Nutritional effects of autoxidized fats in animal diets

2.* Beef fat in the diet of broiler chicks†

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1. Beef fat oxidized to a peroxide value of 109 μmoles/g (218 m-equiv./kg), with a reduction in iodine value of 3·4 units, was incorporated at a 5% level in the diet of day-old chicks (diet 2) and stored for 8 weeks at room temperature during feeding. Similar diets containing fresh fat (diet 1, peroxide value zero) or oxidized fat in which the peroxide had been largely destroyed by heating (diet 3, peroxide value 2 μmoles/g) were also used. The diets contained adequate but not excessive levels of all vitamins, including stabilized vitamin A, except that for half of the chicks the supplement of stabilized vitamin E was omitted. 2. No further oxidation of the dietary lipid occurred during storage, and the initially high peroxide value in diet 2 decreased rapidly. The natural vitamin E decreased by more than 50% in diet 2, but remained unaffected in diets 1 and 3. 3. The chicks grew normally, with no difference in weight gain or feed conversion between the groups, the only differences attributable to the diets being marginally lower vitamin A levels accumulated in the livers of the birds on diet 2 and a just detectably higher liver weight in the birds on diet 3. 4. One of the twelve chicks receiving oxidized fat (diet 2) without synthetic vitamin E developed encephalomalacia. There was no other suggestion of performance being inferior as a consequence of the absence of the vitamin E supplement. 5. No difference could be detected in the flavour of the chickens, either freshly roasted or reheated.

In recent years the inclusion of beef tallow at levels up to about 5% in commercial poultry rations has become common practice. Numerous reports claim that this amount of fat eases the compounding and the feeding of diets; although reports are conflicting as to whether or not it improves the growth rate of chicks (Baldini & Rosenberg, 1957; Donaldson, 1962), it has been shown that properly balanced rations containing levels of animal fat up to 33·8% support normal growth in chicks (Donaldson, Combs, Romoser & Supplee, 1957).

There is, however, some controversy as to how the quality of the tallow used can be characterized. In the past this has frequently been done by the determination of free fatty acids and peroxide value, with rejection of materials that gave values above certain limits. In a previous paper (Lea, Parr, L'Estrange & Carpenter, 1964) it was pointed out that there seemed to be no evidence that free fatty acids as such were harmful, and that the 'peroxide' values permitted were only a small fraction of those that had been demonstrated in laboratory experiments to be harmful. It was shown (Lea *et al.* 1964) that beef fat oxidized to a peroxide value of 93 μmoles/g, which is a level much higher than is permitted in commercial practice, and incorporated at a level of 5%

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† Some of these results have been communicated in a preliminary form (L'Estrange, Carpenter, Lea & Parr, 1965).

in a broiler-type ration containing only normal amounts of stabilized vitamins A and E had no adverse effects on the health or growth of weanling rats during a 6-week feeding trial, though they stored marginally less vitamin A in their livers. A modification of that experiment has now been carried out on broiler chicks.

The vitamin E requirement of chicks can vary from 0 to 92 mg DL-α-tocopheryl acetate/kg diet (Agricultural Research Council, 1963); although it is widespread practice to add stabilized vitamin E to chick diets, this is not always done. It was decided therefore to study the effects of oxidized fat in the diet of chicks, with and without a supplement of stabilized α-tocopherol. The effects on fast-growing broiler cockerels, from day-old to 8 weeks, have therefore been compared of: (1) 5% fresh beef fat, (2) 5% beef fat autoxidized to a peroxide value of 109 μmoles/g, and (3) 5% beef fat oxidized as in (2) but subsequently heated in the absence of air to destroy its peroxides. The basal diet used contained adequate but not excessive levels of supplements, including stabilized vitamin A, and the experiment was run with and without stabilized vitamin E (4 mg DL-α-tocopheryl acetate/g diet; the level chosen is that recommended by Lewis (1963) for broiler chicks), making six groups in all. As in the experiment with rats (Lea *et al.* 1964), 0·02% BHT (2,6-di-*t*-butyl-4-methyl-phenol) was added to each fat before mixing with the diet.

