Studies in Vitamin A

26. THE VITAMIN A-REPLACING EFFECT OF LARD

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Although vitamin A deficiency causes profound changes in epithelial tissues, vitamin A as such is not detectable in epithelia of normal animals and its mode of action in protecting these tissues is unknown. The possibility that other substances may elicit a similar response cannot be ruled out. Considerable importance therefore attaches to verifiable claims for the cure of xerophthalmia and restoration of growth in vitamin A-deficient rats given 'curative' diets which contain neither preformed vitamin A nor known carotenoid provitamins.

Randoin & Netter (1934) used a diet made up of casein 15, lard 20, dried brewer's yeast 5, dextrin 56 and salt mixture 4%; they found it capable of curing xerophthalmia and restoring growth. Le Gallic (1949, 1950) regarded both lard and casein as essential in this diet and further stated (Le Gallic, 1951) that they should be present in the proportions: lard 20–21·5 and casein 18%. Le Gallic has himself devoted special attention to the need for casein. Mayer & Krehl (1948) had also noted the vitamin A-like activity of lard. Kaunitz & Slanetz (1950*a*, *b*) recently claimed that an active material could be concentrated by molecular distillation of lard. The first 7% to be eliminated in the process contained most of the vitamin A-replacing factor. Vitamin A itself was not present as judged by tests sensitive to 0·1 μg./g., nor could carotenoids be detected. When the distillate was added at the level of 2% to a vitamin A-free diet, the test animals fared better than litter mates on the same diet (without lard distillate) corrected by vitamin A palmitate (7·6 i.u./week, by injection).

The implications of the work of Kaunitz & Slanetz make it necessary to test the reproducibility of their findings.

EXPERIMENTAL

Materials

Lard. Freshly rendered lard was obtained on a Ministry of Food permit from Gaskell Bros. of Warrington.

Lard distillate. The lard was subjected to molecular distillation at very low pressure and the material eliminated at 210–220° (7–8%) was collected. We are indebted to Dr R. E. Stuckey and Mr A. W. Davidson of the Research Laboratories of British Drug Houses Ltd., for kindly carrying out the distillation.

Vitamin A solution. Vitamin A, administered to some of the animals, was in the form of a tested cod-liver oil diluted with arachis oil to 12 i.u./ml.

Examination of lard and lard distillate

Lard. Lard dissolved in *cyclo*hexane shows an absorption spectrum with inflexions at 315–335 mμ. ($E^{1\%}_{1\,cm.}$ 325 mμ. 0·02) and 220–235 mμ. ($E^{1\%}_{1\,cm.}$ 225 mμ. 4·5) and $\lambda_{max.}$ at 269–273 mμ. ($E^{1\%}_{1\,cm.}$ 0·147). A 10% (w/v) solution of lard in *cyclo*hexane was colourless and gave no colour with the $SbCl_3$ reagent.

The lard used yielded 0·23% unsaponifiable material as a very pale yellow, low-melting solid which in concentrated solution fluoresced pale blue under ultraviolet illumination.

A solution of the unsaponifiable material in *cyclo*hexane (8·6%, w/v) was yellow and gave a very pale-green colour with the $SbCl_3$ reagent. No absorption band could be detected in this green solution. The absorption curve of lard unsaponifiable material showed inflexions at 225 mμ. ($E^{1\%}_{1\,cm.}$ 14·0), 250–300 mμ. ($E^{1\%}_{1\,cm.}$ 270 mμ. 5·5), 315–365 mμ. ($E^{1\%}_{1\,cm.}$ 330 mμ. 0·9), 445–460 mμ. ($E^{1\%}_{1\,cm.}$ 450 mμ. 0·06) and 470–495 mμ. ($E^{1\%}_{1\,cm.}$ 480 mμ. 0·04).

The unsaponifiable material was chromatographed on watered Al_2O_3 (Table 1), but neither preformed vitamin A nor carotenoid precursors could be found in any fraction.

