohol. The free cholesterol was then precipitated as digitonide as for total cholesterol.

The results were found to be reproducible usually within 3%, though occasional deviations as large as 7 - 10% were found with total cholesterol estimations.

Estimation of Dry Weight of Tissues.

In case variations in lipide content might be accompanied by marked changes in the moisture content and thus give a misleading picture of the magnitude of the changes involved, occasional determinations were made of the dry weight of the tissues. Some very thin slices, amounting in all to about 1 gm. were removed from various parts of the specimen and weighed rapidly in a covered glass dish. The dish was then placed, minus the cover, in a hot-air oven at 110°C. for 4 - 5 days, and the percentage loss of weight determined at the end of this period.

Calculated Values.

A number of check experiments showed that free fatty acids were small in amount. Moreover, no reliable method was available for the estimation of galactosides. These two fractions, therefore, were included in the neutral fat fraction. The following values were calculated from the data obtained by the above methods.

(a) Bound cholesterol = total - free.

(b) Cholesterol esters (as oleate) = $\frac{650}{386} \times$ (a)

(c) Fatty acids, combined with cholesterol (as oleic acid) = (a) x $\frac{282}{386}$

(d) Phospholipides (as di-oleo-lecithin. P = 3.86%)

1 mgm. lipoid P. = 25.9 mgm. phospholipides.

(e) Phospholipide fatty acids (as oleic acid) = 70.4% of (d).

(f) Neutral fat fatty acids (as oleic acid) = Total fatty acids - [(c) + (e).]

(g) Neutral fat (as triolein) = (f) x 1.045.

Results of a typical extraction.

Tissue:- Sheep's liver - appeared normal macroscopically and histologically.

Data measured.

Wt. of liver extracted	=	20.88 gm.
% moisture present	=	68.
Total lipides present	=	1.65 gm. = 7.9% moist tissue
Total fatty acids	=	1.095 gm.
Mean molecular weight of total fatty acids	=	293
Iodine value of total fatty acids	=	90.
Lipoid P.	=	34.1 mgm.
Total unsaponifiable material	=	0.191 gm.
Total cholesterol	=	0.0770 gm.
Free cholesterol	=	0.0605 gm.

Data Calculated.

Unsaponifiable material other than cholesterol	=	0.0114 gm.
Combined cholesterol	=	0.0165 gm.
Cholesterol esters	=	0.0278 gm.
Fatty acids combined with cholesterol	=	0.0122 gm.
Phospholipide present	=	0.882 gm.
Phospholipide fatty acids	=	0.621 gm.
Neutral fat fatty acids	=	0.462 gm.
Neutral fat	=	0.485 gm.

Amount/

Amount of the various fractions present in 100 gm. liver.

Phospholipide	= 4.21 gm.
Free cholesterol	= 0.29 gm.
Cholesterol Esters	= 0.13 gm.
Other Unsaponifiable material	= 0.54 gm.
Neutral fat	= 2.32 gm.
Total lipides	= 7.49 gm. i.e. about 5% less than direct estimation.

This discrepancy between the total lipides as estimated direct and as calculated has been noticed before (9 and 41), and it has been suggested that the difference may be due to the method of extraction used removing substances which are not soluble in alcohol or ether but are soluble in ethereal solutions of phospholipides. That some such explanation is probably correct can be shown by comparing the weight of total lipides with the weight of unsaponifiable material and total fatty acid obtained. Saponification and subsequent removal of water-soluble substances will cause loss of glycerol, choline and phosphoric acid. On the assumption that the phospholipide is lecithin, the expected loss can be calculated for any sample if the amount of phospholipide present is known --- the loss of glycerol from glyceride is usually small compared to the amount of total lipide.

Total/

Total lipides calculated from the sum of fatty acids plus unsaponifiable material, a correction being introduced for the water-soluble products of saponification of phospholipide. (Assuming it is all present as di-oleo-lecithin with a water-soluble fraction of 29.6%).

Total unsaponifiable material + total fatty acids = = 1.286 gm.

Total lipides = 1.286 + (29.6% of 0.882) = = 1.548 gm. = 7.41% of moist liver.

Thus, the amount of total lipide calculated as above is within about 1% of the amount derived from adding together the different fractions estimated. This confirms the view that the method of extraction adopted, though having advantages in the completeness of extraction obtained, suffers so far as direct estimation of total lipides is concerned, from the extraction of certain non-lipoid materials. In subsequent investigations, therefore, the direct weighing of total lipides has usually been dropped (being done only occasionally as a check). The data obtained have been expressed as the amounts of the various fractions present in 100 gm. moist liver, and the total lipides estimated as the sum of the various fractions isolated.

Reproducibility/

TABLE II.

	Sample 1	Sample 2	Sample 3	Mean ± S.D.
Phospholipides	3.75	3.56	3.45	3.59 ± 0.15
Free Cholesterol	0.27	0.25	0.25	0.257 ± 0.011
Cholesterol Esters	0.23	0.19	0.15	0.19 ± 0.04
Other Unsaponifi-:able material	0.22	0.15	0.27	0.21 ± 0.06
Neutral Fat	1.25	1.4	1.73	1.46 ± 0.24
Total Lipides	5.72	5.55	5.85	5.71 ± 0.17

Reproducibility of Results.

As a check on the reproducibility of results, as affected by sampling variations and experimental errors, a large specimen of liver was obtained from a young non-pregnant ewe slaughtered for food. Three different lots of liver were taken, each being approximately 20 gm. in weight, and the lipide analysis carried through as described on previous pages. The results are shown in Table II.

It will be seen that the greatest variation occurred with cholesterol esters and unsaponifiable material other than cholesterol. This is associated with variability in the results for total cholesterol which were sometimes unsatisfactory, as prolonged hydrolysis with alkali tended to interfere with the precipitability of the cholesterol as digitonide. In spite of all precautions, this remained the least satisfactory of the estimations. Neutral fat, which is obtained by difference, also showed considerable variation, but results for phospholipides and free cholesterol could be reproduced with considerable accuracy and the figures for total lipides (which depended mainly on the results for phospholipides and total fatty acids) were also reproducible within reasonable limits. From the results it seems unlikely that unequal sampling would give rise to more than a 4 or 5% variation in the results for total lipides/

lipides, though the much smaller quantities of unsaponifiable material present might show considerably greater percentage variations.

TABLE III.

Analyses of Livers from 25 non-pregnant ewes.

	Moisture Content ⁂ %	Lipides present in gm. per 100 gm. moist tissue.						Mean Molecular Weight	Iodine Value
		Phospho-lipides	Free Cholesterol	Cholesterol Esters	Other unsap. material	Neutral Fat	Total Lipides		
Mean	70	3.56	0.26	0.17	0.15	1.86	6.04	300	101
Range	68 - 73	2.9-4.2	0.15-0.35	0.08-0.30	0.01-0.40	0.7-4.0	4.25-8.67	290-310	87-112
Standard Deviation	18.2	0.5	0.087	0.064	0.1	0.4	0.78	70	28
Standard Error of the Mean	4.7	0.1	0.017	0.013	0.02	0.08	0.16	18.3	7.3

⁂ From analyses of 15 specimens only.

The Effect of Pregnancy on Liver and Kidney Lipides.

Considerations of space render it impossible to give a detailed account of the large number of individual analyses performed, but a summary of the results is presented in the following pages along with a statistical survey and discussion. The tissues studied can be considered in six groups.

GROUP I.

Liver specimens from 25 non-pregnant ewes.

The specimens from non-pregnant ewes were random samples obtained over two successive years at intervals during the period when pregnancy normally occurs, viz., October to April. Animals of all ages have been included and some specimens came from the same flocks as the pregnant animals studied. A preponderance of specimens, however, came from animals about one year old, which were being slaughtered for human consumption.

The results obtained are summarised in Table III. They are in fairly close agreement with the few observations of previous workers and with the findings for other species (Table IV). The moisture content is somewhat smaller than that found by Snook (63), but both sets of results show considerable variation, and the difference between the means is practically equal/

TABLE IV

Comparison with results of previous workers.

Worker and Reference	Animal	Moisture Content %	Phospho-lipides %	Free chole-sterol %	Cholesterol Esters %	Total Fatty Acid %	Total Lipides %	Mean Molecular Weight	Iodine Value
Turner (68)	5 sheep					3.9		300	120 (93-124)
Hilditch et al. (42)	22 sheep		2.4	0.17	0.07		5.4	267	98
Snook (63)	3 sheep	74	3.2	0.4%			5.6		95
Aylward & Stott (3)	6 rabbits	67	3.35	0.28	0.09		4.27		
Best et al. (9)	21 rats.		3.36	0.19	0.36		5.42		
Author	25 sheep	70	3.56	0.26	0.17	4.8	6.04	300	101

equal to the standard error of the difference, so that it is probably of no significance. The average phospholipide content and average total lipides are somewhat greater than found by other workers, but the difference is probably accounted for by the more thorough methods of extraction adopted.

TABLE V.

Liver specimens from 90 pregnant ewes - all stages.

	Moisture Content * %	Lipides present in gm/100 gm moist tissue.						Mean Molecular Weight ××	Iodine Value ××
		Phospho-lipides	Free Cholesterol ‡	Cholesterol Esters ‡	Other unsap. material ‡	Neutral Fat ‡	Total Lipides		
Mean	66.8	3.36	0.23	0.141	0.24	6.59	9.74	292	91.7
Range	59-72	2.46-4.7	0.12-0.33	0.07-0.30	0.05-0.45	0.13-16.0	4.3-19.53	250-310	70-115
Standard Deviation	14.3	0.33	0.053	0.055	0.124	4.09	3.61	59.4	22.3
Standard Error of the mean	2.52	0.035	0.0075	0.008	0.013	0.58	0.383	11.9	4.5

* Average of 32 specimens only.
‡ Average of 50 specimens only.
×× Average of 25 specimens only.

TABLE VI.

Comparison between Groups I & II.

Difference between means Gp.I & II	3.2	0.2	0.03	0.03	0.09	4.73	3.70	8	10
Standard error of the diff. between the means	5.3	0.104	0.0175	0.0133	0.024	0.82	0.415	21.8	8.56

GROUP II

Liver specimens from 90 pregnant ewes. (Table V)

The ninety specimens obtained from apparently healthy pregnant ewes were also spread over successive breeding seasons. When compared with the previous group, the most obvious change is an increase of about 60% in the average total lipides, and this increase is due practically entirely to an increase in the neutral fat fraction.

Table VI shows the difference between the means of the two groups along with the standard error of the difference between the means. It will be seen that the most significant difference is certainly in total lipides, where it is practically nine times the standard error. The neutral fat fraction, which is mainly responsible for this, naturally also shows a very significant difference. In the case of phospholipides and the various unsaponifiable fractions the difference is barely large enough to be significant unless for the unsaponifiable material other than cholesterol, where the difference between the two means is practicallyfour times the standard error. As the nature of this fraction is unknown, the significance of the finding is obscure, but the difference forms only about 2% of the increase in total/

Fig. I.