EXPERIMENTAL

Chicks and management

Broiler-type cockerels of the Chunky strain (W. D. Evans Ltd, Market Harborough) were used. They were randomized according to weight at day-old into fourteen similar groups of six chicks each. The six experimental treatments were randomized among twelve of these groups, and the remaining two groups were slaughtered and their livers stored at −20° for subsequent analysis.

Each group occupied a single cage. At 3 weeks the birds were transferred from galvanized wire-mesh cages with dropping tray to larger cages, made from hardboard and wire mesh, resting on a floor bedded with sawdust. Both sets of cages had outside feeders which allowed food intake to be recorded accurately. The temperature was maintained at 32°, 29° and 27° for the 1st, 2nd and 3rd weeks respectively, and thereafter at 21°.

Diets

The diets, which were rather similar to those given to the rats in the previous experiment (Lea *et al.* 1964), had the percentage composition: beef fat (prepared as described below) 5, ground whole wheat 40, ground barley 15, extracted soya-bean meal 20, white fish meal 10, dried skim milk 3·33, unextracted dried yeast 1·67, steamed bone flour 1·13 and a premix containing minerals, antibiotics and vitamins 3·87. The premix contributed to the final diet: manganese (as $MnSO_4.H_2O$) 70 ppm, zinc (as zinc carbonate) 56 ppm, oxytetracycline (as Terramycin; Pfizer Ltd, Sandwich, Kent) 40 ppm, sulphaquinoxaline (as Embazin premix; May and Baker Ltd, Dagenham, Essex) 125 ppm, riboflavine 4 ppm, menaphthone 2 ppm, pantothenic acid (as calcium pantothenate) 10 ppm, vitamin A (as Rovimix A 50; Roche Products

Ltd, Welwyn Garden City, containing 50000 i.u. vitamin A/g in a gelatin-based powder) 3000 i.u./kg, vitamin D_3 (as Rovimix D_3-100; Roche Products Ltd, containing 100000 i.u. vitamin D_3/g) 1500 i.u./kg. In the vitamin E-supplemented diets vitamin E (as Rovimix E; Roche Products Ltd, containing 10% DL-α-tocopheryl acetate), 4 mg/kg, was also present. Because of an outbreak of perosis when the chicks were 3 weeks old, each chick was given 0·4 mg folic acid orally and 12500 i.u. vitamin D_3 by intramuscular injection, and to the diets from then on were added manganese (as $MnSO_4.H_2O$) 30 ppm, biotin 0·05 ppm, choline (as choline chloride) 700 ppm, riboflavine 1·4 ppm and folic acid 0·3 ppm.

The diets were made up 3 days before feeding began and were stored throughout the experiment in large polythene bags with some head space in a dark room at a temperature ranging from 18° to 24°. The moisture contents of the complete diets were all between 11·3 and 11·9%.

Fats used

A beef tallow, dry-rendered in a stainless steel vessel from approximately three parts fresh subcutaneous to one part internal fatty tissue, had a peroxide value of zero. Two-thirds of this material was oxidized by bubbling with oxygen at 90° for 52 h to reach a peroxide value of 109 μmoles/g. Half of the oxidized fat was then deoxygenated by passing CO_2 through it and then heating it under a blanket of CO_2 at 200° for 20 min to decompose its peroxides. BHT 0·02% was dissolved in each of the prepared fats before it was incorporated into the diet.

Characterization of the fats

The three beef fats used were characterized by determination of their iodine, peroxide and carbonyl values and by gas chromatographic analysis of their methyl esters, as previously described (Lea *et al.* 1964), but using a magnetic injection system with the argon chromatograph.

As a measure of non-volatile oxidation products 'oxidized fatty acids' (acids insoluble in light petroleum) were determined as before, but acids not forming urea adducts (NAF) were also measured. For this purpose the method of Sahasrabudhe & Bhalerao (1963) was first used, but then even the fresh fat gave an obviously too high value of 5–6% NAF acids, an error traced to the presence of unremoved urea in the precipitate. The earlier method of Firestone, Nesheim & Horwitz (1961) was therefore adopted, but modified to use saponification with alkali rather than interesterification with boron trifluoride–methanol. With this somewhat less simple procedure consistent and reproducible results were obtained.