As it was conceivable that very small amounts of vitamin or provitamin might escape detection because of destruction during the saponification process, attempts were made to detect vitamin A and carotene by chromatography of lard itself on 2% watered Al_2O_3. Table 2 gives detailed results of such chromatography.

In an attempt to ascertain whether the inflexions in the region of 330 mμ. in chromatographic fractions 3–5 (Table 2) were due to vitamin A, it was decided to bulk these fractions and saponify. Fractions 1 and 2, and 6 and 7 were also bulked and saponified. In each case saponification was carried out by adding the required volume of 60% (w/v) KOH to the fat, warming until a solution was obtained and then adding 2 vol. of boiling ethanol. To minimize destruction of possible traces of vitamin A, the mixtures were then boiled for 2 min., after which time they were cooled, diluted with water and the unsaponifiable material extracted. The soaps were acidified and the liberated acids extracted. The absorption spectra of both the unsaponifiable materials and the acids were investigated, results being shown in Table 3.

No fraction from lard has given a positive test for the presence of vitamin A or carotene, but it is clear that the lard contains traces of conjugated diene, triene and tetraene acids and that the ultraviolet absorption shown by lard unsaponifiable fractions are of some interest. The failure to obtain a blue colour with the $SbCl_3$ reagent makes it very improbable that vitamin A is present in any fraction.

Table 1. *Chromatography of lard unsaponifiable matter*

(Al_2O_3 (10 g.) (P. Spence and Co., grade 0) weakened with water (0·5 ml.) used. 172 mg. lard unsaponifiable material placed on column. Length of column, 7 cm.; diameter, 1·5 cm. Recovery from column, 113·6 mg.)

Fraction	Eluant	Eluate (mg.)	Absorption spectra (*cyclo*hexane)	Remarks
1	Light petroleum (60 ml.)	8·4	General absorption	No colour with $SbCl_3$ reagent
2	2% Ether:light petroleum (100 ml.)	5·6	~250 mμ., $E^{1\%}_{1\,cm.}$ 50·5 ~265–300 mμ., $E^{1\%}_{1\,cm.}$ 280 mμ. 39·7	No colour with $SbCl_3$ reagent
3	4% Ether:light petroleum (150 ml.)	11·0	$\lambda_{max.}$ 235 mμ., $E^{1\%}_{1\,cm.}$ 52·3 flat 240–250 mμ., $E^{1\%}_{1\,cm.}$ 250 mμ. 51·1	No colour with $SbCl_3$ reagent Fraction contains material fluorescing pale blue
4	8% Ether:light petroleum (300 ml.)	46·6	~258 mμ. $E^{1\%}_{1\,cm.}$ 7·4 ~269 mμ., $E^{1\%}_{1\,cm.}$ 5·8	No colour with $SbCl_3$ reagent. Fraction contains material fluorescing green-blue
5	16% Ether:light petroleum (100 ml.)	1·2	Not examined	No colour with $SbCl_3$ reagent
6	32% Ether:light petroleum (100 ml.)	Nothing eluted	—	—
7	64% Ether:light petroleum (100 ml.)	7·8	General absorption	No colour with $SbCl_3$ reagent. Contains material fluorescing pale blue
8	Ether (100 ml.)	Nothing eluted	—	—
9	Al_2O_3 extracted with boiling ethanol (3 times)	33·0	In ethanol ~270 mμ., $E^{1\%}_{1\,cm.}$ 6·75. General absorption in visible region (400–500 mμ.)	A pale-yellow, low-melting-point solid. No colour with $SbCl_3$ reagent; contains a material fluorescing pale blue

Table 2. *Chromatography of lard*

(Al_2O_3 (200 g.) (P. Spence and Co., grade 0) weakened with water (4 ml.) used. 21·87 g. lard placed on column. Length of column 31 cm.; diameter 2·5 cm. Recovery from column, 19·78 g.)

