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Studies on the Effects of the Nutrition on Antioxidant Levels of the Body

I. Tissue Antioxidants in Chicks on an Encephalomalacia-producing Diet

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The content of water-soluble antioxidants in the brain is decreased in encephalomalacia. The antioxidant content of blood plasma of chicks is larger than that of mammals. Uric acid occurs in higher concentrations in chicken plasma and has been found to possess free-radical scavenging properties.

The function of vitamin E as an *in vivo* antioxidant was early postulated and was established beyond doubt by the demonstration of lipoperoxides in the adipose tissues of chicks and rats fed vitamin E-deficient diets containing highly unsaturated fatty acids.[1]

The study of the mode of action of vitamin E became complicated by the discovery that several of the deficiency symptoms could also be prevented by factor 3 and selenium. One of the diseases not influenced by factor 3/selenium is encephalomalacia in chicks. It is prevented by vitamin E and by certain redox compounds and antioxidants, *e.g.*, methylene blue, but so far all attempts to demonstrate the presence of lipoperoxides in the encephalomalacic brain have been without result.

A study on the antioxidant level in vitamin E-deficient chicks was carried out by Budowski and Mokadi.[2] The method used was an adaption of the novel principle for determination of antioxidants by means of a stable free radical indicated by Blois.[3] Antioxidants were determined in trichloroacetic acid filtrates of livers and brains of chicks reared on an encephalomalacia-producing diet. No difference was observed in the livers, but in extracts of the cerebella the amount of antioxidants was decreased in comparison with control animals receiving tocopherol. Soluble thiols as well as non-SH antioxidants were decreased suggesting that the decrease was due to the oxidizing action of free radicals.

Methods for determination of antioxidants, based on Blois' principle, were also worked out in this laboratory and used in a study on the occurrence

of antioxidants in mammalian blood and livers.[4] Three groups of substances reacting with the stable free radical α,α-diphenyl-β-picrylhydrazyl are present: Fat-soluble antioxidants, water-soluble antioxidants, and proteins containing, *e.g.*, thiol-groups.

The methods have been used for the determination of the antioxidants in blood plasma, liver and brain of chicks given an encephalomalacia-producing diet and of a control group supplemented with vitamin E. Three sets of determinations were carried out, *viz.*, (1) directly on plasma and aqueous organ extracts (total amount of antioxidants), (2) on protein-free filtrates (non-protein antioxidants), and (3) finally, fat-soluble antioxidants were determined in lipid extracts of brains.

EXPERIMENTAL

Animals. Day-old chicks were reared on a vitamin E-low starter ration for one week and then allocated to two groups. One of the groups was given the encephalomalacia-producing diet while the other received the same diet supplemented with D,L-α-tocopherol acetate, 0.1 mg/g diet.

The encephalomalacia-producing diet had the percentage composition: casein ("Vitamin-test", Genatosan Ltd., Loughborough, England), 30; gelatine, 3; salt mixture,[5] 5.17; vitamin mixture,[5] 0.1; choline chloride, 0.2; corn starch, 31.53; and lard 30, and was supplemented with 2-methyl-1,4-naphthohydroquinone diphosphoric ester dicalcium salt, 10 μg/g, and selenium dioxide, 1.4 μg/g. Vitamins A and D were given orally in aqueous solution,[6] 0.1 ml twice a week, corresponding to vitamin A, 250 i.u., and vitamin D_3, 20 i.u. per chick daily.

The chicks were inspected daily. As soon as a chick showed signs of encephalomalacia, a sample of blood was taken from the jugular vein into heparin. The chick was killed by decapitation, and cerebrum and a portion of the liver removed for analysis. Control samples from a vitamin E-supplemented chick were taken simultaneously. Encephalomalacia was diagnosed by the occurrence of the gross symptoms of ataxia and by macroscopic examination of the brain.

Preparation of blood plasma and organ extracts. The heparinized blood sample was centrifuged and the plasma diluted with 4 parts of distilled water. A part of the solution (0.8 ml) was used for the determination of total antioxidants while the rest was deproteinized with tungstic acid. After adjusting pH to 5.6 with saturated aqueous sodium acetate, 1 ml was used for determination of non-protein antioxidants.

The cerebra were weighed, homogenized with 30 parts of water in a tissue homogenizer and gently centrifuged. Two ml of the supernatant was taken for determination of dry-matter, 0.5 ml for determination of total antioxidants, and the remainder deproteinized in the same manner as plasma. One ml of the protein-free filtrate was used for the determination of non-protein antioxidants.

Weighed portions of liver were treated in exactly the same manner as the brains. Antioxidant determinations were carried out on 0.3 ml of the homogenates and on 0.6 ml of the protein-free filtrates.