Changes in the diets during storage

Extraction and examination of the lipids. The total lipid of the basal diet was determined, by extraction with chloroform–methanol (4:1, v/v) and re-solution in chloroform, before mixing with the beef fat, and at intervals during storage the total lipid extracted from the mixed diets was examined for peroxide value, free fatty acid and carbonyl group content. The total fatty acids of the mixed diet were also prepared at the beginning and the end of storage, for determination of their iodine values as a

more valid index of oxidative change than direct determination on the extracted lipid itself (Lea *et al.* 1964).

Tocopherol determination. At intervals α-tocopherol was determined in lipid obtained by Soxhlet extraction with diethyl ether and subjected to a preliminary purification process by saponification in the presence of pyrogallol and chromatography on Decalso F (Diplock, Edwin, Bunyan & Green, 1961). The method used was the two-dimensional paper chromatographic technique recommended by the Society for Analytical Chemistry: Analytical Methods Committee (1959).

To ensure identification of the α-tocopherol spot on the paper chromatogram and to measure recovery, three subsamples (25 g) of each sample of diet were analysed together, after adding to the ether extract of one of them a known quantity of α-tocopherol somewhat in excess of that already present. The percentage recovery of α-tocopherol was then calculated from the difference between the amount determined in this subsample and the mean of the other two.

Total reducing substances were also determined in the extracts before the paper chromatographic separation.

Performance of the chicks and examination of the carcasses

Weight increase and food consumption were measured throughout the 8 weeks of the experiment, and water consumption over limited periods. At the end, the livers were weighed and their vitamin A contents determined as previously described (Lea *et al.* 1964).

Fatty acid composition of the body fats

Such fatty tissue as was visible in the body cavity was removed and combined in each of the six dietary groups for extraction with peroxide-free ether and gas–liquid chromatographic analysis as previously described (Lea *et al.* 1964). The amounts of fatty tissue available were small and variable, and stability tests could not be done on them.

Flavour of the cooked meat

The carcasses were roasted under aluminium foil, which was opened for the last $\frac{1}{2}$ h, by a standardized procedure in an oven at 186°: the breast and leg meats were then tasted hot by a panel of ten persons and ranked in order of preference. In a subsequent test the cooked carcasses were held, either for 1 day at room temperature or for 3 days at domestic refrigerator temperature, and the breast and leg meats were then diced and reheated for 45 min at 100° before submission to the panel. The whole series was repeated a second time, to give eight tests in all. In addition, twelve persons tasted three birds each under home conditions.

RESULTS

Lipids of the diet

The basal diet contained 3·19% lipid and the mixed diets (95% basal, 5% beef fat) 8·03%. The fatty acid composition of a basal diet of the type used has been reported in the previous paper (Lea *et al.* 1964). The characteristics of the fats added

are given in Table 1 and their fatty acid compositions, together with those of the chicken abdominal fats, in Table 2. For simplicity, nine acids present in amounts < 1%, and sometimes of uncertain identity, have been omitted from the table, and the compositions of fats from chickens receiving diets with and without extra tocopherol, which showed no consistent differences, have been averaged.

Table 1. *Characteristics of the fats incorporated at the 5% level into the diets**

Diet no.	Iodine value	Free fatty acids (%)	Peroxide value (μmoles/g)	Carbonyl† value (μmoles/g)	'Oxidized' fatty acids (%)	Acids not forming urea adducts (%)
1	47·8	0·9	0	5	0·1	0·5
2	44·4	1·3	109	158	0·2	3·8
3	44·4	1·7	2	87	0·3	5·8

* Lipid content of the basal diet 3·19%, of the mixed diet 8·03%.

† Direct determination; the figure found for diet 2 is too high, because of interference by the peroxide present.

