Nutritional effects of autoxidized fats in animal diets

Beef fat in the diet of rats

By C. H. LEA and L. J. PARR
Low Temperature Research Station, Cambridge
and J. L. L'ESTRANGE and K. J. CARPENTER
School of Agriculture, University of Cambridge

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There are many reports in the literature, some of them considered in a recent series of reviews (Schultz, Day & Sinnhuber, 1962), demonstrating that feeding with rancid fat can in some circumstances have growth-depressing or other unfavourable effects on experimental animals. Thus the practice has grown up of characterizing the fatty fraction of animal feeding-stuffs by simple procedures (in the past these have been the determination of free fatty acids and peroxide value) and rejecting materials that gave values above certain limits.

However, there seems no evidence that free fatty acids as such are harmful to animals (though they may reflect the existence of mouldiness in certain classes of materials); moreover, the permitted levels of 'peroxide' have been only a small fraction of the high levels found harmful in diets under experimental conditions.

It is known that the presence of oxidizing fat in the diet may cause severe losses of easily oxidizable vitamins, particularly E and A, when these are not provided in stabilized form. However, there is little direct experimental evidence for harmful effects arising from the use of fats, or fat-containing ingredients, within the range of oxidation likely to be encountered in commercial samples and in well-balanced rations prepared with the precautions now available to the compounder. Practical people, nevertheless, have the impression from field observations that poor results are often seen with rations containing unsaturated fat that have been stored for prolonged periods.

In a previous paper (Carpenter, Lea & Parr, 1963) we have reported the last of a series of experiments with herring meal (containing approximately 17% lipid, that is, the chloroform–methanol extract), which had been allowed to oxidize during storage before it was given at a level of about 18% to chicks. There was no evidence in these short-term experiments with generous vitamin supplementation of any direct toxic effect of the oxidized fish oil; the slight depressing effect on weight increase observed with the stored, compared with the fresh, meal accompanied the non-lipid rather than the lipid fraction on separation. Small losses of available lysine had previously been shown to result from an interaction between the protein and lipid oxidation products in stored herring meals (Lea, Parr & Carpenter, 1958, 1960). Moreover, addition to the chicks' diet of 2·5% of herring oil pre-oxidized to a peroxide value of 142 μmoles/g

(284 m-equiv./kg) failed to depress weight increase. Weanling rats receiving 14% stored herring meal as sole source of protein did, however, show a reduced appetite quotient, about 90% of that found with the fresh meal, as well as a slightly lower protein retention efficiency (60, compared with 64).

Several recent investigations quoted in the earlier paper (Carpenter *et al.* 1963) have shown that lipids extracted from stored herring meals and consumed at reasonable levels do not depress the rate of weight gain of chicks and that solvent extraction of commercial herring meals has little or no effect on their value in chick diets. Dreosti & Wiechers (1962) have also found that vitamin A accumulation in the liver was not affected when stored pilchard meal (containing 8% hexane-extractable oil) was given at a 10% level to chicks.

There is no doubt, however, about the adverse effects on health and nutrition when fats that have been oxidized or heated or both are consumed at high levels. March, Biely, Claggett & Tarr (1962) depressed increase in weight of chicks by adding 10% of a chloroform–methanol extract of herring meal to a vitamin-deficient diet, though the effect could be overcome by adequate fortification with vitamins. Rasheed, Oldfield, Kaufmes & Sinnhuber (1963) produced steatitis and enlarged livers in rats with menhaden oils of peroxide value 125–310 (units not stated, but believed to be m-equiv./kg) consumed at a 10% level and found that even the 'fresh' clay-bleached oil at a 15% level caused similar signs and ultimately death, apparently owing to its rapid autoxidation in the 'synthetic' diet during exposure in the feeding pans before consumption. No vitamin A was added to these diets. In similar experiments with pigs (but with added vitamin A), Oldfield, Sinnhuber & Rasheed (1963) found lower food intakes and weight gains with fresh menhaden oil (peroxide value 2·6) at the 10% level, compared with lard (peroxide value zero), and these differences increased progressively as the degree of oxidation of the fish oil was increased to peroxide values 15·5 and 61. Mokadi & Budowski (1963) produced a high incidence of encephalomalacia in chicks with oxidized sunflower oil, but the oil used had been oxidized at 180° until its iodine value was reduced by at least 13 units, and it was given at a 20% level.

The ingestion of any considerable quantity of polyunsaturated fat, whether of marine-animal or of vegetable origin, is known to increase greatly the tendency towards in vivo peroxidation of tissue lipids and vitamin E deficiency and to increase the requirements for tocopherol or other biologically active antioxidant. The total quantity of fat contributed by the fish-meal component to any practical diet is, however, usually only of the order of 1–1·5%. Fats incorporated into compound rations (usually for broiler chickens) are generally of land-animal origin and much less highly unsaturated, and the amount used rarely exceeds 5%. Depression of weight increase has, indeed, been demonstrated by giving autoxidized beef tallow to rats, but the amount used and the degree of oxidation of the fat greatly exceeded anything likely to be encountered in animal feeding: Kreier, Reber & Norton (1961) used 20% of beef fat of peroxide values > 500 m-equiv./kg.

It seemed of interest, therefore, to compare the performance of animals raised on diets containing (*a*) 5% of fresh beef fat, (*b*) 5% of fat autoxidized to a peroxide value

considerably higher than any likely to be encountered in fats prepared commercially for animal feeding, but less severely treated than most fats that have been used to demonstrate toxicity in the literature, and (*c*) fat oxidized as in (*b*) above but subsequently treated to destroy its peroxide content, either by steam deodorization or by the cheaper process of heating to a sufficiently high temperature in the absence of air. Since an antioxidant would normally be incorporated in a fat used for animal feeding, 0·02% of BHT (2,6-di-t-butyl-4-methyl-phenol) was added to each before mixing it in the diet.