Fraction	Eluant	Eluate (g.)	Absorption spectra (*cyclo*hexane)	Remarks
1	Light petroleum (500 ml.)	4·05	Steep end absorption (230–260 mμ.) flattening from 265 mμ.	—
2	2% Ether:light petroleum (500 ml.)	11·56	Steep end absorption (230–260 mμ.) ~270 mμ., $E^{1\%}_{1\,cm.}$ 0·04 ~280 mμ., $E^{1\%}_{1\,cm.}$ 0·03	—
3	5% Ether:light petroleum (500 ml.)	1·05	~265–270 mμ., $E^{1\%}_{1\,cm.}$ 270 mμ. 0·2 ~310–330 mμ., $E^{1\%}_{1\,cm.}$ 320 mμ. 0·023	No colour with $SbCl_3$ reagent
4	10% Ether:light petroleum (500 ml.)	0·52	Broad ~270–280 mμ., $E^{1\%}_{1\,cm.}$ 275 mμ. 0·4 ~316–354 mμ., $E^{1\%}_{1\,cm.}$ 330 mμ. 0·09	No colour with $SbCl_3$ reagent
5	20% Ether:light petroleum (400 ml.)	0·08	Definite ~265–280 mμ., $E^{1\%}_{1\,cm.}$ 272 mμ. 2·35 Very slight ~320–350 mμ., $E^{1\%}_{1\,cm.}$ 330 mμ. 0·27	No colour with $SbCl_3$ reagent
6	Ether (500 ml.)	2·20	Very definite flat portion 265–270 mμ., $E^{1\%}_{1\,cm.}$ 267 mμ. 8·5	—
7	Ethanol (500 ml.)	0·28	$\lambda_{max.}$ 227 mμ., $E^{1\%}_{1\,cm.}$ 20·6 Very definite ~265–270 mμ., $E^{1\%}_{1\,cm.}$ 265 mμ. 4·12	—
8	Top 1 cm. of Al_2O_3 extracted with boiling ethanol	0·003	Not investigated since it gave opalescent solutions in petrol, *cyclo*hexane, ether, ethanol, $CHCl_3$ and CS_2	Pale yellow solid
9	Remainder of Al_2O_3 extracted with boiling ethanol	0·06	$\lambda_{max.}$ 230 mμ., $E^{1\%}_{1\,cm.}$ 43·0 ~270 mμ., $E^{1\%}_{1\,cm.}$ 3·8	—

Table 3. *Saponification of chromatographic fractions of lard*

Fractions	Weight used (g.)	Unsaponifiable material (g.)	Ultraviolet absorption of unsaponifiable material (*cyclo*hexane)	Recovered acids (g.)	Ultraviolet absorption of acids
1 and 2	15·44	0·14	$\lambda_{max.}$ 228 mμ., $E^{1\%}_{1\,cm.}$ 3·5	13·6	$\lambda_{max.}$ 269 mμ., $E^{1\%}_{1\,cm.}$ 1·05 ~225 mμ., $E^{1\%}_{1\,cm.}$ 4·5
3–5	1·51	0·023	~225 mμ., $E^{1\%}_{1\,cm.}$ 15·3 flattens out at 250 mμ. Very slight ~315–330 mμ., $E^{1\%}_{1\,cm.}$ 320 mμ. 1·5 (No colour with $SbCl_3$ reagent)	1·37	$\lambda_{max.}$ 230 mμ., $E^{1\%}_{1\,cm.}$ 11·2 $\lambda_{max.}$ 269 mμ., $E^{1\%}_{1\,cm.}$ 4·5 $\lambda_{max.}$ 320 mμ., $E^{1\%}_{1\,cm.}$ 0·40
6 and 7	2·27	0·057	~225–230 mμ., $E^{1\%}_{1\,cm.}$ 225 mμ. 15·5 ~255–275 mμ., $E^{1\%}_{1\,cm.}$ 265 mμ. 5·9 Very slight ~315–325 mμ., $E^{1\%}_{1\,cm.}$ 320 mμ. 1·1 (No colour with $SbCl_3$ reagent)	1·99	$\lambda_{max.}$ 233 mμ., $E^{1\%}_{1\,cm.}$ 170 $\lambda_{max.}$ 269 mμ., $E^{1\%}_{1\,cm.}$ 91 $\lambda_{max.}$ 320 mμ., $E^{1\%}_{1\,cm.}$ 0·93