Table 2. *Iodine values and major fatty acid components of the beef fats included in the three diets and of the abdominal fat of chickens receiving the diets*

Type of fat	Diet no.	Iodine value		Fatty acid composition (%)								
		Fats	Esters	14:0	14:1	16:0	16:1	17br.	17:0	18:0	18:1	18:2
Beef fat in diet	1	47·8	48·4	3·4	1·5	26·7	5·2	0·9	1·0	15·7	39·1	3·4
	2	44·4	44·2	3·4	1·6	27·4	5·2	1·2	1·2	16·3	37·7	2·5
	3	44·4	44·5	3·4	1·6	28·5	5·3	1·8	1·3	16·8	35·9	2·8
Chicken fat	1	—	66·5	2·4	0·9	25·1	6·7	0·9	0·7	8·7	41·6	10·3
	2	—	64·0	2·4	0·9	25·7	7·3	0·8	0·7	8·3	41·3	9·5
	3	—	64·0	2·6	0·9	25·9	6·2	0·8	0·7	9·9	40·1	10·0

Changes in the diets during storage

Lipids. As in the rat experiment, the highly peroxidized beef fat in diet 2 lost its peroxide during storage in the mixed diet, rapidly at first and then more slowly (Fig. 1), and in the other diets initially low peroxide values decreased a little further. Since a falling peroxide value is not itself conclusive evidence that lipid autoxidation is not proceeding, iodine values were also determined on the total fatty acids recovered after saponification of the dietary lipids at the beginning and end of storage. No decrease in iodine value was observed; in fact, the value determined at the end of storage was in each diet slightly higher than at the beginning, indicating that no significant degree of lipid autoxidation had occurred during storage.

The carbonyl contents (direct determination) of the lipids of diets 1, 2 and 3 decreased during storage from 60, 156 and 112 to 21, 38 and 35 respectively, again furnishing no evidence of lipid oxidation.

As before, extensive hydrolysis of the lipid occurred during storage of the mixed diets, the lipolytic enzyme, apparently, being somewhat inhibited by the oxidized fat present in diets 2 and 3 (Fig. 1).

Vitamin E. The results of the analyses are summarized in Table 3. A mean loss of

12% of added α-tocopherol was found to occur during saponification and separation on Decalso F, and a further loss of 22% during paper chromatography, though losses of α-tocopherol during paper chromatography of mixtures of pure tocopherols alone were never more than 10%. It appeared that there were some interfering substances in the diets not completely removed by saponification and separation on Decalso F.

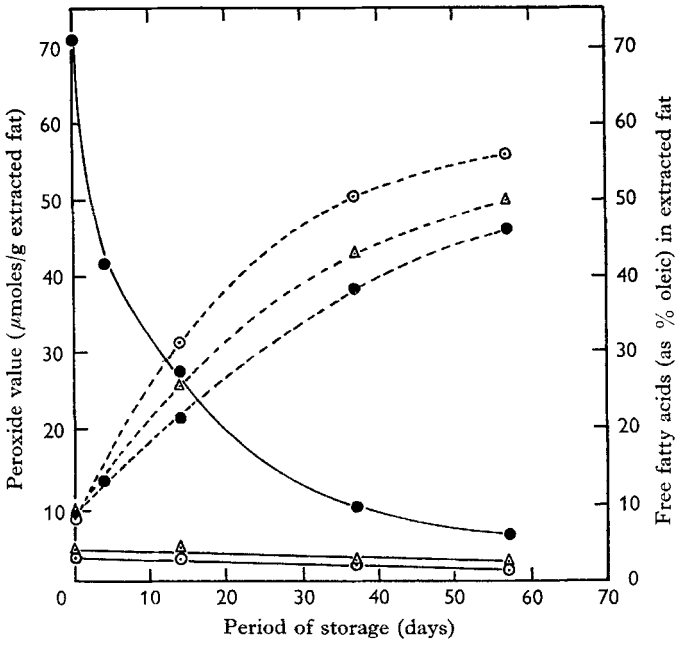

Fig. 1. Chemical changes in the fat of diets (see p. 117) stored at room temperature. ⊙, diet 1; ●, diet 2; △, diet 3. ——, peroxide value; ----, free fatty acids.

