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Irving, J. T.; Budtz-Olsen, O. E.

1955

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Irving, J.T. Budtz-Olsen, O.E.1955. The relation between the action of vitamin E and protein in the body, and the influence of various fish-liver oils in the diet upon vitamin E activity in vivo. British Journal of Nutrition;9(03):301-309

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The Relation between the Action of Vitamin E and Protein in the Body, and the Influence of Various Fish-liver Oils in the Diet upon Vitamin E Activity in Vivo

By J. T. IRVING

Joint Dental Research Unit of the Council for Scientific and Industrial Research and the University of the Witwatersrand, Johannesburg

AND O. E. BUDTZ-OLSEN

Department of Physiology, Medical School, University of Cape Town

(*Received* 14 *February* 1955)

The experiments reported here were undertaken primarily to investigate further the histological changes occurring in the enamel organ of the rat's incisor tooth during vitamin E deficiency. These changes were originally described by Irving (1942) and have been confirmed in general by Granados, Mason & Dam (1945, 1946) and by Pindborg (1950, 1952). It was, however, found by the present writers, using a diet based on that of Gillman, Gilbert, Gillman & Spence (1952), that extreme changes in the enamel organ, avertable by vitamin E, could be produced in 21 days or less, whereas the changes described by the other authors took much longer to develop and were less extensive.

In an attempt to determine further the effects of other dietary factors upon the enamel organ, certain additional findings seemed to be of significance. The present paper is concerned chiefly with these, and an account of the histological aspect is being prepared for publication. In particular it was found that the vitamin content of the oil containing highly unsaturated fatty acids was of importance and also that the actions of protein and vitamin E, which superficially appeared similar, differed in certain fundamental respects.

EXPERIMENTAL

Animals. The experimental animals were 137 young rats descended from ancestors of the Wistar strain. Both sexes were used indiscriminately, as we got similar results with either. The rats were put on to the experimental diets when they weighed about 50 g. Previously to that both they and their mothers were fed on the stock diet employed in this Department. With one exception the rats were in groups of at least six.

Diets. The diets used were based on that of Gillman *et al.* (1952). They consisted of potato starch, dried brewer's yeast and fish-liver oil, sometimes with dried egg albumin (Hopkins and Williams Ltd) added. The proportions were as shown in Table 1. A thick paste was made of the starch in boiling water. After it had cooled, the other ingredients were thoroughly stirred in. The fish oil used was either cod-

liver oil or hake-liver oil. According to Hilditch (1947) the content of highly unsaturated fatty acids in these two oils does not differ markedly. Their vitamin D contents likewise are not markedly different, but the vitamin A content of hake oil is approximately fifteen times that of cod-liver oil. Except when stated, the diets were fed without stint.

Table 1. *Composition of diets used and times of killing of experimental animals*

		Composition of diet							
Diet no.	No. of rats in group	Potato starch (%)	Cod-liver oil (%)	Hake-liver oil (%)	Yeast (%)	Egg albumin (%)	Protein (%)	α-Tocopheryl acetate (mg/day)	Days on diet before killing
1	9	60	20	—	20	Nil	9·3	Nil	20, 30, 40, 50, 60
2	6	70	10	—	20	Nil	9·3	Nil	20, 30, 40
3	6	77·5	2·5	—	20	Nil	9·3	Nil	20, 30, 40
4	9	60	20	—	20	Nil	9·3	3	20, 30, 40, 50, 60
5	6	70	10	—	20	Nil	9·3	3	20, 30, 40
6	6	77·5	2·5	—	20	Nil	9·3	3	20, 30, 40
7	6	47·5	20	—	20	12·5	18·8	Nil	20, 30, 40
8	6	47·5	20	—	20	12·5	18·8	3	20, 30, 40
9	42	60	—	20	20	Nil	9·3	Nil	2, 5, 10, 15, 20, 30, 35, 40, 50
10	8	70	—	10	20	Nil	9·3	Nil	20, 30, 40
11	15	60	—	20	20	Nil	9·3	3	10, 20, 30, 40
12	14	47·5	—	20	20	12·5	18·8	Nil	10, 15, 20, 30, 40
13	4	47·5	—	20	20	12·5	18·8	3	20, 30, 40

Vitamin E. Vitamin E was given as α-tocopheryl acetate (Ephynal, Roche Products Ltd) in 3 mg doses daily. A 3 mg tablet was crushed and mixed with 3 g of freshly made diet each day, which were then given to the animals in the morning before they were fed, and no more diet was given until the 3 g had been eaten.