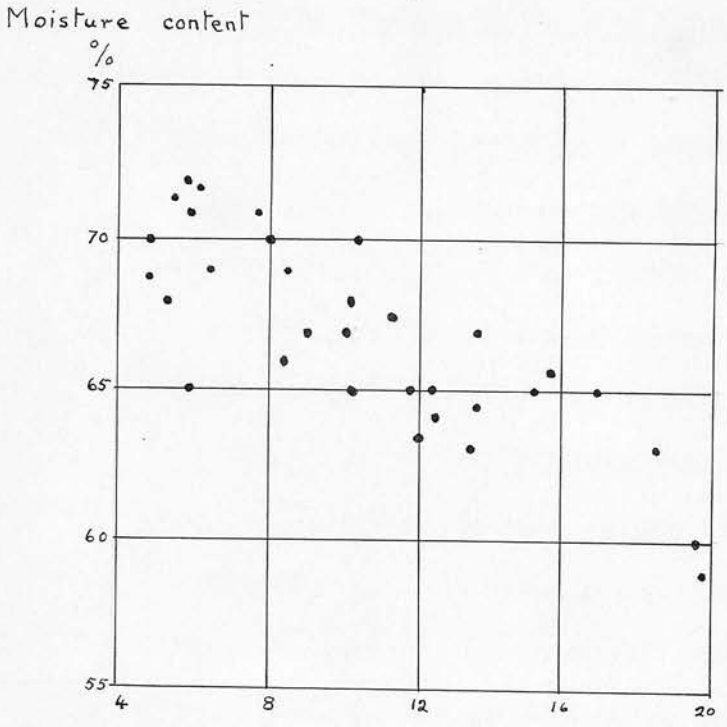
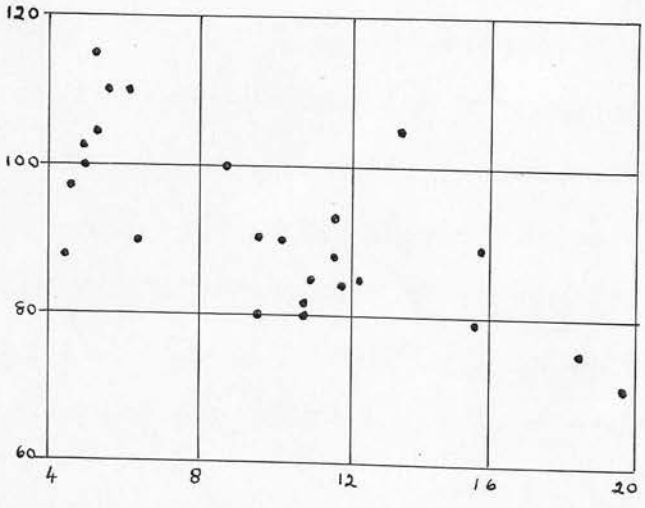

Moisture content
%
75
70
65
60
55
4
8
12
16
20
Total lipides (gm. % moist tissue.)

Fig. II

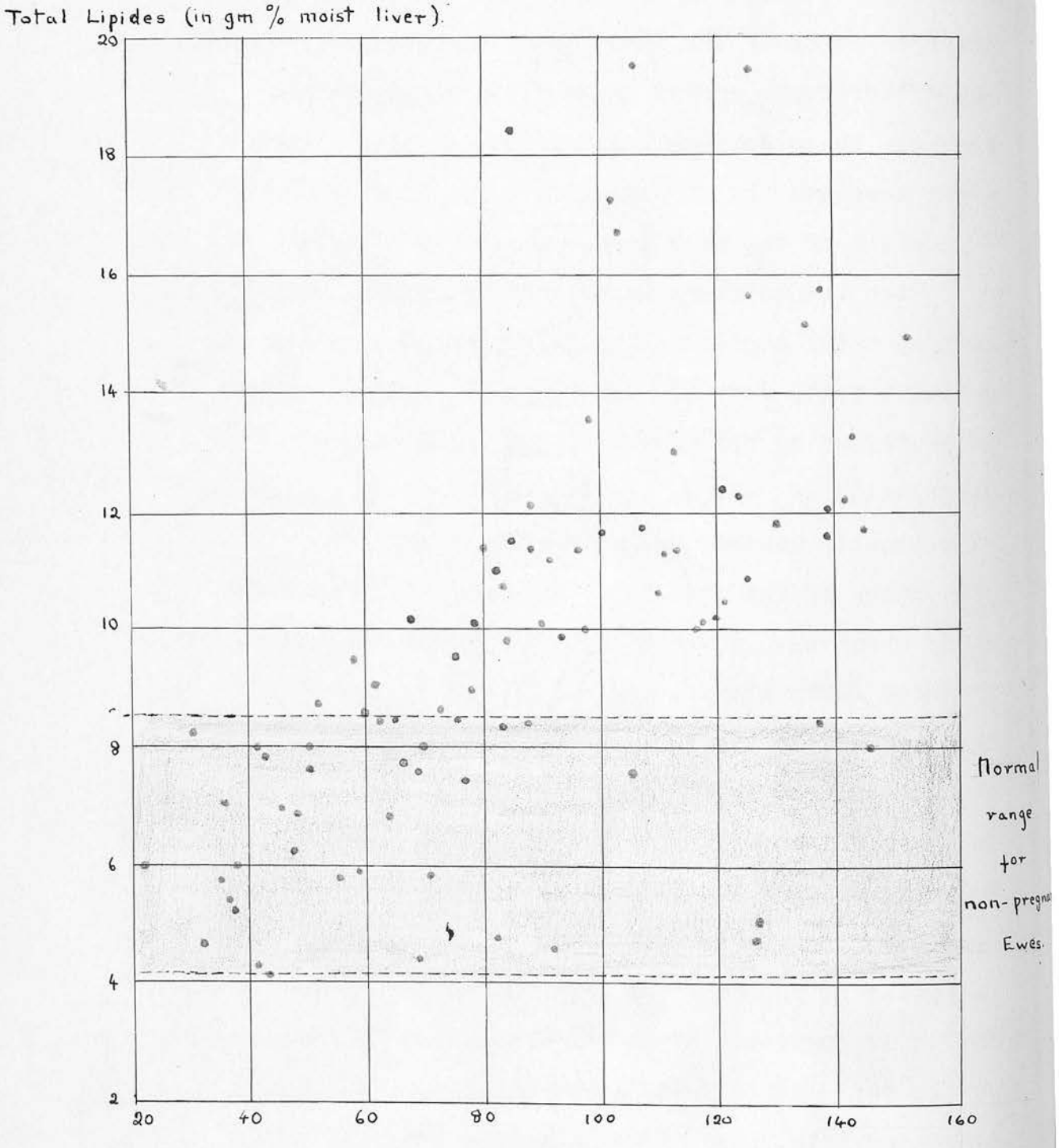

Iodine value.
120
100
80
60
4
8
12
16
20
Total lipides (gm. % moist tissue.)

total lipides.

Moisture content and iodine values show a difference between the means of the same order as the standard error of the difference, and such differences would, therefore, appear to be of no significance. However, these estimations were not carried out in every case and the figures quoted do not give a true indication of the real magnitude of the change.

When the moisture content of the liver is plotted against total lipides (Fig. I), there is a slight but definite fall, more or less linear in nature. This is confirmed by the fact that the co-efficient of correlation is -0.82, showing quite a high degree of relationship between moisture content and the percentage of fat present. This has been found by other observers, who have studied experimentally produced fatty livers, and may be due to the transference to the liver of the more saturated glycerides from the fat depots, causing an increase in liver weight without a corresponding increase in moisture content and thereby producing a fall in the percentage of moisture present. That the fat, which is appearing in the liver, may come from the fat depots is suggested by the fact that the iodine value of the fatty acids falls as the amount of liver fat increases (Fig. II). Here there is more individual variation/

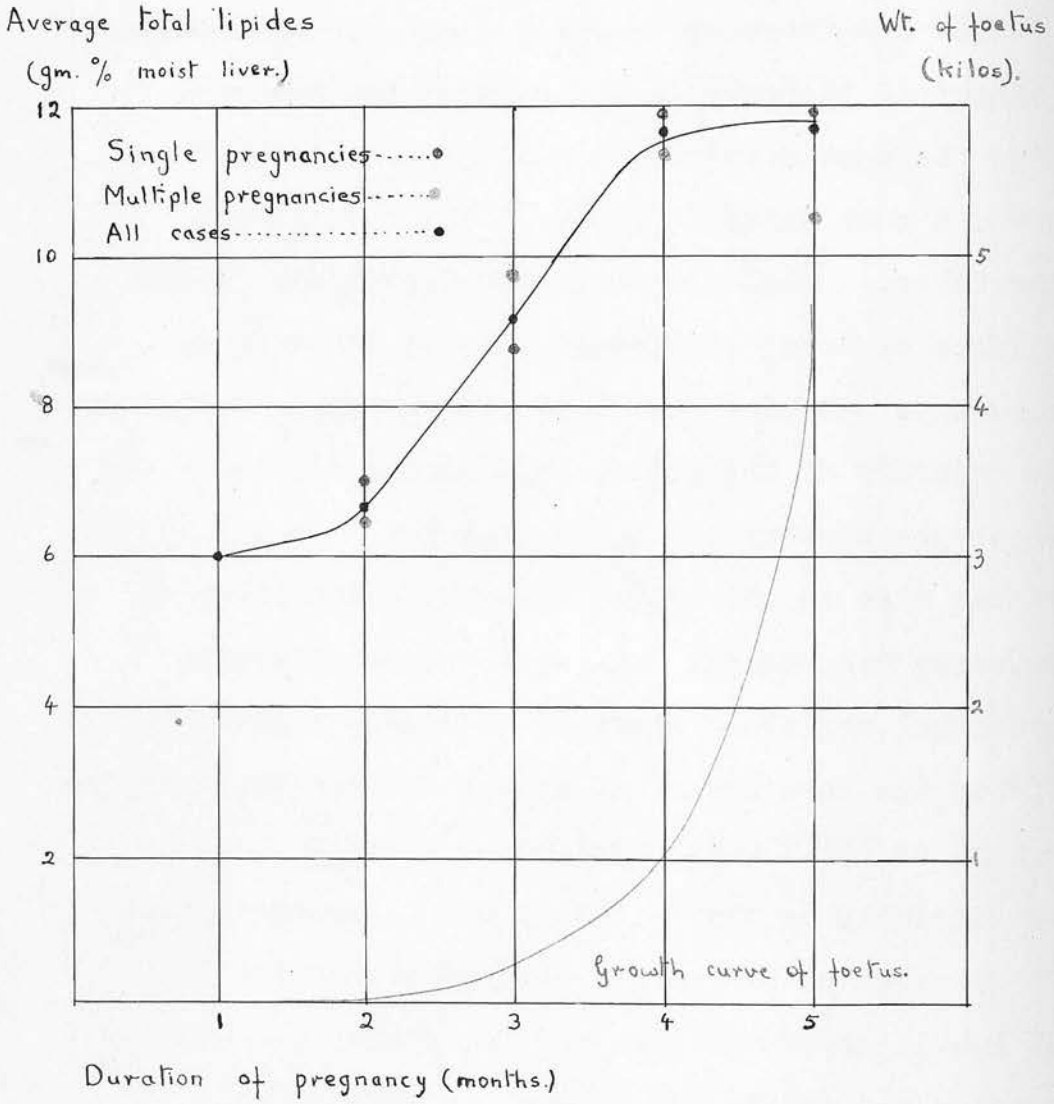
Fig. III


Total Lipides (in gm % moist liver).
20
18
16
14
12
10
8
6
4
2
20
40
60
80
100
120
140
160
Normal range for non-pregn Ewes.
Duration of pregnancy (in days).
Single pregnancies ..... .
Multiple pregnancies ..... .

variation than in the case of the moisture content, but the co-efficient of correlation is -0.72, showing a fair degree of relationship between the two factors.