Table 4. *Diets used in experiments planned to test the vitamin* A*-activity of lard distillate*

	Diet		
Material	1 Vitamin A-low, given to pregnant rats (g./100 g.)	2 Vitamin A-free (g./100 g.)	3 Test (g./100 g.)
Casein (light white)	18	—	—
Casein (ethanol-extracted)*	—	18	18
Starch	65	65	65
Marmite	8	8	8
Arachis oil	5	5	—
Salt mixture†	4	4	4
Lard distillate	—	—	5

* Light white soluble casein (B.D.H. Ltd.) (400 g.) was boiled under reflux with abs. ethanol (2 l.) for 3 hr. It was then filtered at the pump and twice washed with 200 ml. portions of boiling ethanol. The casein was again refluxed with fresh ethanol (2 l.) for 3 hr., filtered and washed as before. It was then dried overnight at 110° in an electric oven and on the next day ground to a fine powder and incorporated in the diet mixtures.

† Osborne & Mendel (1913).

Lard distillate. The absorption spectrum is characterized by inflexions at 220–230 mμ. ($E^{1\%}_{1\,cm.}$ 225 mμ., 5·0), 260–270 mμ. ($E^{1\%}_{1\,cm.}$ 265 mμ. 0·41) and 310–340 mμ. ($E^{1\%}_{1\,cm.}$ 320 mμ. 0·05) in *cyclo*hexane. A 10% (w/v) solution of lard distillate in $CHCl_3$ was colourless and gave no colour with the $SbCl_3$ reagent, while more concentrated solutions (20%, w/v) gave an evanescent green. The available lard distillate was required for the feeding experiment and none could be spared for saponification or chromatographic separation. Such experiments were in any case unnecessary after the tests recorded in Tables 1 and 2.

Animals and diets

Adult female hooded rats were given a vitamin A-low diet after mating. This resulted in litters having relatively small liver reserves of the vitamin and thus shortened the time required for depletion.

The newly weaned animals were transferred to a vitamin A-free diet. The composition of the diets is shown in Table 4.

Procedure

Newly weaned rats from three litters were distributed into groups 1 and 2 (card selection) so that each group contained two animals (one of each sex) from each litter. The animals were separately housed. For the first 4 weeks of the depletion period each rat was given 10 g. food/day; thereafter, and for the whole of the test period, 12 g. food/day were given. Water was provided *ad lib.* throughout.

All animals received diet 2 during the period of depletion which was terminated when there was either a marked loss in weight or definite xerophthalmia or both. Animals belonging to group 1 were then given 6 i.u./day vitamin A (in 0·5 ml. arachis oil), but otherwise remained on diet 2. Animals in group 2 were transferred to diet 3 and in addition were given 0·5 ml. arachis oil/day. The oil and the vitamin A in oil were administered orally from a calibrated tube.

Analysis of tissues

At the end of the experimental period the rats were anaesthetized and as much blood as possible obtained by cardiac puncture. The blood samples from each group of rats were pooled and analysed for vitamin A (Glover, Goodwin & Morton, 1947). Livers and kidneys were removed, washed, roughly dried with filter paper and weighed. The livers from animals in group 1 were pooled and the lipid extracted (Glover *et al.* 1947). The unsaponifiable fraction was then obtained and examined. The same procedure was followed for kidney and for the same tissues from group 2 animals.

RESULTS

Animals in group 1. When placed on the vitamin A-free diet the six rats continued to increase in weight for 35–55 days. Each showed a period of practically steady weight; this persisted for a variable time before there was a rapid decline in weight or definite signs of xerophthalmia. Of the four animals with marked eye lesions three were completely cured and grew quickly on administration of vitamin A. The fourth rat was very weak, although xerophthalmia was only incipient; it died although 100 i.u./day of vitamin A were given for 2 days before death. The remaining two rats did not show xerophthalmia, but vitamin A brought about an excellent growth response. Fig. 1 summarizes the experience with some of these animals, and the results of analyses of pooled tissues are shown in Table 5.