High levels of various dietary supplements, particularly vitamin E, have been reported sometimes to mitigate or prevent the deleterious effects of oxidized fats (March *et al.* 1962; Rasheed *et al.* 1963; Oldfield *et al.* 1963), but not always (Kreier *et al.* 1961). In the work described in this paper, levels believed to be adequate but not excessive under normal conditions, and similar to those that would be employed in practical feeding, have been used. Vitamin A was present in the diets in the gelatin-enrobed form that is known to be one of the most stable and best utilized (Olsen, Harvey, Hill & Branion, 1959; Ascarelli & Senger, 1962).

In brief, little if any adverse effect from the inclusion of oxidized beef fat in the diet under these conditions has been noted. It is hoped in future work to carry out a similar experiment with chicks or turkey poults.

EXPERIMENTAL

Animals

For each of two experiments, twenty-four weanling piebald rats were divided into three sets by weight and sex, and then each set was randomized amongst four treatments, so that each treatment was given to two rats in each of three cages, namely, two pairs of males and one pair of females. A further six rats from the litters used for the experiment were killed immediately and stored at $-20°$ for later analysis of their livers at the same time as those of the experimental animals.

In Expt A the heaviest set consisted of eight rats 29–31 days old and weighing 54–78 g at the beginning of the experiment; the remaining rats were 24–26 days old and weighed 40–48 g. In Expt B all the weanlings were 21–24 days old at randomization and weighed 32–49 g.

Up to the time of randomization the young were kept with their mothers and had access with them to a commercial cubed diet. After randomization the rats received the experimental diets *ad lib.* as dry powders. The temperature of the rat room was maintained at 20°. Each experimental diet was prepared as a single batch on the day before the beginning of Expt A and then stored at 20° in the presence of air, except for small portions canned under nitrogen and held at $-20°$ for subsequent analysis of the diets in their fresh condition. Each experiment continued for 6 weeks, and Expt B began a week after Expt A ended, so that the diets remaining at the end of both experiments had been stored for 13 weeks.

Experimental diets

Each diet had the percentage composition: beef fat (prepared differently, as explained below, for each treatment but always with 0·02% BHT) 5, ground whole wheat 45, ground barley 15, extracted soya-bean meal 15, white-fish meal 10, dried skim milk 3·33, unextracted dried yeast 1·67, ground limestone 1·67, steamed bone flour 1·13, common salt 0·53 and a vitamin–starch concentrate 1·67. The vitamin–starch concentrate was made up of maize starch, pure riboflavine, Rovimix A 50 (Roche Products Ltd, Welwyn Garden City; containing 50000 i.u. vitamin A/g in a gelatin-based powder), Rovimix D3-100 (Roche Products Ltd; containing 100000 i.u. vitamin D_3/lb) and Rovimix E (Roche Products Ltd; containing 10% α-tocopheryl acetate) in proportions designed to contribute 4 mg riboflavine, 2667 i.u. vitamin A, 5333 i.u. vitamin D_3 and 60 mg vitamin E to each kg of diet. These levels were chosen so as to be adequate for the rat ((USA) National Research Council: Committee on Animal Nutrition, 1962) without providing any large excess of either vitamin A or vitamin E.

Fats used

Beef fat was rendered in the laboratory from fresh beef brisket adipose tissue, filtered and vacuum-dried. This fat had a lower setting-point (which facilitated uniform mixing into the diet) and a higher iodine value than the average tallow, which would include a proportion of harder fat from the interior of the carcass. Part was sealed in cans filled to the top and stored at $-20°$ until required for use (fresh fat, for diet no. 1).

The remainder of the extracted beef fat was oxidized by bubbling oxygen through the oil at 100–110°, the peroxide value (μmoles/g) rising from zero to approximately 2 in 5 h, 20 in 8 h and 93·3 in 11·5 h, at which point the fat was cooled and part of it canned for storage at $-20°$ until required (oxidized fat, for diet no. 2).

A second portion of the oxidized beef fat was deodorized in an all-glass laboratory apparatus by blowing steam through the oil heated at 180° in a silicone oil bath for 1·5 h at a pressure of 1 mm mercury, cooling under reduced pressure and canning (oxidized and deodorized fat, for diet no. 3).

A third portion of the oxidized fat was heated at 180° for 1·5 h, under a 'blanket' of carbon dioxide produced by slowly passing the gas through the oil, cooled and canned (oxidized and heated fat, for diet no. 4).

BHT (0·02%) was dissolved in each prepared fat before canning.

Characterization of the fats

Gas–liquid chromatographic analysis. For determination of their fatty acid compositions the four beef fats were first converted into methyl esters by heating at 100° for 4 h in methanol–benzene (10:1, v/v) containing 5% (w/v) H_2SO_4 and 0·01% hydroquinone in an evacuated sealed tube. After recovery, the esters, in iso-octane solution (containing 0·01% BHT), were pipetted on to a fragment of glass fibre paper attached to a platinum wire that (after evaporation of the solvent) was dropped on to the column of the argon chromatograph (W. G. Pye and Co., Cambridge). A secon-

dary inflow of argon from a side tube near the top of the column was used to prevent introduction of air with the sample. The column was packed with polyethyleneglycol adipate (15%) on Celite (85–100 mesh) and operated at 190°.