General treatment. The animals were weighed twice weekly. They were killed between 20 and 60 days after going on to the diet (see Table 1). Blood was taken for the dialuric acid haemolysis test (György & Rose, 1949). The state of pigmentation of the upper incisor teeth was noted and also the presence or absence of pigment in the uterus. The skulls, radii, ulnae, tibiae, kidneys and right median lobe of the liver were removed for histological examination. After decalcification, the upper incisor teeth, the epiphyses of the lower ends of the radii and ulnae and upper ends of the tibiae were cut in longitudinal section. Sections were also made of the kidney and liver. All sections were stained with haematoxylin and eosin.

RESULTS

Growth rate

On the diets with either hake- or cod-liver oil, the animals either lost weight or made very small gains. With 20% cod-liver oil all the animals lost weight, but as the percentage of oil was reduced the animals made up their losses later in the experiment and gained somewhat. In general the same was found with hake oil, but with 20% hake oil the weight losses were not so universal. Vitamin E dosage improved the picture in the rats on cod-liver oil, small weight gains, especially towards the end of

the experiment, being found in all. Vitamin E had hardly any effect on the weight changes in the rats on hake oil.

When given extra protein, all rats on both oils gained considerable weight over the period of the experiment, those on cod-liver oil at the same rate as stock rats of the same age, and those on hake-liver oil at a lower rate. Hake-liver oil thus contained some factor counteracting the beneficial effect of protein. The inclusion of vitamin E made no difference to the weight gains of rats on the diets with extra protein.

In spite of the weight losses or poor gains on the basal diet, only one rat died during the experimental period.

In some of the experiments with rats on the hake-liver oil diet, paired feeding was employed between the rats on the vitamin E-free diet and on the diet with extra protein. The food consumption of the vitamin E-free rats was somewhat less than that of those on the protein diet *ad lib.*, but was surprisingly high in view of the fact that the weight changes were so slight. The food intake of these rats rose slowly as the experiment progressed. The weight gains of the pair-fed rats on the protein diet were naturally less than those of the rats fed on the protein diet *ad lib.*, but were much greater than those of the corresponding rats deficient in vitamin E. In spite of the restriction in food intake, the rats pair-fed on the protein diet showed the same changes in other respects as those on the full protein diet.

Based on the average food intakes, the daily vitamin A intake with cod-liver oil was about 3600 i.u. at the 20% level in the diet and correspondingly less with reduction of the oil level. With hake-liver oil, the intake was 60,000 i.u. at the 20% level in the diet. No signs of hypervitaminosis A were seen, there were no bone fractures, and, as stated below, when the protein content of the diet was raised, the bone structure was normal.

Incisor teeth

The histological change in pure vitamin E deficiency is confined to the enamel organ. With the types of diet described in this paper, the changes occur at the junction of the formative two-thirds and the incisal third. The earliest change is a sudden disappearance of all the layers of the enamel organ; they are replaced by fibrous tissue in which are embedded a number of macrophages laden with translucent granules. This change has been already described (Irving, 1942, 1952). With the lapse of time, the extent of the degenerated enamel organ increases and extends towards the formative end of the tooth: in extreme cases as much as two-thirds of the enamel organ can have disappeared, being replaced by fibrous tissue. The description of the changes by Granados *et al.* (1945, 1946) was brief and not illustrated by microphotographs, but their findings appear to be similar to these. Pindborg (1950, 1952) described changes of a lesser degree, which took longer to occur on his diet and were prevented by the administration of tocopherol. Along with these changes, the incisor teeth and especially the maxillary incisors may lose their normal orange pigment, either becoming paler and finally white or losing the pigment in strips and finally getting completely white. The latter changes were the ones usually seen in these experiments. Moore (1950)

stated that it took about 6 weeks for the teeth to become white on a vitamin E-free diet and Hove, Copeland & Salmon (1949) found teeth white at 10 weeks. There is no enamel hypoplasia (Granados & Dam, 1952).

Diets with cod-liver oil

With no tocopherol, no extra protein (*Table* 1, *diets nos.* 1–3). With levels of cod-liver oil from 2·5 to 20% in the diet, the enamel organ was consistently affected at 20 days, the lesion being more marked with time. The teeth were all white at 30 and 40 days, and two out of three were white at 20 days. It should be remembered that it takes some days for the tooth in contact with the degenerated enamel organ to erupt.