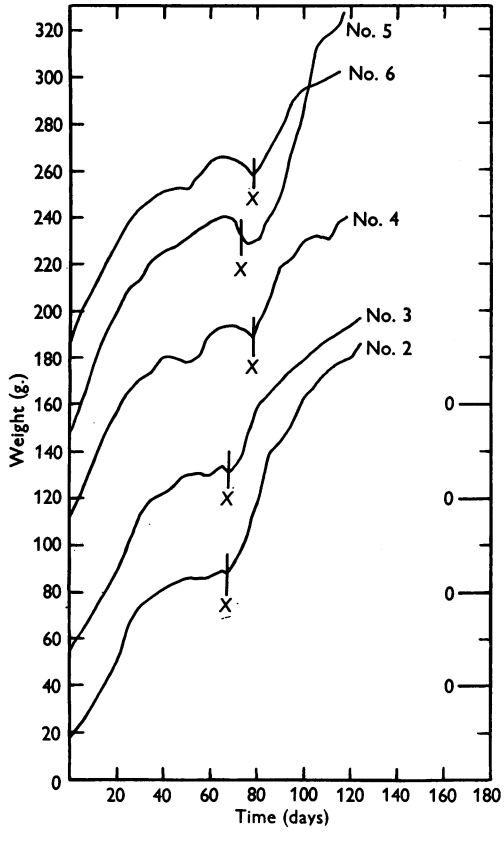

Fig. 1. Growth curves for rats of group 1. × = time at which each individual rat was supplied with 6 i.u. vitamin A/rat/day. Base-line for each growth curve is 40 units above the preceding one to avoid overlapping.

Table 5. *Vitamin* A *in tissues of rats cured of the signs of deficiency*

(Group 1 received 6 i.u. vitamin A/rat/day; group 2 received lard distillate.)

	Group 1	Group 2
Blood vitamin A (i.u./100 ml. plasma)	52·3	None
Liver vitamin A	None	None
Kidney vitamin A (i.u./g.)	7·0	None

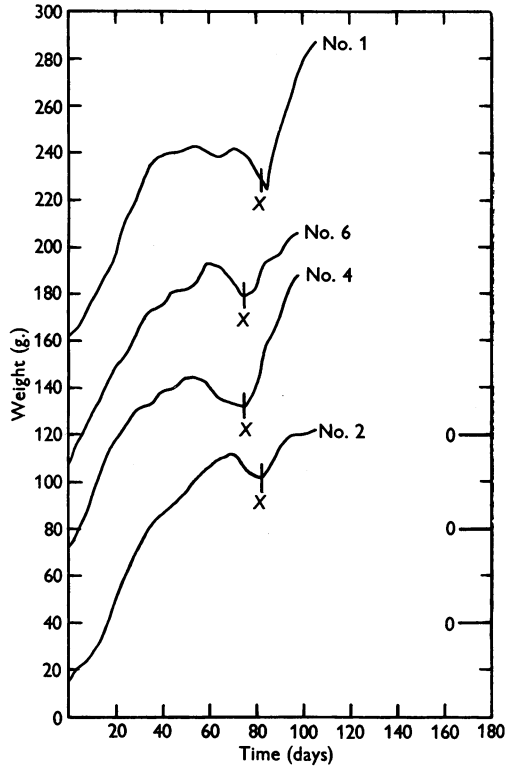

Fig. 2. Growth curves for rats of group 2. × = time at which each individual rat was supplied with diet 3 (test diet), 0·6 g. lard distillate/rat/day. Base-line for each growth curve is 40 units above the preceding one to avoid overlapping.

Table 6. *Comparison of growth responses of vitamin* A *deficient rats to vitamin* A *and lard distillate*

(Group 1 rats received 6 i.u. vitamin A/day; group 2 rats received about 0·6 g. lard distillate/day.)