The 60% increase in total lipides, representing a threefold increase in the neutral fat content, being the most significant feature of the figures shown, a more detailed study of the results was undertaken. Fig. III shows the individual lipide analyses of Group II plotted against duration of pregnancy. During the first two months of pregnancy the majority of the points fall within the range for non-pregnant ewes, but after that there is a definite gradual rise in the lipide content of the liver as pregnancy progresses. In spite of considerable individual variation there is obviously a relationship between the duration of pregnancy and the total lipide content of the liver. This is confirmed statistically by the χ^2 test, where, assuming that the duration of pregnancy does not affect the liver fat level, one finds that the difference between the observed and calculated values, as summed up by χ^2 is much greater than could be ascribed to chance. Thus $\chi^2 = 32.38$ and as $n = 4$, this gives a value of P much less than 0.01. Taking the conventional level of $P = 0.05$ as significant would indicate that/

Fig. IV


Average total lipides
(gm. % moist liver.)
Wt. of foetus
(kilos).
Single pregnancies
Multiple pregnancies
All cases
12
10
8
6
4
2
5
4
3
2
1
Growth curve of foetus.
1
2
3
4
5
Duration of pregnancy (months.)

that the original testing hypothesis was wrong, so that it is statistically highly probable that the duration of pregnancy does affect the liver fat level.

The marked individual variation is well shown in Fig. III, values as high as 19.5% total lipides (practically 60% of the dry matter of the liver) being obtained from apparently normal healthy pregnant ewes. On the other hand, at all periods of pregnancy a few specimens were within the range found for non-pregnant animals. Fig. IV shows the same results taken in monthly groups and the average total lipides plotted against duration of pregnancy. This shows more clearly the gradual increase in lipide content, commencing in the second month of pregnancy and proceeding to a maximum of about 12% in the fourth month, after which the average percentage of lipides remains fairly steady until parturition occurs.

Table VII

Comparison of Single and Multiple Pregnancies

Group II	Moisture Content %	Lipides present in gm./100 gm. moist tissue						Mean Molecular Weight	Iodine Value
		Phospho-lipides	Free Cholesterol	Cholesterol Esters	Other unsap. material	Neutral Fat	Total Lipides		
A Mean	66.6 *	3.36	0.237 ××	0.145 ××	0.245 ××	6.54 ××	9.73	290.3 δ	92.8 δ
63 Range	59-72	2.7 - 4.2	0.12 - 0.30	0.09 - 0.30	0.08-0.45	0.13-.16.0	4.32-19.53	280 - 301	70 - 110
single S.D.	14.2	0.3	0.054	0.051	0.130	4.30	3.91	75.3	26.95
pregnancies S.E. of mean.	1.79	0.038	0.008	0.0064	0.016	0.69	0.493	18.8	6.74
B Mean	67.2 ‡	3.35	0.213 ‡‡	0.141 ‡‡	0.23 ‡‡	6.74 ‡‡	9.75	294.6 δδ	89.8 δδ
27 Range	63 - 71	2.46 - 4.0	0.13-0.26	0.07 - 0.30	0.05-0.35	1.65-15.0	4.8-18.44	282 - 310	84 - 115
multiple S.D.	24	0.77	0.078	0.073	0.109	3.74	3.5	104.6	33.8
pregnancies S.E. of mean.	4.6	0.147	0.024	0.0218	0.033	1.15	0.674	34.8	11.3
Difference between means	0.6	0.01	0.024	0.004	0.015	0.20	0.02	4.3	3.0
Standard Error of difference between means	4.8	0.155	0.025	0.023	0.036	1.34	0.84	39.6	13.2

* Average of 23 specimens only
‡ Average of 9 specimens only
×× Average of 39 specimens only
‡‡ Average of 11 specimens only
δ Average of 16 specimens only

Effect of multiple pregnancy.

Many of the ewes carrying two or more lambs had livers containing large quantities of fat, and it seemed possible that it might be mainly these specimens which were responsible for raising the average fat content of the liver. However, a statistical comparison between the results for 63 ewes with only one foetus each and those for 27 ewes carrying two or more foetuses (Table VII) showed that with every constituent the difference between the means is much less than the standard error of the difference between the means, so that none of the differences observed between these groups can be regarded as having any significance. This is also shown graphically in Fig. IV, where the monthly average total lipides for single and multiple pregnancies are also recorded. It will be seen that the monthly average for multiple pregnancies was sometimes greater and sometimes less than that for single pregnancies. It would appear, therefore, that multiple pregnancy has no significant effect on the liver fat content.

Relationship of liver fat to foetal growth.

The increase in liver fat with the progress of pregnancy might be ascribed to the increasing demands of the foetus causing increasing fat transport from the depots to the liver and accumulation of fat in that organ. But the growth curve of the foetus, as obtained from some forty determinations of foetal weight (Fig.IV) shows that the maximum growth occurs in the last month of gestation, when the liver fat has already reached its maximum. During the third and fourth months of pregnancy, when the liver fat is increasing to its maximum level, there is only a small increase in the weight of the foetus, - viz., about 1 kilo. In the last month of pregnancy the weight of the foetus increases rapidly to 4 or 5 kilos, whilst the average liver fat content remains steady. Moreover, in the case of multiple pregnancies, the demands on the maternal organism are presumably greater than in single pregnancies, yet there is no significant difference between the two groups. A more likely explanation would be that some factor was causing a mobilisation of depot fat in the liver at a greater rate than that organ could deal with until the last month of pregnancy, when the greatly increased foetal demands stimulated utilisa-:tion of fat by the liver at about the same rate as it/

TABLE VIII.

Liver specimens from 6 cases of suspected Pregnancy Toxaemia.

	Moisture Content %	Lipides in gm/100 gm. fresh liver						Mean Molecular Weight *	Iodine Value *
		Phospho-lipides	Free Cholesterol	Cholesterol Esters	Other unsap. Material	Neutral Fat	Total Lipides		
Mean	69	3.49	0.228	0.135	0.15	8.45	12.64	290	93
Range	64-74.5	3.07-4.07	0.15-0.30	0.10-0.18	0.09-0.28	2.8-14.4	6.94-18.76	280-299	87-103
Standard Deviation	27.8	1.59	0.115	0.068	0.067	5.9	7.35	169	53.5
Standard Error of the Mean	11.4	0.65	0.047	0.028	0.027	2.4	3.0	69	21.8
Difference between means of Gp.I & III	1	0.07	0.032	0.035	0.005	6.59	6.6	10	8
Standard Error of Difference	12.3	0.66	0.052	0.031	0.035	2.54	3.2	71.3	22.6
Difference between means of Gps.II & III	2.2	0.13	0.002	0.005	0.009	1.86	2.9	2	1.3
Standard Error of Difference	11.7	0.651	0.052	0.029	0.029	3.47	3.1	70	22.4

* Average of four specimens only.

it was being brought in, so that the fat content then remained comparatively constant.

GROUP III.

Liver lipides in Pregnancy Toxaemia.

Only six cases of suspected pregnancy toxaemia became available during the period of the experiment, and in some of those cases the diagnosis was doubtful. The results are summarised in Table VIII and a contrast with the figures for Group I and II is also provided. The only significant difference between the means occurred in the neutral fat and total lipide fractions of Groups I and III. In spite of considerable individual variation, the average total lipide content of the liver in Group III (12.6%) was only slightly higher than the average (11.8%) for normal healthy ewes in the last month of pregnancy - the period when pregnancy toxaemia of sheep always occurs. However, as the diagnosis was not always certain and as previous observers (63 and 59) quote the liver fat content in pregnancy toxaemia as being usually about 15 - 20%, the significance of the above figures must remain doubtful.

TABLE IX.

Liver specimens from Castrated male ewes.

	Moisture Content × %	Lipides in gm. per 100 gm. fresh liver						Mean Molecular Weight ‡	Iodine Value ‡
		Phospho-lipides	Free Cholesterol	Cholesterol Esters	Other unsap. Material	Neutral Fat	Total Lipides		
Mean	68	3.45	0.27	0.17	0.145	7.55	11.6	297	91
Range	65-72	3.04-4.0	0.22-0.34	0.09-0.30	0.11-0.19	2 - 12.9	6.15-16.4	283-318	73-104
Standard Deviation	39.4	1.57	0.125	0.108	0.07	6.19	71	307	54.8
Standard Error of the Mean	19.7	0.64	0.05	0.044	0.028	2.52	2.9	125	22.4
Difference between means Gps.I & IV	2	0.11	0.01	0	0.005	5.69	5.56	3	10
Standard Error of Difference	20.4	0.65	0.053	0.045	0.034	2.64	2.93	150	23.5

× Only four specimens included.
‡ Only three specimens included.

GROUP IV.

Liver lipides of castrated male sheep.

During the preliminary investigation of non-pregnant animals six specimens were obtained from healthy wedders (castrated male sheep) slaughtered for human consumption. While not of direct interest to the investigation, it was noticed that the liver fat content in these cases (Table IX) was often markedly higher than for non-pregnant ewes, therefore they have been kept in a separate group. It will be seen that the findings were much the same as for pregnant animals (Group II). Here again, considerable individual variation occurred but there was a noticeable and significant increase in the neutral fat fraction, of the same order as that found towards the end of pregnancy. In spite of the small number of specimens in this group and the individual variation, the difference between the means for neutral fat in Groups I and IV is more than twice the standard error of the difference and is, therefore, of the order conventionally regarded as significant.

TABLE X.