With 3 *mg α-tocopherol daily, no extra protein* (*Table* 1, *diets nos.* 4–6). At the 3 mg/day dose level, all teeth were protected at all levels of cod-liver oil intake, both as to the enamel organ and the colour of the teeth.

With no tocopherol, extra protein (*Table* 1, *diet no.* 7). With the possible exception of one rat, the enamel organ was completely protected by extra protein. The colour of the teeth was, however, not preserved; two of the rats had white teeth or teeth with pigment in strips only at 30 days, and all teeth had a similar appearance at 40 days.

With 3 *mg α-tocopherol daily, extra protein* (*Table* 1, *diet no.* 8). All the teeth were completely protected as to colour and histological appearance.

Diets with hake-liver oil

With no tocopherol, no extra protein (*Table* 1, *diets nos.* 9, 10). From 20 days onwards the enamel organ was affected on both diets. The teeth were white or only partly pigmented at 30 days.

With 3 *mg α-tocopherol daily, no extra protein* (*Table* 1, *diet no.* 11). Whereas with cod-liver oil vitamin E had been completely protective, with hake oil no protection was to be seen. Out of four rats two had enamel-organ degeneration by 20 days, and all rats had similar degeneration at 30 and 40 days. The teeth were white at 40 days.

With no tocopherol, extra protein (*Table* 1 *diet no.* 12). The degree of protection (eleven out of fourteen) of the enamel organ by protein was not so good as with the cod-liver oil diet but, as with cod-liver oil, many animals had depigmented teeth. In one series of eight litter-mate rats, half had white teeth from 15 days onwards, and two of these had normal enamel organs.

With 3 *mg α-tocopherol daily, extra protein* (*Table* 1, *diet no.* 13). The number of rats in this experiment was rather small. Protection of the enamel organ was found in all but one, and the tooth colour was normal throughout.

Bones

The epiphyses of the rats on the diets with cod- or hake-liver oil, both with and without vitamin E (Table 1, diets nos. 1–7 and 9–11), showed the changes described by Follis (1950) and by Frandsen, Nelson, Sulon, Becks & Evans (1954) as occurring with diets deficient in protein. The bones were completely or almost completely normal when the protein content of the diet was increased (Table 1, diets nos. 7, 8, 12, 13), and the addition of vitamin E made no difference.

Dialuric acid haemolysis test

Cod-liver oil. Irrespective of the level of oil in the diet, this test was positive in all rats on the diets that contained no vitamin E, both with and without extra protein (Table 1, diets nos. 1–3, and 7). When vitamin E was given, the test was uniformly negative, with both high and low protein diets (Table 1, diets nos. 4–6 and 8).

Hake-liver oil. The test was uniformly positive, irrespective of the level of oil, in all rats on the diet with no vitamin E and no extra protein (Table 1, diets nos. 9, 10, 12). It was negative in all rats on the diet with vitamin E, both with and without extra protein (Table 1, diets nos. 11, 13) and also in the rats receiving extra protein but no vitamin E (Table 1, diet no. 12).

Renal changes

Degeneration of the convoluted tubules, similar to that described by Martin & Moore (1939) and by György & Goldblatt (1949), was found in the rats on the 20% cod-liver oil diet without vitamin E (Table 1, diet no. 1). It was present in one out of two rats at 30 days, and in all the other rats killed before 60 days (seven in all). No rats getting vitamin E or extra protein or both showed this change, nor did the rats on the lower levels of cod-liver oil (Table 1, diets nos. 2–8). The kidneys of the rats on the hake-liver oil diets showed no pathological changes (Table 1, diets nos. 9–13).

Uterus

Martin & Moore (1936) and, more recently, many others have shown that the uterus becomes discoloured in vitamin E deficiency. In the present experiments this change was seen to a slight degree in only four rats at 40 and 50 days on the vitamin E-free diet with cod-liver oil. In all probability the experiments were of too short duration to show this change to any marked extent.

Livers

The changes in the livers were not consistent, but a great number from rats on all the diets showed a slight change characterized by a piecemeal necrosis of cells and a mild fatty change, usually centrilobular. There was no vascular and little cellular response, and no bile-duct hyperplasia. More males than females were affected. No change similar to that observed by Gillman *et al.* (1952), using the diet without the inclusion of fish oil, was observed by the present writers. There was no parallelism with the renal changes.