	Gains in weight (g./23 days)	
Rat	Group 1	Group 2
1	Died	57
2	56	20
3	40	Killed on 77th day
4	45	54
5	26	Died
6	39	26

Animals in group 2. On the vitamin A-free diet the growth rate of the six rats decreased and weights became approximately constant after 35–60 days. One rat was killed after 77 days and no vitamin A could be found in its liver or kidneys (antimony trichloride colour test on unsaponifiable matter). Three animals developed xerophthalmia and were losing weight rapidly when diet 3 was used to replace diet 2. The remaining two rats were transferred to diet 3 when they began to lose weight quickly although xerophthalmia had not appeared. The five rats were also given 0·5 ml. arachis oil daily. After 23 days all the rats were killed and the pooled tissues were analysed (Table 5). The following case histories record briefly the responses to diet 3:

Rat no. 1. Weight was falling quickly prior to treatment; xerophthalmia. On diet 3 the rat continued to lose weight for 3 days, but thereafter gained weight quickly and the xerophthalmia soon disappeared.

Rat no. 2. No eye lesions were seen. Rapid loss of weight was at once reversed on the diet containing lard distillate. After 13 days, growth decreased and became negligible but restarted towards the end of the 23-day period.

Rat no. 3. Killed on 77th day.

Rat no. 4. Rapid loss of weight was promptly changed to rapid increase; eye lesions, which were definite, had disappeared after 7 days on diet 3.

Rat no. 5. Severe xerophthalmia and rapidly falling weight were present before treatment. Although the decline in weight was stopped, the animal died after 2 days on diet 3. On the day before death the rat was very listless, both eyes were closed and encrusted with blood.

Rat no. 6. The rat was losing weight rapidly before being transferred to diet 3; growth was at once resumed and sustained for the entire 23 days. No eye lesions were seen at any stage.

Growth curves of animals in group 2 are shown in Fig. 2. Table 6 shows the gains in weight of group 1 animals given 6 i.u. vitamin A/day and of group 2 animals given about 0·6 g. lard distillate/day. For both groups the gains refer to 23 days after beginning the treatment.

DISCUSSION

In preliminary experiments on the same lines as those recorded above it was found that a diet containing 2% of lard distillate brought about a temporary halt to the decline in weight of vitamin A-depleted rats, but failed to restore growth. When, however, the proportion of lard distillate was raised to 6%, xerophthalmia was cured and normal growth was promptly resumed. The distillate used by Kaunitz & Slanetz (1950*a*, *b*) thus seems to have been more potent than ours. This fact is relatively unimportant; the significant thing is that the existence of the 'lard factor' is confirmed, since the lard distillate administered at the level of 0·6 g./rat/day elicited a response similar to that of 6 i.u. preformed vitamin A/rat/day. Although a vitamin A-replacing potency of about 10 i.u./g. is not high it seems impossible to attribute it to preformed vitamin A or a carotenoid provitamin A.

A few comments on the experiment are necessary. The food intake of each rat was strictly controlled. Diets 2 and 3 were isocaloric since the only difference was the replacement of arachis oil by an equal weight of lard distillate. The extra calories made available to the rats in group 1 in the form of 0·5 ml./day arachis oil (containing vitamin A) were balanced by 0·5 ml./day of unfortified arachis oil to the rats in group 2. This amount of arachis oil was less than that already in the vitamin A-free diet (Table 4) which rules out the possibility that the growth response was due to the oil itself.

The mean gains in weight in the first 23 days of treatment were 41·2 g. for the vitamin A-treated rats and 39·25 g. for those given lard distillate. The difference is not significant. It seems unlikely, moreover, that the concentration effected by distillation is merely a matter of accumulating tocopherols which could act as anti-oxidants preserving minute amounts of vitamin A. At no stage in the examination of lard or distillate was any spectroscopic evidence of tocopherols observed. The evidence is clearly against attributing the response to vitamin A or carotene.