Kidney lipides in non-pregnant ewes (10 specimens)

	Moisture Content %	Lipides present in gm/100 gm. moist tissue.						Mean Molecular Weight	Iodine Value
		Phospho-lipides	Free Cholesterol	Cholesterol Esters	Other unsap. material	Neutral Fat	Total lipides		
Mean	80	2.41	0.33	0.12	0.14	0.405	3.26	323	128
Range	74-86	1.86-2.8	0.19-0.61	0.08-0.22	0.05-0.27	0.08-1.88	2.85-4.08	317-328	120-132
Standard Deviation	27.1	0.85	0.126	0.06	0.083	0.44	0.115	162	90.4
Standard Error of the mean	8.58	0.27	0.04	0.02	0.026	0.14	0.036	72	40.4
Mean for Rabbit Kidney (3)	-	2.43	0.37	0.01	-	2.06	4.87		

Pregnancy and kidney lipides.

Fatty changes in the kidney are much rarer than those in the liver, though a diffuse fatty degeneration is occasionally met with in the general conditions which lead to fatty degeneration of other organs, e.g. anaemias, fevers, various septic and toxic conditions and various poisonings such as chloroform and phosphorus. Fatty infiltration has seldom been recorded.

According to some workers, in cases of pregnancy toxaemia of ewes the kidneys are also frequently observed to be pale and fatty. In view, therefore, of the findings with the livers of pregnant ewes, it was thought desirable to record the effect of pregnancy on kidney lipides.

GROUP V.

Kidney lipides in non-pregnant ewes.

The results of analyses of specimens from ten non-pregnant ewes are given in Table X. There are no previous figures for kidney lipides in the ewe, but the results obtained are similar to the findings in other species. (The figures quoted for six rabbits by Aylward and Stott(3) are appended for comparison).

Table XI.

Kidney Lipides in Pregnant Ewes (35 specimens).

	Moisture Content % ×	Lipides present in gm/100 gm. moist tissue						Mean Molecular Weight ×	Iodine Value ×
		Phospho-lipides	Free Cholesterol	Cholesterol Esters	Other unsap. material	Neutral Fat	Total Lipides		
Mean	79	2.23	0.32	0.125	0.132	0.607	3.36	323	121
Range	74.5-85	1.62-3.16	0.24-0.40	0.02-0.28	0 - 0.29	0.02-2.06	2.21-4.77	287-348	93 - 134
Standard Deviation	18.9	0.511	0.048	0.059	0.078	0.509	0.794	75	30.6
Standard error of the Mean	4.23	0.086	0.008	0.01	0.013	0.086	0.134	16.8	6.85
Diff. bet. means of IX & X	1	0.18	0.01	0.005	0.012	0.202	0.1	0	7
Standard Error of Difference	9.6	0.28	0.041	0.021	0.029	0.145	0.138	74	41

× Average of 20 samples.

TABLE XII.

Kidney lipides in suspected Pregnancy Toxaemia (2 specimens)

Mean	80.4	2.3	0.31	0.13	0.09	0.52	3.32	321	122

Kidney lipides in pregnant ewes.

Table XI shows the results obtained with 35 specimens from healthy pregnant ewes. There is a slight increase in the neutral fat fraction, mainly due to a few specimens near the end of pregnancy which showed a tendency to fatty infiltration. But the difference between the means is not much greater than the standard error of the difference, so that the change would appear to have little significance. In two cases of suspected pregnancy toxaemia also examined (Table XII) the analyses come within the normal range.

Histological Observations.

In the absence of direct determination of the total amount of fat present in the liver it is difficult to express an opinion as to whether the apparent increase in liver fat was absolute or merely relative due to shrinkage of the organ owing to loss of water and glycogen. This problem has been encountered by previous workers (29, 30 and 53) and various methods have been tried to throw light on the problem. The most suitable method available in the present experiment was the histological one. Small portions of tissue were examined using Sharlach Red or Sudan III to stain the fat globules. The picture obtained conformed to the biochemical findings. In nearly every case examined, an increased percentage of fat was associated with a like increase in the fat visible on histological examination, bearing out the likelihood that the increase in percentage was due to an actual increase in the fat content of the organ. The photographs shown in Plates 1 - 4 are typical examples of liver sections at different stages of pregnancy, showing gradually increasing fatty infiltration associated with a parallel rise in the percentage of liver fat as estimated chemically. They are from frozen sections of liver stained with Scharlach/

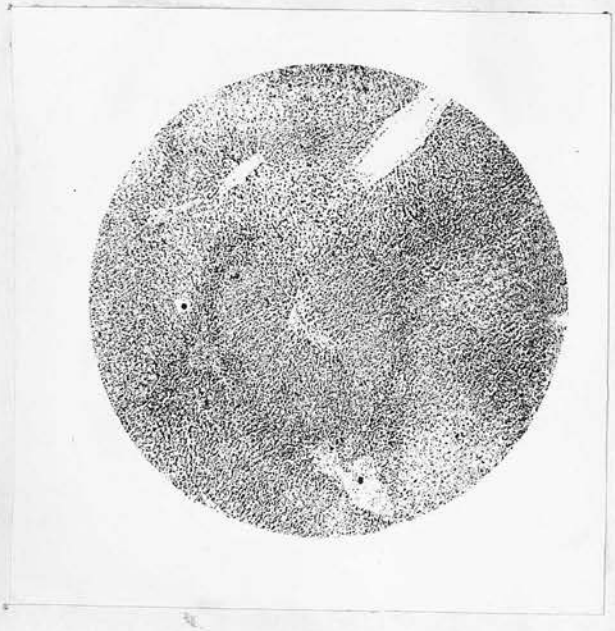


Plate I

Plate II

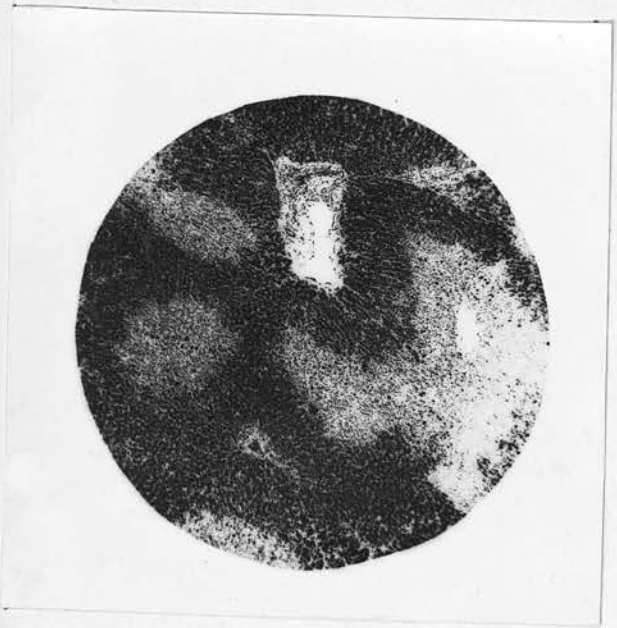


Plate III

Plate IV

Scharlach Red and photographed through a green filter so that the fat globules appear black. Plate I shows a section from the liver of a ewe sixty days pregnant where the liver fat(8.64%) was just at the upper range of normality for non-pregnant ewes. Only a slight trace of fat is visible at the edge of the lobules. Plate II is from a ewe eighty days pregnant where the liver lipides amounted to 11.6%. Here the accumulation of fat at the periphery of the lobules is much more definite. In Plate III, a section from the liver of a ewe 103 days pregnant in which the liver fat amounted to 16.8%, the fatty change extends half-way into the lobule. Whilst in a 125 day pregnant ewe, with total liver lipides amounting to 19.5% (Plate IV), practically the whole of the lobule is infiltrated with fat. The specimens thus show varying degrees of true fatty infiltration, commencing in the liver cells adjacent to the portal tracts, i.e. at the periphery of the lobule, and spreading gradually inwards, till in advanced cases the bulk of the lobule is infiltrated. No signs of cell degeneration were noticed in any of the specimens examined.

In the few kidney sections which showed fatty changes, viz., those from ewes near the end of their gestation period, the fat globules appeared mainly in the cells of the proximal convoluted tubules and in those of the limbs of Henle.

The effect of Pregnancy on Blood Lipides.

Investigation of fat metabolism during pregnancy has been approached largely through a study of blood lipides. Many different species have been examined and the effect has been found to vary greatly from species to species. Thus, Boyd (18) found quite a marked lipaemia in pregnant women, due almost entirely to an increase in plasma lipides, with over 100 per cent. increase in neutral fat and about 25 per cent. increase in phospholipides and free cholesterol. Similarly in guinea pigs it has been shown by Boyd and Fellows (19) and Harrison and McKay (40) that gestation produced quite a marked increase in all the plasma lipides except ester cholesterol, the lipaemia being much more marked than in human pregnancy; and in the mare, Brocq-Rousseau et al.(20) found quite a definite increase in total cholesterol, which was the only fraction studied.

On the other hand, in rabbits, according to Baumann and Holly (6) and Boyd and Fellows (19), a lipopaenia develops during pregnancy with quite marked decreases in the amounts of phospholipides and free cholesterol but little change in the neutral fat fractions. And cattle show a definite decrease in the lipoid P. (51) and in the cholesterol content (61) of the blood during the period of pregnancy. Whilst/

Whilst gravid rats (46) and dogs (6) appear to show little or no change in the blood lipides.

No records are available of the changes in the blood lipides of pregnant ewes, nor for that matter on the blood lipides of the non-pregnant ewe, and in view of the above species variation, it was impossible to forecast what changes might be encountered. Accordingly, it was decided to carry through a series of studies on the blood lipides of a number of pregnant and non-pregnant ewes kept under identical conditions. Twelve ewes, six being tupped and six untupped, were obtained and kept on coarse grazing supplemented with turnips and hay, whilst concentrates were also provided when grazing was poor.

Methods.

A blood sample was taken from each animal once a fortnight from January to April, six animals (three from each lot) being bled on alternate weeks. A Bloor extract (17) was prepared from each sample and determinations were made of:-

(a) Lipoid Phosphorus by the method of Stewart and Hendry (65)

(b) Free and Total Cholesterol by the method of Schoenheimer and Sperry (60).

(c) Total fatty acids by the titration method of Stewart and Hendry (65).

At/

TABLE XIII.

Blood constituents in Mg.% averaged over the period of experiment - non-pregnant ewes.

Animal No.	Glucose	Lipoid P.	Free Cholesterol	Total Cholesterol	Total fatty Acids	Haemoglobin	Ester Cholesterol	Phospholipide Fatty Acids.	Cholesterol Fatty Acids	Remaining Fatty Acids
1	63	8.4	90	109	204	10.1	19	109	13.3	82
2	57	8.8	89	112	183	10.3	23	114	16.7	52
3	59	8.5	98	106	194	10.2	8	110	5.8	78
4	59	8.6	87	99	202	10.1	12	112	8.7	81
5	59	8.7	93	98	181	10.2	5	113	3.6	64
6	64	8.4	87	99	199	10.7	12	109	8.7	81
11	54	8.2	85	88	202	11.7	3	107	2.2	93
12	57	8.8	91	108	197	11.2	17	114	12.3	71
Mean	59	8.55	90	102	195	10.6	12.4	111	9	75
S. D.	3.2	0.2	4.1	7.9	8.8	0.4	7	2.45	5	12.6

At the same time blood sugar estimations were made on the fresh blood by the Hagedorn and Jensen (31) method, and haemoglobin was estimated by the acid haematin method using a Klett colorimeter and Newcomer disc (54).