DISCUSSION

Hake-liver and cod-liver oils. One of the chief conclusions to be drawn from these results is the importance of using a suitable oil containing highly unsaturated fatty acids when investigating vitamin E activity. Dam & Grenados (1945) and Granados *et al.* (1946) pointed out originally that highly unsaturated fatty acids must be present in the vitamin E-free diet to produce degeneration of the enamel organ and dental depigmentation. Cod-liver oil has been usually used, but it is apparent that both the level

and type of oil are of great importance. The chief and probably only difference between hake- and cod-liver oils lies in their vitamin A content. With cod-liver oil, our results fell into line with those of other observers; the dental lesions and colour change were completely prevented by vitamin E. With hake-liver oil, on the other hand, vitamin E had no protective action, either on the enamel organ or tooth colour. Cod-liver oil has been considered destructive of vitamin E in vitro (Mattill, 1940; Mackenzie, Mackenzie & McCollum, 1941) and also metabolically antagonistic in vivo (Mason & Filer, 1947; György & Goldblatt, 1949). In the present experiments, however, cod-liver oil did not prevent the action of vitamin E, and it must be some constituent of hake-liver oil, other than highly unsaturated fatty acids and possibly vitamin A, that had this effect.

It must be pointed out that many workers have produced white teeth in rats on vitamin E-free diets containing no, or very little, fat with highly unsaturated fatty acids. One such diet, that used by Moore & Wang (1947), caused enamel-organ degeneration as well as incisal depigmentation (Irving, 1954), but it required about a year for the diet to produce this effect. It would appear that the presence of cod-liver oil greatly speeds up the process, though as little as 2·5% in the diet, in the present experiments, was in this respect as effective as 20%. When the cod-liver oil was omitted from the diets used in the present experiments, the animals had severe signs of vitamins A and D deficiency, which quite obscured any changes related to vitamin E.

Influence of protein. Within recent years many workers have shown protein to have a partial protective action on the incisal depigmentation developed on vitamin E-free diets (Hove, 1946; Hove & Harris, 1947; Hove, Copeland & Salmon, 1949; Granados, 1949; Granados, Aaes-Jørgensen & Dam, 1949; Moore, 1949). Hove & Harris (1947), judging from effects on the efficiency of protein utilization, considered α-tocopherol and protein to act by separate and independent mechanisms, but Lindan & Himsworth (1950) considered from their protective action on the liver that vitamin E and protein 'focus on a common point in metabolism'.

None of the above quoted workers who reported on the teeth examined them microscopically, and it seems to have been tacitly assumed that the colour losses in the incisors are due to histological changes in the enamel organ. The present results show that this is not invariably so. Thus, with cod-liver oil diets at all levels of the oil, the colour and histological changes followed closely the use of the vitamin E-free diet, the diet plus vitamin E and the diet with vitamin E and protein. With one rather doubtful exception, there was no correlation between these two changes in the animals on the vitamin E-free diet plus protein. The teeth were depigmented, but the enamel organ looked entirely normal histologically. Since vitamin E can protect against enamel-organ degeneration in the absence of adequate protein in the diet, it is apparent that it can replace protein in this respect, but only vitamin E can prevent enamel depigmentation. The loss of pigment on high-protein diets must be due to some biochemical derangement of the process of laying down the pigment, a condition that vitamin E can avert even with low concentrations of protein in the diet. Hove (1946), Hove *et al.* (1949) and Moore (1949) have also found that the protein level of the diet is immaterial for the pigment-protecting action of vitamin E.

The question therefore arises whether the degeneration of the enamel organ has anything to do with depigmentation of the enamel. It seems difficult to believe that the two processes are entirely unconnected, especially as with the other diets a close correspondence was observed. Irving (1952) brought forward histological evidence that there was a connexion and suggested that the pigment was removed from the enamel by macrophages after degeneration of the enamel organ. It is at present impossible to draw further conclusions on this matter, save to say that vitamin E and protein act in part by a similar mechanism, protein having some of the action of vitamin E. The fact that both extra protein and vitamin E are necessary for the protection of tooth colour and of the enamel organ, when the diet contains hake-liver oil, supports this proposition.

Dialuric acid haemolysis test. The results with this test, for the cod-liver oil diets, were in line with the results reported above. Thus the test was negative when vitamin E was given and positive when it was not. In particular, it was positive in the animals on the vitamin E-free diet with extra protein, showing that in this respect, too, protein has not got complete vitamin E activity.