Table 3. *Changes in total reducing substances and α-tocopherol during storage of diets containing fresh, oxidized or oxidized and heated fat, not supplemented with stabilized vitamin E, and recovery of added α-tocopherol*

	Fresh fat diet		Oxidized fat diet				Diet containing oxidized and heated fat	
Days stored...	0	57	0	14	37	57	0	57
Total reducing substances (μg/g diet)	17·3 (3)	19·3 (3)	15·2 (4)	13·5 (2)	9·4 (2)	9·3 (4)	14·2 (2)	14·3 (2)
α-Tocopherol* (μg/g diet)	9·6 (2)	10·7 (2)	8·5 (4)	7·7 (2)	3·0 (1)	3·3 (4)	8·4 (4)	8·4 (4)
% recovery of added α-tocopherol, determined:								
(*a*) Before paper chromatography	93	93	97	92	—	93	71	70
(*b*) After paper chromatography	76	58	66	61	—	73	61	50

* Values are corrected for estimated loss during the analytical procedure. Figures in parentheses are the numbers of determinations.

However, the results were sufficiently reproducible to show that neither total reducing substances nor α-tocopherol decreased during storage of the diets containing fresh fat or oxidized and heated fat, but that both decreased by more than 50% during storage of the oxidized fat diet. In the oxidized fat diet all the loss took place during the first 37 days of the 57-day period and none towards the end when the peroxide present had fallen to a low level.

It was not practicable to determine the content of α-tocopherol in diets to which the stabilized supplement had been added, because the stabilizing matrix, though easily removable by saponification from pure Rovimix E, could not be removed satisfactorily when the other constituents of the diet were also present.

Performance of the chicks

The results of the feeding experiment are summarized in Table 4. One of the seventy-two chicks died and six were removed from the experiment, all before they were 3 weeks old. The chick that died showed an unidentified heart condition *post mortem*. Of the six removed, five showed signs of perosis, none on the oxidized fat diet; one, on the oxidized fat diet without added vitamin E, developed encephalomalacia.

Table 4. *Performance of chicks (two cages of six chicks/treatment) receiving diets containing fresh, oxidized or oxidized and heated fat with or without a supplement of α-tocopherol*

	Fresh fat diet		Oxidized fat diet		Diet containing oxidized and heated fat		
	Without tocopherol	With tocopherol	Without tocopherol	With tocopherol	Without tocopherol	With tocopherol	SE of treatment means*
Live wt at 8 weeks (g/chick)	1550	1534	1628	1617	1568	1515	±49
Food intake to 8 weeks (g/chick)	3187	3166	3433	3355	3283	3274	±100
Food conversion ratio to 8 weeks	2·08	2·05	2·13	2·11	2·07	2·21	±0·039
Water intake 6th–7th week (ml/kg chick)	119	115	140	119	126	117	±5·87
Liver wt (as % body-weight)	1·44	1·43	1·44	1·43	1·55	1·49	±0·028
Vitamin A reserves (i.u./liver)	617	548	439	353	574	475	±48·7
No. of disorders†	2	2	1	0	1	1	—

* Significance of difference between treatment effects. The only significant effects were: (*a*) the liver weights with the diet containing oxidized and heated fat were significantly higher ($P < 0.05$) than with the other diets; and (*b*) the vitamin A liver reserves were significantly less ($P < 0.05$) with the oxidized fat diet than with the fresh fat diet.

† All perosis, except one case of encephalomalacia on the oxidized fat diet not supplemented with α-tocopherol, and one case of heart failure (cause unknown) with the fresh fat diet supplemented with α-tocopherol.

The remaining chicks grew extremely well, and there was no significant difference between treatments in growth rate, food intake or food conversion efficiency from day-old to 8 weeks (Table 4). Nor was there any significant difference between treatments in water consumption from the 6th to the 7th week.

The livers of chicks on the diets with oxidized and heated fat were slightly but significantly heavier ($P < 0.05$) than those of the chicks on the other diets, but there was no effect of any other dietary factor.

The vitamin A reserves accumulated in the livers were significantly less on the oxidized than on the fresh fat diet ($P < 0.05$), but there was no significant difference between the chicks on the diets with fresh fat and with oxidized and heated fat. Vitamin A reserves were not improved by vitamin E supplementation, the (non-significant) tendency being for the values to be less on the vitamin E-supplemented diets.