Results.

The results were somewhat limited by two factors. In the first place, it proved impossible to commence sampling before the second month of pregnancy. However, this was not necessarily serious, as the changes which have been recorded in other species have mainly been limited to the latter half of pregnancy; and the liver changes, on which it was hoped the investigation might throw some light, did not occur to any extent in the first two months of the gestation period. Secondly, at the end of the experiment it was found that only four of the tupped ewes had conceived, so that the balance of the groups was upset, being eight non-pregnant and four pregnant animals.

Blood lipides in non-pregnant ewes.

To get a rough idea of the significance of the results obtained, the figures for each animal were taken separately and the results for the individual constituents over the whole period of the experiment averaged. Table XIII shows this for non-pregnant ewes and includes both observed constituents and those calculated from the experimental results using the factors/

TABLE XIV.

Blood constituents in Mg.% averaged over the period of experiment - - - -pregnant ewes.

Animal No.	Glucose	Lipoid P.	Free chole- Psterol	Total chole- :sterol	Total Fatty Acids	Haemo- :globin	Ester chole- :sterol	Phospho- lipide Fatty Acids	Chole- sterol Fatty Acids	Remaining Fatty Acid
7	55	9.0	93	110	204	9.9	17	117	12.3	75
8	54	9.0	97	112	186	10.4	15	117	10.9	58
9	58	9.1	89	114	192	10.7	25	118	18.1	56
10	54	7.5	81	89	196	8.9	8	97.5	5.8	93
Mean	55	8.65(9.03)	90(93)	106(112)	194.5	10.0(10.3)	16.3(19)	112(117)	11.8(13.8)	70.5(63)
S. D.	2	0.77(0.06)	6.86(3.87)	11.6(2)	7.55	0.8(0.4)	6.7(5.3)	10.8(0.6)	5 (3.8)	17.7(10.4)

(Figures in brackets give averages omitting No. 10)

factors given for the lipides of human blood by Stewart and Hendry (66). (Whilst these factors are not necessarily strictly accurate for the sheep, they are probably near enough to give a basis for comparison.)

I have been unable to find any previous references to the lipides of sheep's blood. When compared with human blood, it is found that the majority of the constituents are present in amounts ranging from 60 - 80% of the corresponding figures for man. An exception is ester cholesterol, which is very low in sheep's blood, being only about one-quarter of the amount found for man.

Blood lipides in pregnant ewes.

The corresponding data for the four pregnant ewes are given in Table XIV. The comparison between the two groups of animals, already disproportionate in number, was somewhat upset by the fact that about the fourth month of pregnancy sheep number 10 developed marked clinical signs of anaemia and had to be treated with haematinic compounds. Even then it remained weak until the end of pregnancy and had to be assisted to get rid of a dead lamb. Accordingly, there were only three pregnant ewes which could be regarded as completely normal, so, in addition to giving the average figures for the four pregnant ewes, the corresponding data for the three normal animals (i.e. omitting No. 10) are included in brackets in those columns where they are significantly different.

Table/

TABLE XV.

Comparison between pregnant and non-pregnant groups.

	Glucose	Lipoid P.	Free cholesterol	Total Cholesterol	Total Fatty Acids	Haemoglobin	Ester Cholesterol	Phospholipide Fatty Acids	Cholesterol Fatty Acids	Remaining Fatty Acids
Difference between Means	4	0.1(0.48)	0 (3)	4 (10)	0.5	0.6 (0.3)	3.9 (6.6)	1 (6.3)	2.8 (4.8)	4.5 (12)
Standard Error of the Difference	1.49	0.38(0.08)	3.7(2.47)	6.5 (3)	4.9	0.4 (0.27	4.2(3.9)	5.5(0.94)	3.3 (2.6)	9.9 (6.8)

Table XV shows the difference between the means for pregnant and non-pregnant groups, along with the standard error of the difference between the means. Taking the averages for all four pregnant ewes, the most significant difference between the groups lies in the lower blood sugar and haemoglobin of the pregnant ewes, where the difference between the means is about twice the standard error of the difference. The difference in blood sugar level is unaltered if No. 10 be omitted, but the effect of such a procedure on the haemoglobin is to raise the value so that it is now not much lower than that of the non-pregnant controls. This was, of course, to be expected in view of the marked anaemia of No. 10 over part of the experiment. But the anaemia of No. 10 also involved a lowering of the average blood lipides in the case of those constituents which are present to a considerable extent in red blood corpuscles, viz., lipoid phosphorus, phospholipide fatty acids and free cholesterol. The exclusion of sheep No. 10 from the average in the case of these latter constituents reveals that the phospholipide content of the blood of the three normal pregnant animals was significantly greater than that of the non-pregnant group. The difference in the case of free cholesterol/

cholesterol was not so marked and is hardly large enough to be regarded as significant.

Ester cholesterol was also slightly larger in the three pregnant animals, but the difference again was barely significant. Total fatty acids remained much the same in both groups but, when allowance is made for fatty acids present as phospholipides and cholesterol esters, the remaining fatty acids - present presumably as triglycerides and soaps - are seen to be lower in the pregnant group, and almost significantly so if No. 10 be excluded.

Averaged over the whole period of the experiment, therefore, the only really significant differences between the three normal pregnant and eight control non-pregnant ewes lay in a 5 - 6% increase in the phospholipides and a 7% decrease in the blood glucose. The increases recorded in free and ester cholesterol and the decrease in neutral fats and soaps can hardly be regarded as significant in view of the small number of pregnant animals.

Graphical studies of the effect of pregnancy on the various constituents are recorded in Figs. V - X where the averages for the eight non-pregnant ewes are shown in green, those for the four pregnant ewes in red (unbroken line) and for the three normal pregnant ewes/

Fig. V

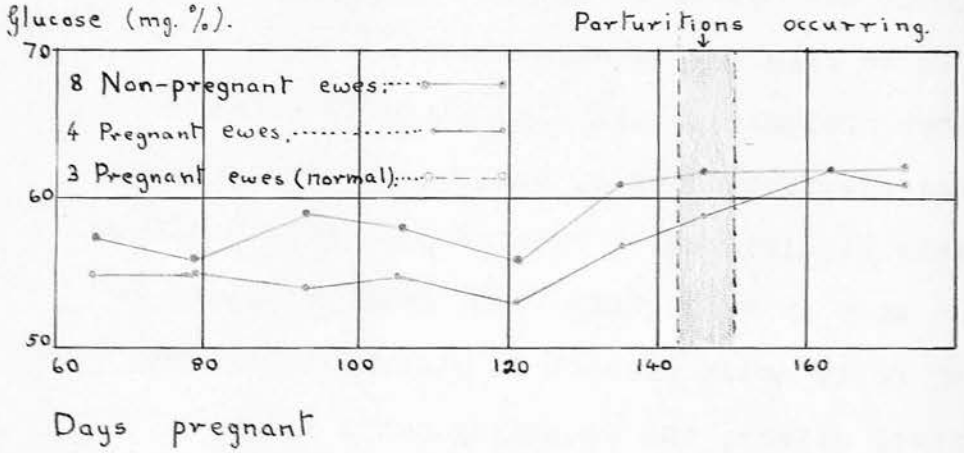


Fig. VI

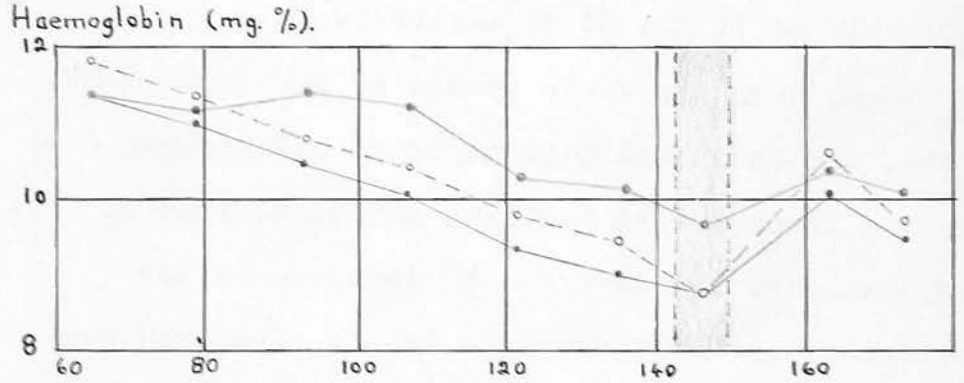
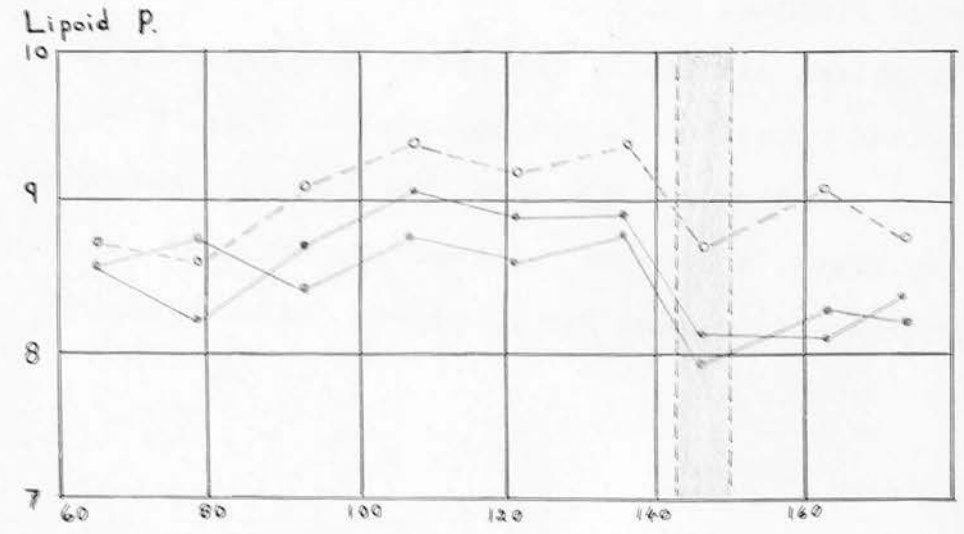


Fig. VII



ewes in red (interrupted line).

Blood glucose in the pregnant ewes (Fig.V) was consistently a few mgm. % below the level of the controls which it followed closely. There was no hypoglycaemia associated with the anaemia of No. 10, so that omitting the figures for this animal makes no difference to the pregnant group.