Other determinations. The free fatty acid contents and iodine values of the fats added to the diets, and subsequently of those recovered from the stored diets, were determined by standard procedures.

Peroxide values were determined by the method of Lea (1952) and total carbonylic substances by the colorimetric 2,4-dinitrophenylhydrazine (DNPH) method of Henick, Benca & Mitchell (1956), as previously described (Lea & Swoboda, 1962).

Volatile carbonyls were determined by distillation from the fat on to a liquid oxygen-cooled cold finger, with determination of the carbonyls in the condensate by the colorimetric DNPH procedure (Lea & Swoboda, 1962).

'Oxidized fatty acids' (fatty acids insoluble in light petroleum) were determined in some of the fats by the British Standards Institution (1958) method.

Changes in the diets during storage

Gas exchange. Portions of 15 g of each diet in 50 ml flasks, connected to mercury manometers, were kept in an air jacket in a constant-temperature room at 20°, and changes in pressure (corrected for barometric variations) were noted at intervals. After 13 weeks, samples of the residual gas from each flask were analysed for O_2, N_2 and CO_2 by gas chromatography.

Extraction and examination of the lipid. The total lipid content of the basal diet (without added beef fat) was determined by continuous extraction for 6 h with boiling chloroform–methanol (2:1, v/v), removal of the solvent and re-extraction of the crude extract with chloroform.

The complete extraction procedure was considered too drastic for routine determination of analytical characteristics, particularly the peroxide values, of the lipids during storage; for this purpose, the diet (50 g) was shaken with de-aerated chloroform (300 ml) under N_2 for 30 min and filtered through sintered glass under a slight positive pressure of N_2. Portions of the extract were then taken for dry-weight determination; after concentration under reduced pressure at laboratory temperature peroxide value and free fatty acids were determined.

Because progressive and extensive hydrolysis occurred during storage, and because digalactosyl glycerides, which are a major constituent of the lipids of cereals, contain a much smaller proportion of fatty acid than ordinary triglycerides (p. 375), simple extraction proved inadequate for ascertaining, by means of changes in the iodine value, the extent of oxidation of the lipids during storage. The iodine values of the lipids after storage of the diets were therefore determined on the free fatty acids, prepared by complete extraction, saponification and recovery of the fatty acids, with precautions against further oxidation during the process.

The analytical methods used for characterization of the extracted fats have already been listed (p. 372).

Vitamin A. The vitamin A content of the diets was measured by a modification of a method used by Roche Products Ltd for the determination of vitamin A in animal

feeding-stuffs fortified with their Rovimix A (A. R. Moss, private communication). The vitamin A from 25 g samples was extracted with a 1:1 (v/v) diethyl ether:40/60° light petroleum mixture after it had been released from the gelatin powder by shaking with hot water, ammonia and ethanol in presence of hydroquinone. The dried extract was saponified (in presence of pyrogallol), taken up in ether and, after washing and evaporation, taken up in 80/100° light petroleum. This solution was passed through a column of alumina deactivated with 15% of water, which retained the vitamin A. The vitamin A was then eluted with a 15% solution of diethyl ether in 80/100° light petroleum and after evaporation was taken up in chloroform and determined by the Carr–Price method.

Performance and condition of the rats

Weight increase and state of vitamin A and vitamin E nutrition. Weight increase and food consumption were measured throughout the experiments and water consumption over limited periods at intervals. At the end of the experiments the livers were weighed and sampled for determination of vitamin A by the method described by Moore (1957, p. 582), with the modification that pyrogallol was added before saponification of the tissue (T. Moore & I. M. Sharman, personal communication).

As an indication of the state of vitamin E nutrition, the resistance to haemolysis of the erythrocytes was measured by the dialuric acid test (Bunyan, Green, Edwin & Diplock, 1960) on four rats receiving diet 2 in the last week of Expt B and on two rats from each of the other dietary treatments.

Fatty acid composition and stability of the body fats. Immediately after death the abdominal adipose tissue was taken from the rats of Expt B and frozen at $-78°$ in an atmosphere of CO_2, material from the individual rats of each group being combined to give four composite samples. Half of the adipose tissue from each dietary group was extracted three times with peroxide-free ether in a top-drive macerator for a total time of 10 min, and the combined extracts were dried over Na_2SO_4 before removal of the solvent at $< 40°$ under reduced pressure. The remaining portion of each tissue sample was extracted twice with chloroform–methanol (2:1, v/v), the extract was filtered, evaporated to dryness under reduced pressure, taken up again in ether, dried and filtered, and the solvent was finally removed at $< 40°$.

Portions of the extracted fats were used for the determination of iodine values and, after conversion into methyl esters, of fatty acid composition by gas–liquid chromatographic analysis. On further portions of the fats susceptibility to autoxidation was measured by following the rate of development of peroxides in 0·2 ml portions pipetted into small glass cups and held at a constant temperature of 60° (ether extracts) or 70° (chloroform–methanol extracts), as previously described (Lea, 1960), but omitting the peroxide 'starter'.

RESULTS

Lipids of the basal diet

Complete extraction of the fresh basal diet (i.e. without added beef fat) gave 2·95% lipid, but only a little more than half of it (1·6%) was removed by the cold extraction procedure with chloroform alone. From the complete diets (basal 95, beef fat 5) the

figures were 7·8% and 6·5–6·6%, respectively, indicating that all the added beef fat was recovered by the cold extraction process.

Since the fatty acid content of the lipids of the basal diet was 73%, compared with 95·5% for the added beef fat, the basal diet contributed 30% and the beef fat 70% of the total fatty acid contents of the complete diets (6·8%).