Fat-soluble antioxidants were determined in the brains of chicks raised on the same diets, but from a second experiment. The following procedure was used for the extraction: One cerebrum or 2-3 cerebella were weighed and homogenized with 3 ml benzene; 5 ml acetone was added, the mixture ground again, centrifuged, and 3 ml of the supernatant used for the determination.

Determination of antioxidants. The methods for determination of water-soluble antioxidants were as described in the previous paper [4] with the only modification that a more concentrated solution of the reagent was used (optical density of the blanks about 1.0) This was done in order to cover a broader field of antioxidant levels. The results were calculated as μequiv. per ml blood plasma and per g dry matter of liver and brain.

Fat-soluble antioxidants were determined by the addition of 1 ml reagent of a suitable strength to 3 ml of the extract mentioned above. The optical densities were

read after 10 min and compared with a blank (3 ml benzene-acetone + 1 ml reagent). The results were calculated as μequiv. per g fresh weight of cerebrum or cerebellum.

Physico-chemical examination of plasma. Plasma of chicks and calves was dialyzed for 1 h at room temperature in a Viscose tubing membrane against 5 volumes of distilled water. The tubing was moved up and down mechanically during the dialysis.

The ultra-violet absorption spectra of the dialysates were registered by means of a Beckman Ratio Recording Spectrophotometer. An estimate of the uric acid content was made from the optical density at the absorption maximum about 290 mμ (molar extinction coefficient 12.200 [7]).

Ascorbic acid was determined in the dialysates by the photometric 2,6-dichlorophenol-indophenol-xylene method.[8] The colorimetric nitroprusside procedure [9] was used for the determination of glutathione.

RESULTS

Encephalomalacia occurred, after periods varying from 9 to 32 days, in all the animals on the vitamin E-deficient diet. No symptom occurred in the

Table 1. Mean values with their standard errors for the content of water-soluble antioxidants in organs of chicks reared on an encephalomalacia-producing diet.

Addition to the diet	Number of determinations	Antioxidants μequiv./ml Total	Non-protein
		Blood plasma	
None	18	1.28 ± 0.07	0.78 ± 0.07
Vitamin E	19	1.46 ± 0.08	0.87 ± 0.06
Addition to the diet	Number of determinations	Antioxidants μequiv./g dry matter Total	Non-protein
		Liver	
None	17	117 ± 5	66 ± 3
Vitamin E	18	106 ± 2	65 ± 2
		Brain	
None	19	64 ± 5	27 ± 2
Vitamin E	20	69 ± 3	39 ± 1

Table 2. Mean values with their standard errors for the content of fat-soluble antioxidants in brains of chicks reared on an encephalomalacia-producing diet.

Addition to the diet	Number of determinations	Antioxidants μequiv./g fresh weight
	Cerebrum	
None	19	0.30 ± 0.01
Vitamin E	14	0.29 ± 0.01
	Cerebellum	
None	9	0.21 ± 0.02
Vitamin E	8	0.21 ± 0.02

Table 3. Antioxidants in plasma of chicks, calves and rats.

Substance	Animal	Antioxidants μequiv./ml plasma	Reference
Whole plasma	Chicken	1.4	Present paper
	Calf	0.3	» »
	Rat	0.3	4
Non-dialyzable	Chicken	0.3	Present paper
	Calf	0.2	» »
	Rat	0.18	4
Ascorbic acid	Chicken	0.13	Present paper
	»	0.22	10
	Calf	0.04	»
	Rat	0.04—0.1	»
Uric acid	Chicken	0.5—0.9	Present paper
	» (whole blood)	0.5	11
	Cow	0.11	»
	Rat	0.18	»

animals on the vitamin E-supplemented diet. Encephalomalacia occurred, in 13 to 35 days, in all animals of a third group fed the vitamin-deficient diet supplemented with 2.5 % uric acid.

The results of the determinations of antioxidants in the tissues of the animals on the deficient and the vitamin E-supplemented diets are presented in Tables 1 and 2.

A comparison of the ultraviolet spectra of dialysates of chicken and calf plasma revealed a great absorption at 290 mμ in plasma of chicks, whereas in calves only a minor inflection occurred. Since uric acid has absorption maximum at 293 mμ, a solution of uric acid was tested by means of the antioxidant determination method. Uric acid was found to react quantitatively (1 mole = 2 equiv.) with diphenyl-picrylhydrazyl.

Representative results of antioxidant determinations, together with the contents of uric and ascorbic acids found by the physico-chemical methods, in plasma of chicks and calves are presented in Table 3. For comparison, data from the literature on the content of uric and ascorbic acids in plasma, calculated as μequiv. per ml plasma, are included in the table. No measurable content of glutathione could be found in the plasma samples.