Fig. VI shows the haemoglobin figures for the various groups. There was a progressive fall in the haemoglobin level in all groups until the supply of concentrates was markedly increased about the middle of April. The haemoglobin level of the pregnant animals is lower than that of the controls, even when the aberrant No. 10 is excluded, but the difference is not significant in view of the individual variation. Other workers (4) have recorded a slight rise in the haemoglobin of pregnant ewes in the last three months of pregnancy, so that the fall recorded above is probably associated with dietetic deficiency.

In spite of the low haemoglobin and presumably associated low red blood corpuscle content of the blood, the lipoid phosphorus (Fig. VII) is higher in the pregnant animals than in the controls, even when sheep No. 10 is included. It would appear, also, that variations in lipoid phosphorus were roughly inversely related to those in blood glucose, the one usually increasing in amount as the other fell.

Ester/

Fig VIII

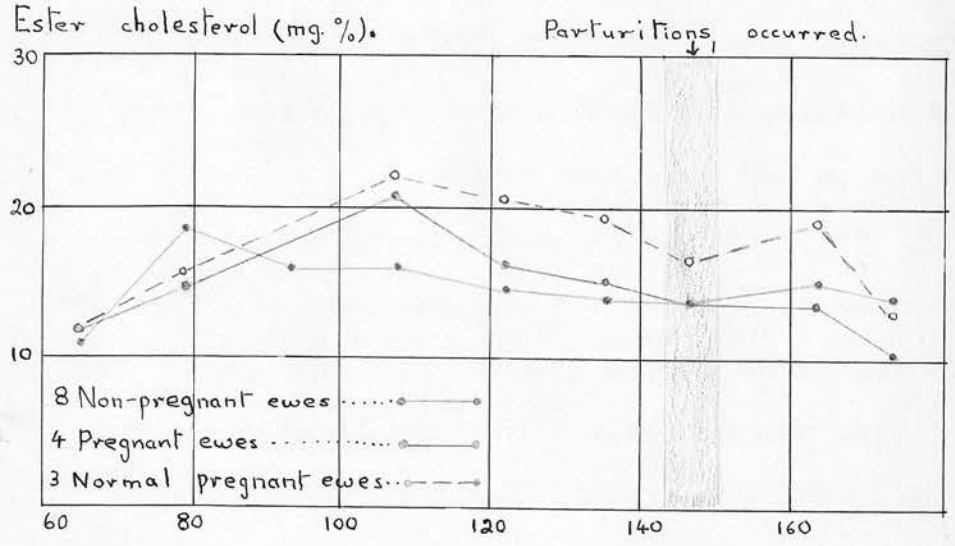
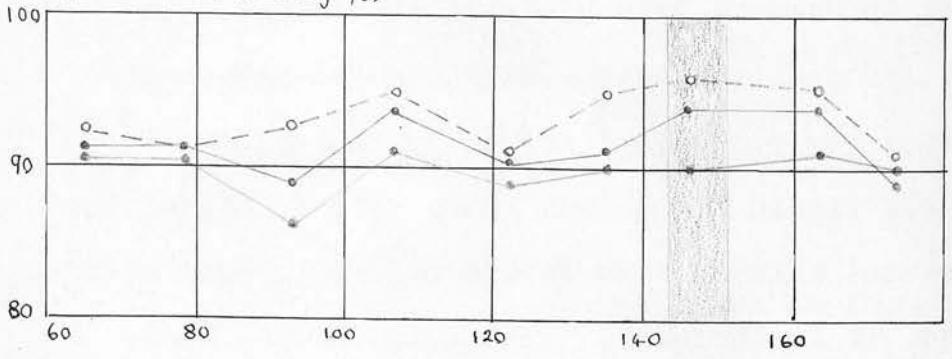


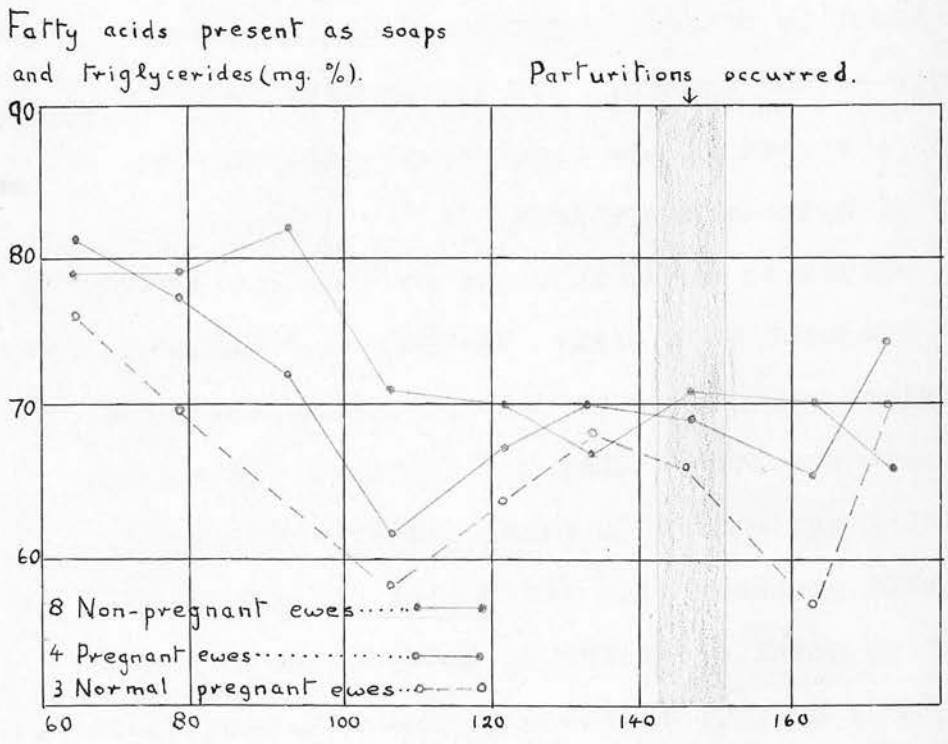
Fig IX



Ester cholesterol (Fig. VIII) would appear to be somewhat higher in the pregnant animals during the last two months of pregnancy, though this is somewhat masked by the fact that No. 10 had a low ester cholesterol as well as a haemoglobin deficiency. It seems unlikely that the low haemoglobin and low ester cholesterol can be directly related, since ester cholesterol is usually regarded as being present entirely in the plasma. It is possible that the changes observed in the blood of No. 10 were the result of hydraemic plethora, for an increase in plasma volume is known to occur to a slight extent in normal pregnant ewes (4). On the other hand, No. 10's blood glucose remained normal and neutral fats and soaps were somewhat higher than normal. Moreover, individual variations in ester cholesterol values, associated probably with defects in the method of estimating total cholesterol, are such as to render differences in this fraction of doubtful significance.

Free cholesterol (Fig. IX) is also slightly higher in the three normal pregnant ewes than in the controls, the low figures for No. 10 being presumably associated with the low haemoglobin, since probably some two-thirds of the free cholesterol is present in the red blood corpuscles. But the difference between the groups /

Fig. X


Fatty acids present as soaps
and triglycerides (mg. %).
Parturitions occurred.
90
80
70
60
8 Non-pregnant ewes
4 Pregnant ewes
3 Normal pregnant ewes
60
80
100
120
140
160
Days pregnant.

groups is even less well-marked than in the case of previous constituents and can probably be disregarded.

As will be seen from Fig. X, fatty acids present as soaps and triglycerides fell in both groups during February, rising again in March. The reason for this fall is obscure, but it may be a seasonal variation as Houchin and Turner (43) have shown that with rats the plasma fat was influenced by environmental temperatures, a low temperature leading to a fall in blood fat; and certainly during February, the sheep were exposed to long spells of cold weather with severe snowstorms. But it is unsafe to argue from species to species, and cattle, which are much more closely allied to sheep, do not appear to show any significant seasonal trends (1). The values for pregnant ewes were in the main somewhat lower than those for the controls, the difference being more marked when the high figures for No. 10 are excluded, and coming close to the range usually regarded as significant.

So far as the effect of pregnancy on blood lipides is concerned, changes which can definitely be ascribed to pregnancy are slight and therefore the sheep would seem to be most akin to those species such as rats and dogs which are said to show little or no change. The data obtained throw little light on the phenomena underlying the fatty infiltrations of the liver recorded/

recorded on previous pages, though the slightly higher blood phospholipides of the pregnant ewe might be taken to indicate increased transport or increased metabolism of fat.

The effect of fasting on Tissue Lipides.

As referred to in the introduction (page 3) evidence has accumulated to show that certain dietetic factors such as choline and various analogues of choline are of importance in preventing fatty infiltration of the liver and deficiency of these factors may lead to marked fatty changes in the livers of animals fed on diets rich in fat. Certain proteins and amino-acids have also a marked lipotropic action when added to diets which would otherwise produce fatty livers.

It seems unlikely that deficiency of any of these factors could be the cause of the fatty infiltration observed in pregnant ewes. The diet of the sheep concerned contained less than 2% of ether-soluble material and there was an adequate supply of protein provided. Moreover, the majority of non-pregnant ewes from which samples were obtained were on the same diet as the pregnant ewes or closely similar diets.

One factor which must not be overlooked is that of under-nutrition. The general conclusions of Snook (63) and of Fraser and his colleagues (33, 34) were that Ketosis and fatty infiltration of the liver in pregnant ewes are associated with continued under-nutrition or temporary lack of food, that they are inversely/

inversely correlated with the calorific value of the diet, and that multiple pregnancy accentuates the susceptibility of the ewe to dietetic deficiency. Thus, Snook (63) who obtained a few liver samples from the Cambridge sheep and noted quite an intense fatty infiltration on histological examination, suggested that the cause was "unsuspected under-nutrition associated with variable food consumption by the ewes". A scrutiny of the diet of the animals concerned shows that this is unlikely. They were grazed on good pasture all through the winter, and in addition each received a daily ration consisting of 1 lb. crushed oats, 3/4 lb. bran and 4 lb. mixed hay and chaff. The starch and protein equivalents of this extra ration amount to 1.8 lb. and 0.25 lb. respectively, which is ample for the requirements of a ewe even in the absence of grazing and is more liberal than the diet supplied by Fraser and his colleagues (33) to ewes fed to over-fatness.

All the pregnant sheep from which we obtained liver specimens throve well and put on weight normally throughout the duration of pregnancy. Further, they were fed until just before death, so that there is no question of fasting causing a sudden mobilisation of depot fat. Thus, though there was no check on the amount of food consumed by individual ewes, it is reasonably certain that the flock/

flock as a whole received ample supplies of food, so that the steady rise in liver fat, which is shown on an average of the large number of specimens examined, is not likely to be due to malnutrition. Also there was no evidence that multiple pregnancy accentuated the condition.