The fatty acid compositions of the lipids of the basal diet, of the fresh beef fat and of whole diet no. 1 are given in Table 1. The moisture content of the (complete) diets was 10·9%.

Table 1. *Composition of the methyl esters of the fatty acids of (fresh) diet no.* 1

Fatty acid* (%)	Basal diet (30% total)	Fresh beef fat (70% total)	Whole diet no. 1 (calculated)
14:0	1·8	3·2	2·8
15 br	—	2·3	1·6
15:0	—	0·4	0·3
16:0	19·7	23·3	22·2
16:1	5·3	9·6	8·3
17 br	—	1·3	0·9
17:0	—	0·6	0·4
17:1, 18 br	—	1·2	0·8
18:0	2·7	8·5	6·8
18:1	15·1	44·1	35·4
16 tr	—	2·0	1·4
18:2	44·0	1·1	14·0
18:3	4·5	0·4	1·6
C19–22†	7·0	2·0	3·5

* Shorthand designation suggested by Farquhar, Insull, Rosen, Stoffel & Ahrens (1959). br, a branched-chain acid; tr, a *trans* acid.

† Acids with retention volumes greater than 18:3 were not identified. The basal diet esters showed three major and several minor peaks in this region, probably derived in part from the fish-meal constituent.

Composition of the supplementary beef fats

The composition of fresh beef fat (Table 1) is complicated by the presence of minor proportions of odd-numbered, branched-chain and *trans* acids derived from the metabolism of the rumen bacteria. The even-numbered acids of the n-series were identified by comparing their retention volumes with those of pure reference compounds, but tentative identification of the remaining minor constituents had to be based on retention data quoted for these acids in the literature and supplied in a private communication by Dr F. B. Shorland.

The oxidized fats were, on the average, about 4 units lower in iodine value (Table 2), and their gas chromatograms, which were not significantly different, showed small reductions in unsaturated fatty acid content (oleate from 44·1 to 41·5, linoleate from 1·1 to 0·6, linolenate from 0·4 to 0·1%), but there were no obvious new peaks attributable to oxidation products.

Oxidized fatty acids, carbonylic compounds and free fatty acids, as well as peroxides, were all in greater amounts in the autoxidized fats than in the fresh control (Table 2).

Changes in the diets during storage

Gas exchange. For many dry and semi-dry fat-containing foods, measurement of the amount of O_2 absorbed is a convenient way of following oxidation of the lipid during storage, the amount of CO_2 produced in the early stages of fat autoxidation being relatively small. With the rat diets, however, the pressure in the manometric vessels increased during storage; after 13 weeks the headspace gas contained 11·5–14·3% O_2 and 6·3–12·6% CO_2. The mean values for each diet are given in Table 3.

Table 2. *Composition of the beef fats added to the diets at the 5% level*

	Diet and type of beef fat			
Characteristic	No. 1, fresh	No. 2, oxidized	No. 3, oxidized and deodorized*	No. 4, oxidized and heated
Iodine value	58·1	54·3	53·9	54·5
Peroxide value (μmoles/g)	0·0	93·3	1·6	2·3
Oxidized fatty acids (%)	0·0	0·3	0·4	0·2
Carbonyl content (μmoles/g):				
Total	3·6	—†	67·3	73·0
Volatile	0·03	12·6	1·3	12·6
Free fatty acids (as % oleic)	0·2	0·9	0·7	1·0

* Steam-deodorized for 1·5 h. Deodorization for 5·5 h gave peroxide value 0·9; total carbonyl 65·4; volatile carbonyl 0·4.

† A high peroxide content invalidates total carbonyl determination by the 2,4-DNPH method.

Table 3. *Oxygen absorption and carbon dioxide production by the four diets during storage for 13 weeks at 20°*

	Diet			
	No. 1	No. 2	No. 3	No. 4
O_2 absorbed (ml s.t.p./15 g diet)	2·9	3·7	3·3	4·0
CO_2 produced (ml s.t.p./15 g diet)	5·1	3·4	4·7	6·8
Ratio, CO_2:O_2	1·76	0·92	1·42	1·70
O_2 absorbed (μmoles/g dietary lipid)	111	141	126	152

It is apparent that sufficient O_2 had been absorbed to increase the degree of autoxidation of the dietary lipid by 100–150 μmoles/g, but the large amount of CO_2 produced makes it doubtful whether, in fact, the absorbed O_2 had reacted with lipid.

Changes in the lipid. Initial peroxide and free fatty acid values for lipid extracted from the basal diet by the cold extraction process (p. 373), together with calculated and observed initial values for the four diets containing beef fat, are given in Table 4.

Even during mixing with the basal diet, the highly peroxidized beef fat used in diet no. 2 began to lose its peroxide, as indicated by the difference between the calculated and observed values before storage, and this process continued at a gradually decreasing rate throughout storage (Fig. 1). The small initial peroxide values for the other three diets remained almost unchanged.