The effects of vitamin A deficiency on epithelial tissues are so definite that it is reasonable to expect some trace of the vitamin in those tissues which in avitaminosis exhibit the characteristic keratinizing metaplasia. In fact, however, Popper (1941, 1944) and his colleagues failed to discover any evidence for the presence of vitamin A in the epidermis or epithelium of the mucous membranes, tissues which are considered to be the first sites of vitamin A deficiency. The fluorescence technique used might well have revealed anything more than minute traces. The only claim to have found vitamin A in epithelia is that put forward by Edwards, Finklestein & Duntley (1951) for its presence in intact human skin, but even these workers admit that they do not know whether the vitamin is in the skin structure proper or in the circulating blood.

There is a clear choice between (*a*) assuming that the amount of vitamin A needed in normal epithelial tissue is extremely minute and (*b*) considering a new hypothesis that the keratinizing metaplasia is brought about by the presence of a harmful resultant of generalized avitaminosis A. Vitamin A need not necessarily be the unique antagonist of such a product.

The function of vitamin A in visual processes depends ultimately on the interconversion of vitamin A alcohol and its aldehyde (retinene) in the presence of alcohol dehydrogenase. The protein of rhodopsin, opsin, seems to have a very highly

specialized role. The function of vitamin A in preventing xerophthalmia, in maintaining healthy epithelia and in promoting growth, is so little understood that the work of Kaunitz & Slanetz (1950*a*, *b*) on the lard factor may provide a new approach if the active substance can be characterized.

SUMMARY

1. The claim that lard contains a vitamin A-replacing factor has been confirmed.

2. A molecular distillate (210–220°) of lard, administered at the level of 0·6 g./day, cured xerophthalmia and restored normal growth in avitaminotic rats.

3. Neither preformed vitamin A nor carotenoid provitamin A could be detected by spectrophotometric or colorimetric tests on lard or lard unsaponifiable matter before or after chromatography, or on lard distillate.

We wish to thank Dr H. Jasperson, Dr R. E. Stuckey and Mr A. W. Davidson for valuable help. One of us (J. S. L.) has participated in this work as holder of a University Fellowship.

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The Lipids of Fish

4. THE LIPIDS EXTRACTED BY AN ETHANOL:ETHER MIXTURE FROM HADDOCK FLESH PREVIOUSLY EXTRACTED WITH ACETONE

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The material discussed in the present paper was the lipid fraction extracted at room temperature from the flesh of the haddock by ethanol:ether (3:1, v/v) after the tissue had been exhaustively extracted with acetone, also at room temperature, and was the second in the series of extracts described in part 1 of this series of papers (Lovern, 1953). The total crude extract available weighed 94 g., being 0·22 % of the wet weight of the haddock flesh. Although this had been heated on a steam can under vacuum for about an hour it was later found still to contain 16 g. of solvent. The difficulty of removing the last traces of solvent or water from phospholipids is well known, but it was considered inadvisable further to prolong the original heating. A large mass of sticky phospholipid could obviously trap some residual solvent almost indefinitely. The corrected weight of the present crude lipid extract is, therefore, 0·18 % of the wet weight of the tissue.

ANALYTICAL METHODS

The analytical methods used were as described in previous papers (Lovern & Olley, 1953; Olley & Lovern, 1953), with the following exceptions.

Inositol. We have found that the filter-paper chromatographic examination of lipid hydrolysates, using an ammoniacal silver nitrate spray reagent (Hough, 1950), is unreliable for the detection of inositol in very low concentrations. Thus in examining the hydrolysates of various fractions of the acetone extract by the chromatographic method we were unable to detect any inositol (Olley & Lovern, 1953), whereas we have since found that it was present to the extent of 1·1 % in a product corresponding to the combined fractions *B* and *C* (Lovern & Olley, 1953) of the acetone extract. In the present work inositol was determined microbiologically after hydrolysis by refluxing for 6 hr. with 6 N-HCl, using Northam & Norris's (1951) procedure and the organism *Kloeckera brevis* with basal medium I plus a bacteriological yeast extract prepared as by Northam & Norris (1952). There are different views on the