It is well known, however, that undernutrition has a bearing on liver fat content. Fatty changes in the liver associated with fasting were first demonstrated histologically by Statkewitsch (64) in guinea pigs, dogs and cats, less obvious in rabbits and least of all in pigeons. Nikolaides (55) confirmed his findings for dogs, and Gilbert and Jomier (36) had similar results with dogs and rabbits. An extensive series of experiments were carried out by Mottram (53), who found that in the rabbit and guinea pig starvation caused a great increase in the visible fat in the liver, which on chemical examination proved to represent an actual increase of from two to five times the normal content. He further showed that in many cases the increase in fat was an absolute one and not due to any shrinkage of the organ, and he expressed the opinion that its source was an infiltration of mobilised fat from the storage depots. Similarly, Dible (29) showed that in the case of the rat, starvation produced a considerable increase in the visible liver fat which a quantitative examination showed to represent a real and absolute increase.

Later/

Later (30) he demonstrated that the same condition prevailed in rabbits, whilst Best and Ridout (14) had similar findings with rats.

Whilst from the available evidence it was probable that the fatty infiltrations observed in pregnant ewes were not due to undernutrition, it seemed desirable to determine the effect of fasting on the liver fat of pregnant and non-pregnant ewes to see if there was any decided difference between the two groups which might help to account for the previous findings. A factor which hampered an extensive survey, however, was the expense of the animals involved and this rendered it undesirable to use methods involving slaughter if they could possibly be avoided. It was decided, therefore, to try and obtain the necessary specimens of liver using the technique of partial hepatectomy.

Partial Hepatectomy in Sheep.

Partial hepatectomy is a procedure quite commonly employed on dogs and smaller experimental animals and has also been tried out on pigs (70). It has not, however, been regarded as a feasible proposition for ruminants, partly because they are not good subjects for general anaesthesia, so that abdominal operations are regarded as involving considerable risk; partly because of the anatomical position of the liver - pushed cranially and to the right under cover of the ribs by the bulky complex stomach.

In view of the important results which might accrue from the successful adaptation of this technique to ruminants - for it places a valuable extra tool in the hands of biochemists studying the various aspects of metabolism in which the liver is involved - it was thought worth while experimenting with the technique. The question of anaesthetics was solved by the use of intravenous injections of nembutal. A 7% solution was employed and injections were made very slowly into the saphenous or jugular vein until complete relaxation occurred. Usually 15 - 20 c.c. were necessary, representing 30 mg. nembutal per kilo of body weight, which is of the same order as for other species. The injection was spread out over several minutes so as to minimise the risk of sudden fall in blood pressure/

pressure which intravenous injections of nembutal tend to produce. A satisfactory degree of anaesthesia could be readily attained and, though the effects tended to wear off in about 35 - 45 minutes, it was found possible - after a little practice - to complete the operation without further administration of anaesthetic.

The anatomical problem proved more difficult. It was impossible to reach the liver from the left side owing to the interposition of the bulky rumen and reticulum. Operations in the midline also proved unsatisfactory, as the liver seldom reaches there and also, since the sheep was usually on its feet and moving about normally within an hour or so after the operation, there was considerable risk of the sutured muscles giving way under the weight of the abdominal contents, thus producing a ventral hernia. It was found necessary to confine the operation to the right flank and even there the scope was limited by the fact that in the dorsal region mobility of the liver was greatly impeded by attachment to the right kidney and the surface of the reticulum. The greatest mobility of the organs was found to be about midway down the right flank, and even here it was seldom possible to bring it out beyond the costal arch. However, in this /

this region the right border of the liver was fairly thick, rendering it possible to remove several grammes of liver without going deeply into the tissue. The procedure finally adopted therefore was as follows:-

An incision 2 - 3 in. long was made through the skin on the right hand side, parallel to the costal arch and about half-an-inch caudal to it, in the region of the last costo-chondral junction. The skin incision revealed the fibres of the external oblique muscle running more or less horizontally underneath. The incision through this muscle was made parallel to the fibres, i.e. at right angles to the previous incision, and the fibres retracted to expose the underlying aponeurosis of the internal oblique muscle. This was incised parallel to the costal arch as also the underlying transverse abdominal muscle. Finally, the peritoneum was opened taking care not to damage the viscera. The liver lay slightly cranial to the costal arch and was not mobile enough to be brought out through the opening. But it was possible to grasp it with the fingers and bring it fairly near the opening, where a piece weighing some 4 - 5 gms. could be snipped off with scissors. In the case of fatty livers, it could be easily broken off by hand, as the tissue in those cases proved very friable and, indeed, pieces often came/

came away when attempts were made to bring the edge of the liver close to the abdominal opening.

After the portion of liver had been removed, the cut surface was gripped tightly with gauze swabs for a few minutes to control haemorrhage, which was usually slight. Then the abdomen was closed by suturing the several layers separately, the skin wound being finally protected by a large swab soaked in Friar's Balsam and lightly sutured to the skin. The animal was usually on its feet again and feeding in a few hours. The whole procedure was carried through with stringent precautions against infection and, in spite of the difficulty of protecting wounds in animals from contamination, no trouble with sepsis was encountered. Adhesions occasionally occurred, which hampered subsequent operations a little, but the only untoward sequel was the development in two cases of abdominal herniae which required further surgical intervention to restore the animals to normal.

The effect of fasting on liver lipides.

Sheep are not very suitable animals for use in experiments where it is desired to estimate with accuracy the duration of a fast, for the capacity of the stomach is such that it continues to digest food for a considerable and very variable period after this has been withdrawn. According to Ritzman and Benedict (58) the post-absorptive condition is not usually reached in the ruminant until the fourth day of fasting. This appeared to be confirmed by a preliminary series of experiments which showed that on the fourth day of fasting the liver fat of sheep A (Table XVI) was quite normal in amount, whilst six days fasting produced a marked increase in liver fat. A standard fasting period of six days was therefore adopted, as it was felt that this would usually give one or two days of actual fasting. Moreover, it was found impossible to carry out the operative technique without some reduction in the contents of the rumen and therefore, in order to get specimens of normal liver, the animals were usually starved for 24 hours prior to operation. During this period the animal would undoubtedly be deriving nourishment from the food stored in its rumen, and therefore such a procedure should have little effect on liver fat.

Method/

TABLE XVI.

Effect of Fasting on Liver Fat.

Non-pregnant Sheep.

Number	Type	Duration of Fast	Liver fat in gm/100 gm. moist liver.		Increase (in gm/100 gm. moist liver)	Increase as % of original liver lipides.
			Before fasting	After fasting		
A	Castrated male	4 days	-	6.4	-	-
A	ditto	6 days	6.3	20	13.7	218
16	Lactating ewe	6 days	7.4	7.5	0.1	1
12	Non-pregnant ewe	6 days	7.5	9	1.5	20
Mean					5.1 ± 7.5	80
Pregnant Sheep.						
11	100 days pregnant	6 days	8.1	10.1	2	25
6	110 days pregnant	6 days	18.8	20.4	1.6	8.5
2	135 days pregnant	6 days	8.3	24.4	16.1	194
Mean					6.6 ± 5.3	76

Method.

The animal was starved for 24 hours and then 4 - 5 gm. of liver were removed under nembutal anaesthesia and the total lipides estimated. The animal was then allowed to recover from the operation for about a week before the actual fasting began and during this time it fed normally. Then it was fasted for six days, at the end of which period a second piece of liver was removed and the total lipides once more estimated. Three pregnant and three non-pregnant ewes were thus examined.

Results.

It will be seen from Table XVI that the mean increase in liver fat for the pregnant ewes was somewhat greater than that for the non-pregnant group, though when expressed as a percentage of the liver lipides originally present the reverse is true. But the standard deviation in both groups is large, so that the difference between the means (1.5 gm.%) is much less than the standard error of the difference (5.4gm.%) and can therefore be regarded as insignificant.

It will be observed that there is a great variation within each group. This has been found also by workers with other species. Thus Mottram (53) encountered some irregularities in his results with rabbits and concluded that there were periodical variations/

variations in the fat content of the liver during starvation. Similarly Dible (29) found with rats that the increases were inconsistent and uncertain and seemed to bear no relation to the duration of the fast. His results indicated rather that the degree of fatty infiltration in the liver during starvation depended more on the quantity of fat in the storage depots than on the length of the fast. Later (30) he reported similar findings with rabbits. Deuel et al.(28) found that with rats fasting was followed by considerable increase in liver lipides if the rats had previously been fed on a high fat diet, whilst after a normal diet they found only slight increases during fasting. Also Best and Ridout (14) found that accumulation of fat in the liver during starvation was an extremely variable phenomenon in the rat and that it was governed to a considerable degree by the previous diet of the animals.

Their conclusions are borne out to some extent in the above experiments. Sheep A was a large and thriving animal which had been fed exceedingly liberally for a considerable period prior to the experiments and sheep No. 2 was also in very good condition. Sheep No. 6, on the other hand, was in poor condition and very weak, whilst sheep No. 16 was only in moderately good condition. It would appear, therefore /

therefore, that the state of nutrition was more important than pregnancy in influencing the effect of fasting on liver fat.