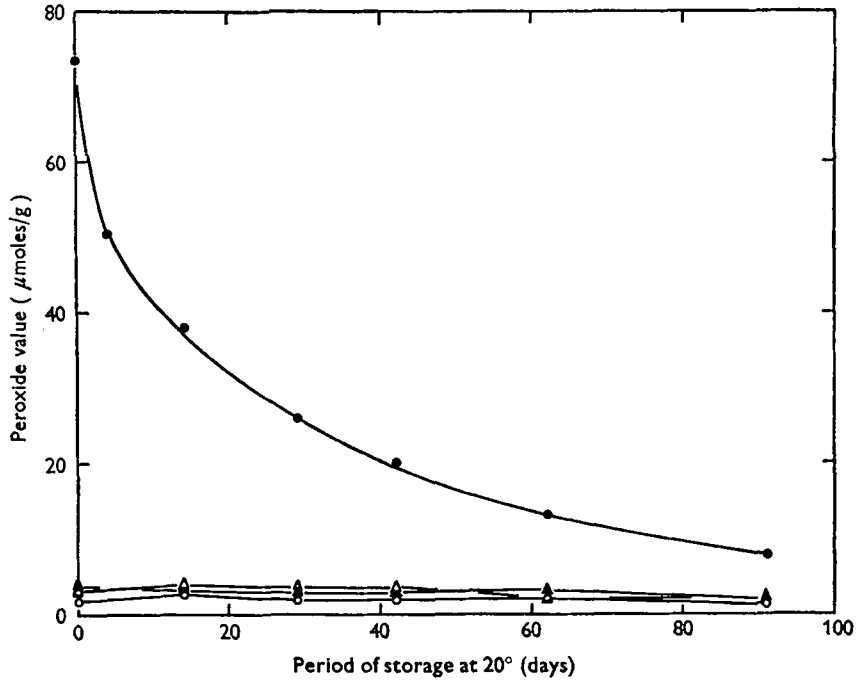

Fig. 1. Changes in peroxide value of the lipids of the diet during storage. ○—○, diet no. 1; ●—●, diet no. 2; △—△, diet no. 3; ▲—▲, diet no. 4 (see p. 376).

Table 4. *Characteristics of the lipids before and after storage (in the diet) for 13 weeks at 20°*

	Diet*				
	Basal	No. 1	No. 2	No. 3	No. 4
Peroxide value (μmoles/g):					
Before, calculated	—	1·8	73·7	3·1	3·6
Before, observed	8·0	2·3	59·1	3·7	4·1
After	—	1·3	7·7	2·1	2·2
Free fatty acids (as % oleic):					
Before, calculated	—	3·2	3·8	3·6	3·8
Before, observed	13·3†	6·3	4·7	5·2	5·6
After	—	52·2	33·1	53·3	49·7
Iodine value‡:					
Before, calculated	131	83·8	79·2	79·0	79·5
After	—	83·2	79·1	79·6	80·2
Carbonyl value (μmoles/g):					
Before, calculated	90	23	—	72	77
After	—	19	—	54	55
Vitamin A (i.u./g diet)§:					
Before	—	2·7 (6)	2·8 (6)	2·4 (5)	2·6 (5)
After	—	2·8 (7)	2·5 (8)	3·1 (7)	2·5 (7)

* Beef fat content as in Table 2.

† Part of this initial acidity may have been due to acidic phospholipids.

‡ Of the total fatty acids of the dietary lipids (see p. 373).

§ Each value is followed in parentheses by the number of determinations upon which the mean is based. The standard error of means based on five, six, seven and eight determinations, respectively, is estimated to be 0·27, 0·25, 0·23 and 0·21. Overall analysis indicated that the observed differences in means could have occurred by chance.

The free acidity of the lipid in all four diets rose appreciably during mixing, before the first analysis could be done (Table 4), and continued to increase rapidly during storage, reaching 50% or over for diets nos. 1, 3 and 4 after 13 weeks, but only 33% for diet no. 2 containing the highly peroxidized fat (Fig. 2).

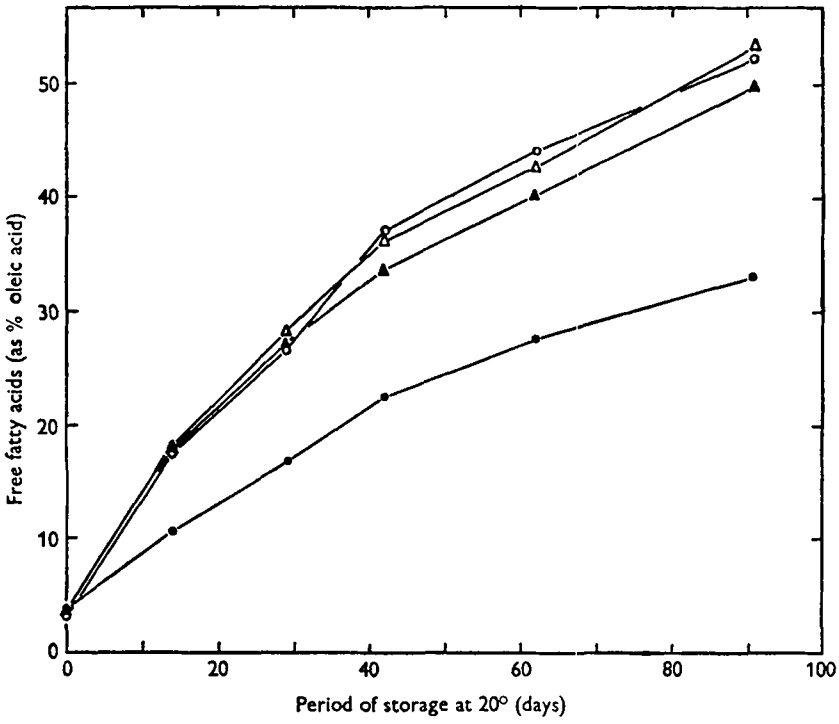

Fig. 2. Hydrolysis of the lipids of the diet during storage. ○—○, diet no. 1; ●—●, diet no. 2; △—△, diet no. 3; ▲—▲, diet no. 4 (see p. 376).