DISCUSSION

The results show that the antioxidative activity of the water-soluble constituents of the brain is lower in encephalomalacia than in the control chicks. The difference between the two groups is due to the non-protein antioxidants and is of a high statistical significance. The results confirm the observations of Budowski and Mokadi.[2]

When the activities of the brain protein fractions of the two groups are calculated by subtracting non-protein from total amount of antioxidants,

no difference is observed. No significant difference is seen between the livers or the blood plasmas, neither with respect to the antioxidant activity of the total nor the non-protein fraction.

No difference was found between the contents of fat-soluble antioxidants of the brains of vitamin E-deficient chicks and those supplemented with tocopherol.

Studies on the occurrence in brains of the most prominent fat-soluble free radical scavengers, the tocopherols and the ubichromenols, are scarce. Edwin *et al.*[12] reported that the content of α-tocopherol and ubichromenol in rat brains varied with diet and sex; a total content of 0.02—0.1 μequiv./g brain can be calculated from their figures. We find a higher level which is the same even though tocopherol is added to the diet. Since the use of our method for brain has not been controlled with other methods, the results may not be entirely reliable.

The antioxidant activity of the total water-soluble and the non-protein fractions of the livers was of the same order of magnitude (total activity about 25 μequiv./g fresh tissue) as that reported earlier for mammalian livers.[4] On the other hand, the activity of the plasma was much greater than that of mammalian plasma (Table 3). The reason for the higher activity of chicken plasma was found to be the much higher concentration of ascorbic acid and, especially, uric acid.

Uric acid reacts quantitatively with diphenyl-picryl-hydrazyl. It seems that the function of uric acid as a free-radical scavenger has not earlier been reported in the literature. The substance has been used as a stabilizer for hydrogen peroxide, and its ability to react with oxidizing substances to form a great variety of oxidation products is well-known. From a quantum-mechanical approach, based on its very low energy coefficient of the highest occupied orbital, Pullman and Pullman[13] have calculated that uric acid should be a very good electron donor.

It was suggested in our previous paper[4] that ascorbic acid is the most abundant antioxidant in plasma. An inspection of Table 3 shows, however, that uric acid accounts for a greater share of the free-radical scavenging potency of plasma, of chicks, rats and calves, than does ascorbic acid.

The biological significance of the free-radical combining power of uric acid is difficult to evaluate. Wyngaarden and Stetten[14] established that although the larger part of uric acid administered to man is excreted unchanged in the urine, about 20 % of labelled uric acid is broken down. Peroxidase, catalase, and methemoglobin have been shown to be capable of catalyzing the oxidation of uric acid in the presence of hydrogen peroxide. The special reactivity of uric acid observed in our studies opens for still more speculation about possible ways for uricolysis.

It remains also to be studied whether uric acid has a function as a biological antioxidant. The addition of 2.5 % uric acid to the diet to one group of chicks influenced very little the onset of signs of encephalomalacia. Evidently uric acid cannot be included in the group of substances which exhibit vitamin E-mimetic activity in feeding experiments. On the other hand, the dietary amount of uric acid should be compared with the amount synthesized in the animal. Creek and Vaisaitis[15] found that approximately

one-fourth of dietary nitrogen was excreted as uric acid in the chick. Since the encephalomalacia-producing diet contained 33 % protein, an excretion of uric acid amounting to about 3.5 % of the food consumption should be expected, *i.e.*, that an amount of 2.5 % added to the diet will probably not produce a very great increment of the uric acid pool of the chick.

Toxic substances, *e.g.*, alloxan, may be formed by the attack of free radicals on uric acid. The amount of such substances may be elevated when vitamin E is lacking, and they may play a role in the development of symptoms of the deficiency, especially those which can be produced in birds only.

As to the etiology of encephalomalacia, the theory has been advanced that toxic metabolites formed through autoxidative processes outside the brain are the causative agents. The fact that the antioxidative activity decreased in brain but not in blood and liver do not lend support to such a theory but makes a local metabolic disturbance seem more likely. It is known from studies on livers of rats on necrogenic diets that the content of water-soluble antioxidants may decrease to about one half without greatly influencing the onset of necrosis.[16] By analogy it seems most probable that a decrease of the content of water-soluble antioxidants of the brain of about one third (Table 1) is not the cause of the encephalomalacia but rather a consequence of the disease. Probably in local sites certain unsaturated fatty acids, when they are not protected by adequate amounts of tocopherol, generate free radicals and initiate chain-reactions whereby the antioxidants are consumed. However, only further studies can explain the seemingly contradictory result: That tocopherol protects against encephalomalacia while dietary cystine is without effect — and at the same time only the *water-soluble* antioxidants are decreased in the affected brains.

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