Fig. XI

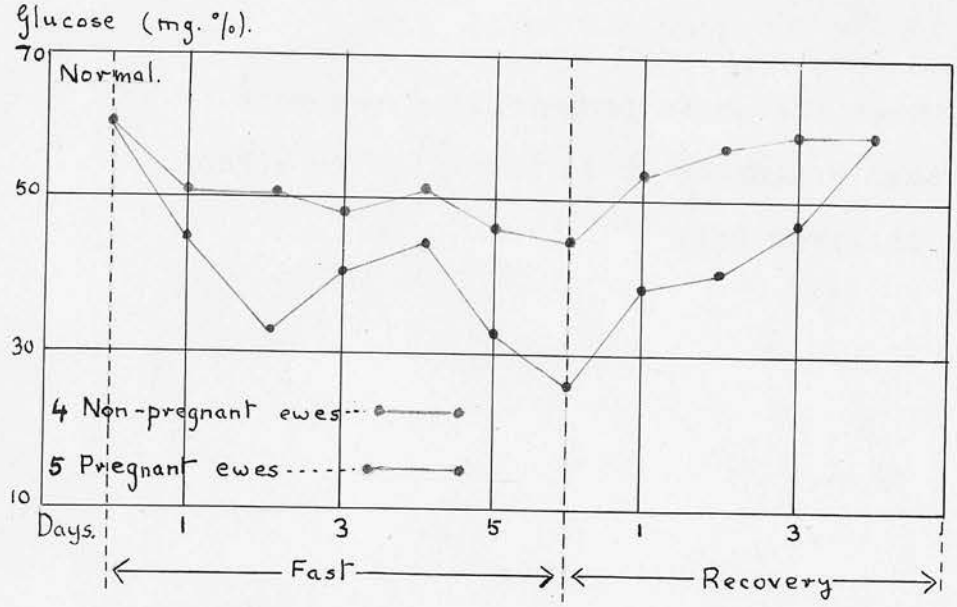


Fig. XII

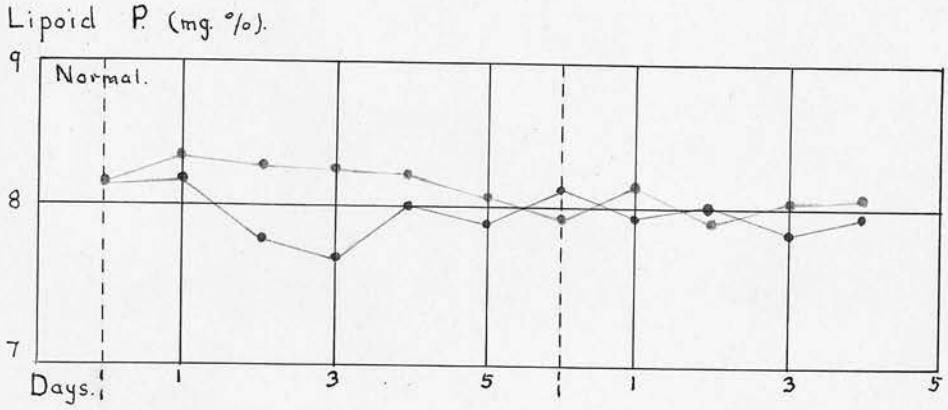
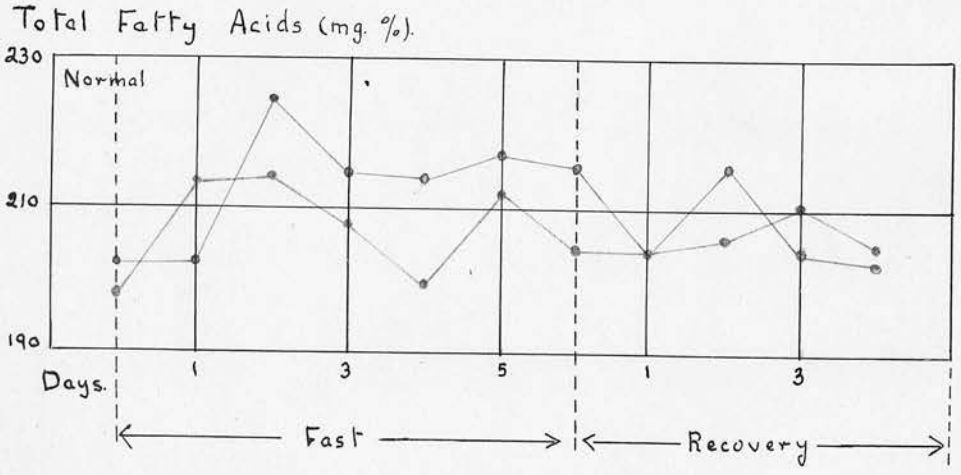


Fig. XIII



The effect of fasting on blood lipides.

Advantage was taken during these experiments of the opportunity to study the effect of fasting on blood lipides. Samples were taken daily during the six-day fast period and for a few days after, and analysed for blood glucose, lipoid phosphorus and total fatty acids. The averages for four non-pregnant sheep (green) and five pregnant ewes (red) are shown graphically in Figs. XI - XIII.

The findings for blood glucose are given in Fig. XI, which shows that the fall during fasting in the case of the pregnant ewes is much greater than for the non-pregnant. Both groups show a distinct rise in the middle of the fasting period, about the time when the post-absorptive state would normally be reached. The cause of this peculiar phenomenon is at present obscure, but the change is quite significant in spite of the small size of the groups.

Lipoid phosphorus (Fig. XII) is variable but never falls below the normal range and is not significantly different in the two groups. Total fatty acids are, however, much greater in the pregnant animals (Fig. XIII) especially in the early part of the fasting period. They would appear to a certain extent to be inversely correlated with the blood glucose, the one rising as the other falls.

These/

These findings are in marked contrast to the results during pregnancy in normally fed animals, in which case, as has been shown previously, the lipoid phosphorus was significantly elevated whilst the total fatty acids remained about normal. It may be, therefore, that the upset in fat metabolism during fasting was different in nature from that occurring during pregnancy, which would be further evidence that some other factor than undernutrition was at work in the latter case.

D I S C U S S I O N.

From the investigations reported, it would seem that in the sheep fatty infiltration of the liver is not necessarily limited to pathological conditions. My findings show that such infiltrations are quite common in apparently healthy pregnant ewes, the average total lipides being about 10%(range 4.3 - 19.5%) of the moist liver tissue as compared with 6% (4.3 - 8.7) for non-pregnant animals, these results being in contrast to the claims made by Snook (63). The increase is mainly in the neutral fat fraction and appears to be a gradual process, commencing about the second month of pregnancy and rising to a maximum about the end of the fourth month, after which the liver fat level remains comparatively constant until parturition at the end of the fifth month.

It is not easy to ascertain to what extent this apparent increase is due to shrinkage of the organ from loss of water and glycogen, but the fall in iodine value of the fatty acids, combined with the histo-:logical evidence of a true infiltration spreading inwards from the portal tracts, suggests that much of the change is due to actual transference to the liver of saturated glycerides from the fat depots. Attempts to verify this by a study of blood lipides during pregnancy were largely negative. The only significant change/

change was a 5 - 6% increase in blood phospholipides during the last three months of pregnancy, which might on current theories be taken as an indication of increased transport or increased metabolism of fat. The sheep would appear, in fact, to be mainly analogous to species such as rats and dogs, which are said to show little or no change in blood lipides during gestation.

In the last few years, research has revealed many unsuspected factors associated with fatty changes in the liver. These have already been discussed but will be rapidly reviewed, in order to see to what extent they might account for this fatty liver of pregnancy. Deficiency of choline and its analogues may lead to intense fatty infiltration of the liver, should the diet be rich in fat, but these factors are hardly likely to be involved in such changes in the sheep where the fat content of the diet seldom exceeds 2 - 3%. Excess of cholesterol in the diet may be dismissed, as, in addition to increasing the neutral fat fraction, it also produces a remarkable rise in the cholesterol content of the liver, whereas in pregnancy, liver cholesterol remains within normal limits. Deficiency of protein or of specific amino--acids cannot be altogether ruled out, but they do not play/

play an important role in the etiology of fatty livers save when the diet is rich in lipides. And, in considering all such dietetic deficiencies, it is well to remember that the non-pregnant controls were on the same diets as the pregnant ewes or on closely similar diets.

There must also be considered the possibility of fasting causing an increase in the liver fat. Some workers such as Snook (63) and Fraser and his colleagues (33, 34) are firmly convinced that fatty infiltrations of the liver, such as are met with in ewes suffering from pregnancy toxaemia or in pregnant ewes with an experimentally-induced ketosis, are associated with malnutrition; and they suggest that fatty changes in the liver during pregnancy are unlikely, unless some such abnormal condition be present.

As regards pregnancy toxaemia, I have found no evidence of the liver lipides in this condition being much above the average for apparently healthy pregnant ewes in the last fortnight of gestation, the period when the disease normally occurs. As there were no cases of illness recorded amongst the pregnant animals from which specimens were obtained, and as the maximum degree of fatty infiltration was reached by the end of the fourth month, i.e. before pregnancy toxaemia usually/

usually appears, it can be dismissed as a causal factor.

In the absence of direct information as to the food consumption of individual animals it is not so easy to rule out the possibility of mild ketosis due to malnutrition, which Snook claims to be associated with fatty infiltration of the liver. There is, of course, a history as to the diet, health and condition of the animals, which there is no reason to doubt, but the possibility is one which warrants further consideration.

Accepting the explanation put forward by Snook (63) that these fatty livers were really the effect of malnutrition, due to "variable food consumption by the ewes", certain consequences follow. One would expect, for example, that the condition would be most acute during the last month of pregnancy when foetal growth is very rapid, but the maximum degree of infiltration has usually been reached before that time. And, according to Fraser et al (32),"a caloric deficiency in the diet will produce ketonaemia in pregnant ewes more easily in multiple than in single pregnancies". But a perusal of the data presented in earlier pages shows that multiple pregnancy has certainly no aggravating effect on fatty infiltration, in spite of the greater demands on the maternal organism.

Snook's/

Snook's theory presupposes that the effect of fasting will be greater in pregnant than in non-pregnant animals and, so far as the production of ketonaemia is concerned, the experiments of Fraser and his colleagues show this to be the case. And it is a fact that fatty livers can occur in pregnant ewes and yet be non-existent in non-pregnant animals under the same conditions of feeding. If Snook's theory of undernutrition as the cause of this also, be correct it means that its effects must be greater in pregnant than in non-pregnant animals - quite a plausible assumption. But a study of the effects of fasting on liver fat by the method of partial hepatectomy has shown that the increase is no more marked in pregnant ewes than in non-pregnant controls. Both groups showed an increase in liver fat over a period of five to six days fasting, amounting to 5 - 6 gm. per 100 gm. moist liver, i.e. about 70 - 80% of the original liver lipides. And great individual variation was observed, the intensity of the change being apparently related to the condition of nutrition of the animal prior to fasting. The blood lipide picture during fasting was very different from that in pregnancy, the fatty acids being greatly increased in amount while the phospholipides remained unaltered. From

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a consideration of these facts along with general evidence as to the diet and condition of the pregnant animals, whose liver lipides were studied, it seems unlikely that undernutrition played any important part in the causation of this fatty infiltration of pregnancy.

One aspect which has not yet been considered is the role of endocrine organs in the control of liver fat in the pregnant ewe. It is well known that anterior pituitary extracts can produce an increase in liver lipides in various species (7, 8, 48, and 54). Moreover, Anselmino and Hoffman (2) found that extracts of the anterior pituitary of sheep contained large quantities of a "fat metabolism hormone" which produced fatty infiltration of the liver. And castration of sheep, which has presumably some associated endocrine disturbances, has been shown in earlier pages to be accompanied by a definite increase in liver fat.

As my findings lend no support to a dietetic cause for the liver changes observed in pregnant ewes, it seems quite possible that they may be associated with hyperactivity of the anterior pituitary during pregnancy. It was intended to investigate this aspect , also, by the method of partial hepatectomy, but/

but owing to war-time restrictions it proved impossible to get supplies of suitable material. All that can be said at the moment is that, of the various factors known experimentally to be associated with fatty infiltration of the liver, this is the only likely one which has not been eliminated.

S U M M A R Y.

(1) Methods of lipide analysis have been surveyed and suitable techniques adopted for a study of tissue lipides in domestic animals.

(2) Data are presented for liver lipides in twenty-five non-pregnant and ninety pregnant ewes, showing that fatty infiltration of the liver is quite common in apparently healthy pregnant animals. These findings are analysed in detail.

Analyses of kidney specimens from the same animals are also recorded.

(3) The effects of castration and of pregnancy toxaemia on tissue lipides have been examined and discussed.

(4) Original data are given for blood lipides in the sheep, along with a survey of the effects of pregnancy and of fasting on the various fractions.

(5) The operation of partial hepatectomy has been successfully adapted for use in ruminants and applied to a study of the effect of fasting on liver fat.

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