As already stated (p. 373), iodine values determined on the lipids extracted from the diets during storage were found to be unsatisfactory as a measure of autoxidation, because the rapid hydrolysis occurring resulted in the presence of an ever-increasing proportion of fatty acid in the extracted lipid: the observed iodine values of the lipids extracted at the end of storage were, in fact, all about 10 units higher than at the beginning. To overcome this difficulty the values at the end of storage were therefore redetermined on the fatty acids recovered from the total lipid extracts. Similar values could not be obtained directly for the freshly mixed diets, and those in Table 4 have therefore been calculated from the initial values for the beef fats themselves, and for the basal diet with which they had been mixed, the value for the basal diet being obtained via the methyl esters that had been prepared by methanolysis for gas–liquid chromatographic analysis. The results are sufficiently close to show that no appreciable autoxidation of the lipid occurred during storage.

A similar procedure could not be applied to the total carbonyl determinations because of the danger of decomposition during saponification, and carbonyl values

were therefore determined directly on the lipid extracts. The results (Table 4) show that the carbonyl contents of the dietary lipids decreased during storage (possibly owing to a 'browning'-type condensation with the amino groups of protein; cf. Lea *et al.* 1960), so that this criterion also provided no evidence of any lipid oxidation during storage of the diets.

Vitamin A. Application of the analytical procedure to the experimental diets gave results with a wide scatter. Each diet was analysed five to six times as freshly mixed and again seven to eight times after 13 weeks of storage. The combined mean values for the four diets were 2·6 i.u. vitamin A/g freshly mixed and 2·7 i.u. vitamin A/g after 13 weeks. The individual values are set out in Table 4. Although the absolute values are open to doubt, it appears that there were no substantial changes in the vitamin A contents of the diets over the storage period. Part of the variability may have been due to sampling errors.

Performance and condition of the rats

The results of the two feeding experiments are summarized in Table 5. Throughout the two experiments, and regardless of the diet, the rats remained healthy in appearance, with sleek fur and no sign of reddening of the feet. They continued to gain weight steadily throughout the whole experimental period. The erythrocyte haemolysis tests were all negative. The values for water consumption recorded in Table 5 were calculated from the changes in weight of water bottles over successive 24 h periods. There was certainly occasional wastage, but the values were reasonably consistent. The biggest difference between the mean values on different diets was 15%, which is small compared with the changes in water intake that have been reported to be associated with fat toxicity (Kaunitz, 1962).

Table 5. *Results of the feeding experiments*

	Expt A Diets stored for 0–6 weeks					Expt B Diets stored for 7–13 weeks				
	No. 1	No. 2	No. 3	No. 4	SE*	No. 1	No. 2	No. 3	No. 4	SE*
Wt gain in 6 weeks (g)	185	179	179	168	±5·5	164	166	181	172	±6·1
Food eaten in 6 weeks (g)	723	688	688	669	±19·8	619	632	648	644	±18·3
Wt gain/g food eaten (g)	0·256	0·260	0·260	0·251	±0·0036	0·266	0·263	0·275	0·267	±0·0051
Water consumed per rat (ml/week):										
Mean of 3rd and 5th weeks	168	167	179	157	±5·8	—	—	—	—	—
Mean of 4th and 6th weeks	—	—	—	—	—	129	149	161	152	±12·7
Liver wt (as % body-weight)	4·62	4·25	4·48	4·70	±0·13	4·68	4·58	4·58	4·92	±0·12

* Standard errors of treatment means, each mean being that for six rats (four ♂ and two ♀). For none of the measures did an analysis of variance show an overall treatment effect.

The full results for liver contents of vitamin A are set out in Table 6. From a statistical analysis of six determinations replicated on different days, we find the coefficient of variation for each value to be approximately ±7%.

Although the overall mean value for Expt B was lower than that for Expt A, this cannot be interpreted as due to the difference in storage period of the diets, since the litter-mates killed at randomization in the two experiments showed equally big differences. The older rats used in the first set of Expt A in particular gave considerably higher values than any others either at the end of the experiment or at the time of

randomization, presumably because of their high intake of commercial cubes in their 4th week of life.

Considering only the results from the other sets, since it was intended that the animals should have low reserves at the beginning of the experimental treatments, it is seen that all (except for one rat receiving diet 2) apparently accumulated vitamin A on the experimental diets, which contained little more than the bare requirements for the vitamin. An analysis of variance of the results showed that differences between dietary treatments were significant, as set out in Table 6. The least significant difference for dietary means was calculated to be 128; the lowest value was obtained with diet no. 2 and the highest with diet no. 1. The analysis also showed that the females had livers of higher vitamin A content, even though they were lighter in weight.

Table 6. *Vitamin A values (i.u./liver) for individual rats that had received the experimental diets and of litter-mates killed at the time of randomization*

Expt	Set	Age at randomization (days)	Litter-mates killed after randomization	Rats killed after receiving diet			
				No. 1	No. 2	No. 3	No. 4
A	1 ♂	29–31	1000	1620	1100	1070	1120
			1030	1240	720	880	1110
	2 ♂	24–26	162	660	340	520	530
			196	680	160	600	330
	3 ♀	24–26	160	660	640	490	570
			195	920	420	900	390
B	1 ♂	21–24	23	630	310	360	480
			32	180	430	390	390
	2 ♂	21–24	24	560	280	480	260
			50	620	330	340	430
	3 ♀	21–24	36	520	480	590	570
			24	550	390	500	430
	Mean values for all but set 1 of Expt A			600	380	520	440
	Comparison of diet means*			a	c	ab	bc

* Two means are significantly different from each other if they do not have a letter in common. The standard error of treatment means, calculated from the residual term of the analysis of variance, is ± 36·8.