Flavour of the cooked meat

The average order of preference by tasters scoring from 1 (best) to 6 (worst) for the six samples submitted in the eight tests to the panel were: diet 1 (fresh fat, with and without tocopherol supplement) 3·8 and 3·3, mean 3·5; diet 2 (oxidized fat, with and without tocopherol) 3·5 and 3·6, mean 3·5; diet 3 (oxidized and heated fat, with and without tocopherol) 3·5 and 3·3, mean 3·4. Statistical analysis indicated no significant effect of the kind of fat or of the tocopherol supplement. The home cooking test, within its limitations, indicated a similar result.

DISCUSSION

Changes in the diet

Though the beef fat used in diet no 2 had the high peroxide value of 109 μmoles/g and was actively oxidizing at the time of mixing, it ceased to do so on contact with the other constituents of the diet and its peroxide value fell rapidly, in agreement with observations made in the previous experiment (Lea *et al.* 1964). Nevertheless, during the early part of the feeding period, when the chicks were less than a month old, the peroxide value of the dietary lipid was much higher than would be considered acceptable by many analysts.

Our estimate of the α-tocopherol naturally present in the diets before storage was 9 μg/g, a figure in general agreement with published values for the tocopherol contents of the main dietary constituents (Hjarde, Lieck & Søndergaard, 1962; Herting & Drury, 1963). This level remained constant during storage in the diets with fresh fat and with oxidized and heated fat, but it fell to less than half within 37 days in the oxidized fat diet. Destruction of tocopherols by autoxidizing fat is well known (Frankel, Evans & Cooney, 1959; Machlin, 1961) but it is of interest to find that even in a diet in which the fat was not oxidizing further, and in which the hydroperoxides were rapidly decomposing, destruction of tocopherols still took place.

Performance of the chicks

The high incidence of perosis in this experiment was not associated with the oxidized fat and remains unexplained. All cases occurred before the chicks were 3 weeks old, at which time those that had not been affected received a supplement of all the dietary constituents known to prevent perosis, and all were transferred from a cage with a wire floor to one with a solid floor bedded with sawdust.

Despite the loss of α-tocopherol in the oxidized fat diet not supplemented with stabilized vitamin E, only one case of encephalomalacia occurred on this treatment, and none of the other chicks showed any other signs of vitamin E deficiency, e.g. muscular dystrophy or exudative diathesis. The diets did not contain a high proportion of unsaturated fat, and the vitamin E requirement was consequently low. We have discussed some of the factors affecting the vitamin E requirements of chicks elsewhere (Carpenter, L'Estrange & Lea, 1966). We certainly would not recommend, as a result of this trial, that supplementary vitamin E was unnecessary in practical broiler diets.

As with the rats in the previous experiment, there was significantly less vitamin A stored in the livers of the birds on the oxidized than on the fresh fat diets, though the oxidized and heated fat did not have this effect. Since it has already been established that vitamin A in stabilized form is not destroyed in the diet during storage, even in the presence of lipid peroxides (Lea *et al.* 1964), it would appear that some destruction of vitamin A occurred through the action of peroxide, in the intestine or during absorption, after removal of the protective gelatin matrix.

The amount of vitamin A in the diet (3000 i.u./kg) was only slightly higher than the required level of 2620 i.u./kg ((USA) National Research Council: Committee on Animal Nutrition, 1960) and it can be concluded that none of the treatments induced the vitamin A deficiency observed by previous workers using rancid fats with non-stabilized vitamin A (e.g. Stoewsand & Scott, 1959).

A second effect of oxidized fat was an increase in the weight of the livers of the birds on the diets with oxidized and heated fat, but the effect was small, and it was not observed with the oxidized fat diet. Since all the chicks grew well, the minor nutritional changes recorded would appear to be of little or no practical significance.

An observed effect of feeding rats with oxidized fat was a slight depression of linoleate deposition in the abdominal adipose tissue (Lea *et al.* 1964). The chicks at 8 weeks of age contained so little adipose tissue and its fat content was so low that observations recorded on this point can be considered as of only limited value. The small quantities of body fat recovered did show a slightly lower overall unsaturation (iodine value) in the oxidized as compared with the fresh fat diet group, but the effect was only one-third to one-quarter as great as in the rat experiment and the accuracy of the gas chromatographic analysis did not suffice to pick up any significant differences in the linoleate content of the body fats. This matter, therefore, remains undecided for chicks.

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