With the hake-liver oil diets, it was negative in the animals receiving vitamin E, although the protective action of vitamin E on the tooth colour and enamel organ was abolished. This is surprising and is the first instance we know in which the validity of this test can be questioned. No explanation is at present forthcoming. Nor is it clear why the test should be negative for the diet with extra protein but no vitamin E.

Bone formation. The results quoted above show that, at least under these experimental conditions, vitamin E deficiency has no effect upon endochondral bone growth. Barrie (1937) had claimed that vitamin E influenced bone structure and development.

Renal lesions. The present results do not confirm in their entirety those of György & Goldblatt (1949), though admittedly the diet used was quite different. Their cirrhosis-producing rations contained *inter alia* a low level of protein (casein), Crisco (a hydrogenated fat) or lard. α-Tocopherol doses up to 30 mg daily did not prevent the renal change. Also, Crisco is stated to contain α-tocopherol. The addition of methionine, but not of cystine, prevented the kidney lesions. Apparently extra protein in the diet was not tested. Daft (1954), using diets with different levels of protein (casein), found that the kidney lesions increased as the protein level was increased to 16% and were less at the 18% level, though still considerable. He also reported that the incidence of lesions was higher if the fat used, at the 10% level, was more saturated and less if the fat was more unsaturated. In the present results both extra protein and α-tocopherol prevented the renal lesions from appearing; the kidney lesion occurred in animals on the 20% cod-liver oil diet and was not seen when the level of oil was reduced. It is apparent that the nutritional background is of great significance in the aetiology of these renal changes.

Liver changes. It is rather surprising that a great number of rats on all the diets in the present experiments showed the slight changes described on p. 305. Gillman *et al.* (1952) observed severe liver necrosis in 10% of the rats on the diet with brewer's yeast, and nothing approaching this picture was seen here, indicating that both fish-liver oils had a protective effect. Many workers have shown that both vitamin E and

protein will protect the liver against necrosis (Schwarz, 1944*a*, *b*; György, 1947; Hove *et al.* 1949; Lindan & Himsworth, 1950; Goettsch, 1951). In the experiments of Gillman *et al.* (1952), α-tocopherol averted the liver necrosis. Admittedly the diets used in the present experiments differed from those used by these workers, and this shows again the vital importance of the animal's nutritional status.

Casein has almost invariably been used by other workers as the protein for supplementing vitamin E-deficient diets. Egg albumin was used as a protein supplement in the present experiments rather than casein, since one aspect of the study was of the hard tissues and it was desirable not to change the phosphorus content of the diet more than necessary. Egg albumin has a higher content of both cystine and methionine than has casein. Fevold (1951) and Block & Bolling (1945), respectively, give the methionine content of egg albumin as 5·4 and 5·0% and the cystine content as 0·52 and 1·7%. The methionine content of casein is placed at 3·1 and 3·5% by McMeekin & Polis (1949) and Block & Bolling (1945), respectively, and the cystine content at 0·34 and 0·36%.

SUMMARY

1. Rats of the Wistar Institute strain were placed when weighing about 50 g on a vitamin E-free diet consisting of potato starch, dried brewer's yeast and variable amounts of cod-liver oil or hake-liver oil. Some rats were given extra protein, and positive control animals received 3 mg α-tocopheryl acetate daily.

2. The protective action of vitamin E upon the incisal enamel organ and tooth colour of rats on vitamin E-free diets containing oils with highly unsaturated fatty acids was influenced by the type of oil used. With cod-liver oil protection was complete, but with hake-liver oil there was no protection, although the dialuric acid haemolysis test was negative. This effect was possibly due to the high vitamin A intake.

3. Protein had a protective action on the integrity of the enamel organ but not upon the tooth colour when the rats were on a vitamin E-free diet containing cod-liver oil, whereas vitamin E protected both, even when the protein content of the diet was low. It is suggested that lack of pigmentation on the vitamin E-free diet is due to a biochemical and a structural change in the enamel organ, and protein can prevent the latter but not the former change.

4. Vitamin E had no influence upon endochondral bone formation.

5. Kidney and liver lesions, the occurrence of which do not conform to those reported in the literature, are described.

The expenses of this work were defrayed by grants from the Staff Research Fund, University of Cape Town, and from the South African Council for Scientific and Industrial Research. The latter body also gave a grant for technical assistance. For these grateful acknowledgement is made. The writers are also indebted to Professor J. Gillman for guidance in the examination of the liver sections and to Miss V. Jenkinson for her skilled technical help.

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