Susceptibility to autoxidation of the rat body fats

The results of two of the stability tests, carried out on fats extracted by both methods (p. 373) from the four groups of rats, are shown in Fig. 3.

The iodine values of the extracted fats ranged from 67·7 to 75·9 and their linoleate contents (as measured by gas–liquid chromatographic analysis of their methyl esters) from 5·5 to 9·2%. Within each group there was a distinct tendency for oxidative stability to decrease when the iodine value and linoleate content of the fat increased, as shown by Fig. 4, in which the induction periods (means of two runs) are plotted against these two criteria of unsaturation.

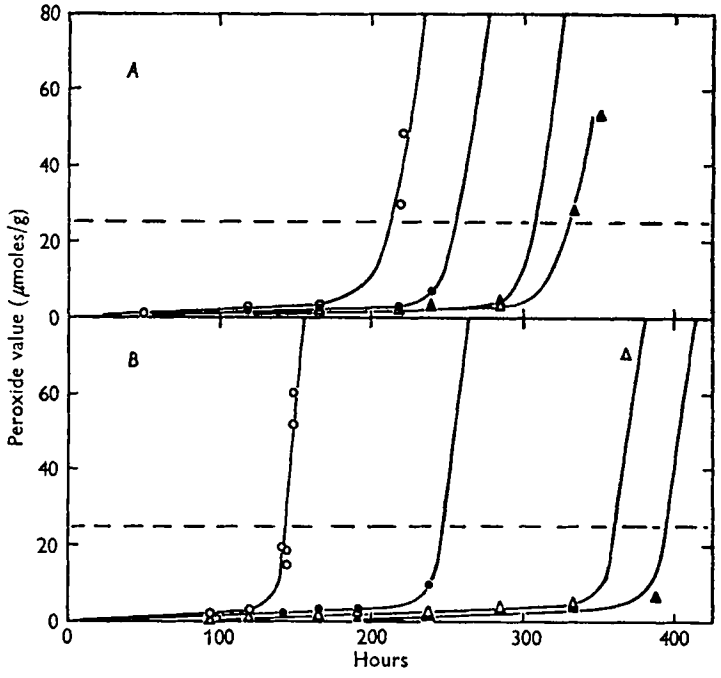

Fig. 3. Relative susceptibilities to autoxidation of lipids extracted from the abdominal adipose tissues of rats by (*A*) peroxide-free diethyl ether or (*B*) chloroform–methanol. Temperature in run *A*, 60°; in run B, 70°. ○—○, diet no. 1; ●—●, diet no. 2; △—△, diet no. 3; ▲—▲, diet no. 4 (see p. 376).

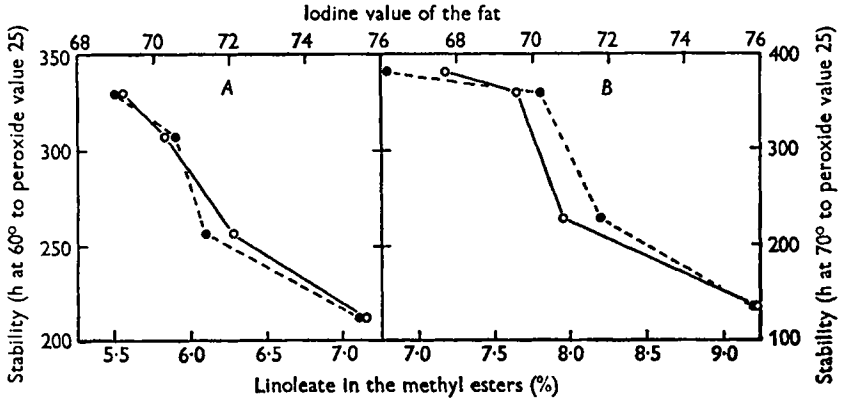

Fig. 4. Relation between unsaturation and oxidative stability of the body fats of rats. *A*, diethyl ether extract; *B*, chloroform–methanol extract; ○—○, iodine value; ●—●, linoleate content.

DISCUSSION

Changes in the dietary lipids. A striking feature of these experiments was the failure of the added beef fat, whether fresh or oxidized, to autoxidize further when dispersed in the diets and stored for 13 weeks at 20°. Appreciable amounts of O_2 were absorbed by all four diets (Table 3), but little or none of it appeared to have reacted with the lipid constituents, as shown by the absence of any decrease in iodine value (Table 4),

increase in peroxide value (Fig. 1) or increase in carbonyl value (Table 4). The initially high peroxide value of diet no. 2 and the initially high carbonyl values of diets nos. 1, 3 and 4 in fact decreased during storage, the peroxide value falling by 90% (Fig. 1). The synthetic antioxidant added with the beef fats no doubt contributed to this stability, but a further and probably major factor would be the presence of the natural antioxidants that stabilize the highly unsaturated lipids of the cereals.

The lipids of all four diets underwent progressive and extensive hydrolysis during storage (Fig. 2), but there was no indication that this change had any adverse effect on the rats, as shown by their generally satisfactory and similiar performances in Expt A (diet stored 0–6 weeks, average free fatty acid content of its lipids 5–32%) and Expt B (diets stored 7–13 weeks, average free fatty acid content 35–47%).

The fat of initially high peroxide content in diet no. 2 considerably retarded lipolysis in this diet, compared with the diets containing either fresh fat or oxidized fat in which the peroxide groups had been largely destroyed. This would appear to be an inactivating effect of the lipid hydroperoxides on the vegetable lipase present, possibly by oxidation of its –SH groups as noted by Wills (1961) for several other –SH containing enzymes but apparently not for pancreatic lipase. Degkwitz & Lang (1962–3) also found only slight inhibition of purified pancreatic lipase by autoxidized soya-bean oil.

The figures in Table 3 suggest that some CO_2-producing enzyme system in the basal diet was also partly inhibited by the peroxidized fat in diet no. 2.

Effects of the diets on the rats. Although the rats receiving 5% of oxidized fat grew well and appeared healthy, they did retain significantly smaller vitamin A reserves in the liver than did the control groups receiving unoxidized fat. We found no evidence for destruction of vitamin A during storage of the diet, though the estimates made were admittedly approximate. But it may still be that there was some destruction in the gut after the release of the vitamin from its protective gelatin matrix.

The mean vitamin A liver reserve appeared to be lowest for the rats receiving diet no. 2, of highest peroxide content, but the value for diet no. 4 containing oxidized heated fat of low peroxide content was not significantly different, so that the effect of oxidized fat in limiting vitamin A deposition cannot be attributed only to peroxide groups as such.

Although these differences were statistically significant, their nutritional significance should not be exaggerated, since all the animals were accumulating reserves on a dietary level of vitamin A close to the recommended allowance for growing rats.

The erythrocyte haemolysis test was negative throughout, and there were no other signs of vitamin E deficiency or of any other abnormality. Ward (1963) has recently shown that the relationship between the in vitro haemolysis test and other signs of vitamin E deficiency in the rat differs between the sexes. In males most of the lesions (except defective storage of vitamin A) can be prevented by from one-sixth to one-third of the amount of tocopherol required to prevent haemolysis of the erythrocytes, whereas in females the fraction is about one-half.

Stability of the body fats. In early work with pigs, Burr (1945) showed that the keeping time of (extracted) adipose tissue fat increased with increasing tocopherol

content when that of linoleic acid was constant and decreased with increasing linoleic acid content when that of tocopherol was constant. A generally similar relationship probably holds also for rats and poultry, and our own (unpublished) observations confirm that giving a vitamin E-deficient diet to rats greatly shortens the induction period of the body fat.

However, in the experiments reported here the rats fed on the autoxidized beef laid down a more stable body fat than did control animals receiving fresh fat, suggesting that no serious loss of tocopherol had occurred, and Fig. 4 shows that the reason for the increased stability was probably the appreciably lower linoleate content of the body fats laid down by the rats on the diets with oxidized fats.

The usual reason for a smaller deposition of linoleate by animals of similar age and growth rate would be a lower linoleate intake in the diet, the relationship being approximately linear, as demonstrated recently by Mohrhauer & Holman (1963) for rats and by Craig & Bell (1963) for mice. But in our experiments even the fresh beef fat contained only about 1% of linoleate, and the intake of this acid derived largely from the vegetable lipid of the basal diet (Table 1), which was always the same and did not oxidize during storage.

We are therefore forced to the conclusion that the presence of autoxidized beef fat in the diet must have depressed the absorption (or deposition) of linoleate from the food and that, unlike the effect on lipase, this depressant effect was more marked for the oxidized fats in which the peroxide had been destroyed by heat than in the fat of high peroxide value. It should be noted in this connexion that Bhalerao, Inoue & Kummerow (1963), comparing the fatty acid compositions of lymph lipids from rats fed on fresh and on thermally oxidized (24 h at 200°) fats, concluded that thermal oxidation lowered absorption of unsaturated fatty acids via the lymph; Kaunitz, Johnson & McKay (1963) similarly found lower linoleate levels in the serum and tissue lipids of rats given the ethyl esters of a molecular distillate from oxidized cod-liver oil.

Since there can be no question of essential fatty acid deficiency at these levels, the more stable and perhaps slightly firmer fat resulting from a lowered efficiency of linoleate deposition would be likely to be beneficial rather than objectionable as a criterion of carcass quality in pigs or poultry. With ruminants the question would not arise, because polyunsaturated acids are anyhow largely destroyed by hydrogenation in the rumen before absorption.

SUMMARY

1. Beef fat oxidized to a peroxide value of 93 μmoles/g, with a reduction in iodine value of approximately 4 units, was incorporated at a 5% level into a broiler-type diet and stored for 13 weeks at 20°. Similar diets containing fresh fat or oxidized fat, in which the peroxide had been destroyed by heating, were also stored. The fats all contained 0·02% 2,6-di-t-butyl-4-methyl-phenol, and the diets were supplemented with 'stabilized' vitamin A and vitamin E at levels necessary to meet the requirements of young rats but with little excess.

2. The free fatty acid contents of the dietary lipids increased progressively during storage, sometimes to above 50%, but oxidation did not occur and the initial high

peroxide value of the one sample decreased rapidly. There was little or no destruction of the added vitamin A.

3. Two groups of weanling rats raised in succession on the diets during the storage period grew normally and accumulated vitamin A reserves in their livers; no adverse effect attributable to the presence of the oxidized fat was detected, apart from marginally lower vitamin A levels in the livers. There were no signs of any deficiency of vitamin A or E.

4. The body (adipose tissue) fats of the animals receiving the oxidized fats contained slightly less linoleate and were consequently more stable towards autoxidation than those of the controls. This appeared to be the result of a depressant effect of the dietary oxidized fat on linoleate absorption or deposition.

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