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ALPHA-OXIDATION OF CEREBRONIC ACID IN BRAINS FROM SCORBUTIC AND
ASCORBIC ACID-SUPPLEMENTED GUINEA PIGS*

by

James F. Mead and Roberta S. Hare

LABORATORY OF NUCLEAR MEDICINE AND RADIATION BIOLOGY

900 VETERAN AVENUE

UNIVERSITY OF CALIFORNIA, LOS ANGELES, CALIFORNIA 90024

AND DEPARTMENT OF BIOLOGICAL CHEMISTRY

UCLA SCHOOL OF MEDICINE, LOS ANGELES, CALIFORNIA 90024

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James F. Mead and Roberta S. Hare

Laboratory of Nuclear Medicine and Radiation Biology, 900 Veteran Avenue, University of California, Los Angeles, California 90024, and Department of Biological Chemistry, UCLA School of Medicine, Los Angeles, California 90024.

SUMMARY

Brains from scorbutic and ascorbic acid supplemented guinea pigs were used as a source of enzyme for decarboxylation of [1-¹⁴C]-2-hydroxytetracosanoic acid. Both in the 10,000xg supernatant fraction and the recombined 110,000xg pellet and supernatant fractions, decarboxylation was greatly reduced. In the latter experiments, the loss of activity was largely due to the supernatant fraction, which contains most of the brain ascorbic acid. Ascorbic acid supplementation, both in vivo and in vitro restored enzymatic activity in the preparations from scorbutic animals; but, in vitro, did not affect the preparations from the supplemented animals.

Recent experiments in this laboratory (1) and that of Radin (2) have demonstrated the existence, in the mammalian brain, of a system of alpha oxidation or one-carbon degradation of the long-chain fatty acids. In vitro studies (3,4,5,6) have indicated that this enzymatic activity is concentrated in the microsomal fraction (or the 110,000xg precipitate) and that it requires the 110,000xg supernatant and certain other cofactors, including oxygen. Unpublished experiments from this laboratory have shown that the only required additive is ferrous ion and that the supernatant factor is indeed ascorbic acid, as had been suspected by Levis (7).

In view of this information and as a further test of the function of ascorbic acid as the true cofactor in vivo, it was considered of interest to test for alpha oxidation activity in the brains of scorbutic as compared with ascorbic acid supplemented guinea pigs.

MATERIALS AND METHODS

Animals Six 275 g guinea pigs were raised on an ascorbic acid-

deficient diet (Nutritional Biochemicals) with ascorbic acid (30-50 mg/100 ml) included in the drinking water of 3 of the animals designated controls. With an average water consumption of 100 ml, each of the control animals received 30 mg of ascorbic acid daily. After 10 days, the experimental animals had begun to show signs of ascorbic acid deficiency (weight loss, tenderness of the joints, unkempt coat) whereas the controls appeared completely normal. At this time one control and one experimental animal were sacrificed. After 14 days a second pair was used. The brains, which weighed the same in control and experimental animals, were homogenized in about 10 volumes of 0.25 M sucrose solution buffered to pH 7.4 with .01 M phosphate buffer. The homogenate thus produced was centrifuged at 1000xg, the precipitate was discarded and the supernatant suspension was centrifuged for 0.5 hours at 10,000xg. This supernatant (S_4) was used directly for enzymatic studies or was centrifuged at 110,000xg. The supernatant, S_5 , and pellet P_5 , from this centrifugation were recombined in equal volumes for enzyme studies.

Materials Cerebronic acid-1- ^{14}C was synthesized by a procedure to be described elsewhere. It was better than 94% pure by TLC and GLC, the impurities consisting of small percentages of neighboring homologs. For use in the enzymatic reactions, it was converted to a 10^{-4} M micellar solution by dissolving .4 mg of the acid in 15.5 mg of monolaurin, treating this mixture with a solution of 28.8 mg taurocholate in 10 ml of 0.1 M phosphate buffer, pH 7.4, and subjecting the mixture to sonication with a Branson Sonifier, model S110. The resulting clear solution was homogeneous and remained so until it was frozen following the experiment. Thawing and sonication served to clarify it before each use.

Other chemicals and solvents were reagent grade and were used without further purification.

Methods The procedures for carrying out the enzymatic reactions and

for collecting and counting the $^{14}\text{CO}_2$ have been described previously (4). Either 1.5 ml of S_4 or 1 ml each of S_5 and sonicated (30 sec at level 1 at 4°C) P_5 were used for the reactions. In addition, each vial contained Fe^{++} ($5.8 \times 10^{-5} \text{ M}$), medium (NaHCO_3 , $3.4 \times 10^{-2} \text{ M}$; phosphate buffer, $2.4 \times 10^{-2} \text{ M}$) and substrate (0.2 ml of sonicated micellar solution containing .02 μmoles , 3300 cpm of cerebronic acid-1- ^{14}C). In some cases 0.1 ml of a $4 \times 10^{-3} \text{ M}$ solution of ascorbic acid (final concentrations, $5.8 \times 10^{-5} \text{ M}$) or the same volume of water was added. Incubations were for one hour at 37° followed by addition of 0.2 ml of 9 N H_2SO_4 to the mixture and 0.2 ml of hydroxide of hyamine solution to the center cup and further incubation for 1.5 hrs at room temperature. The hyamine solutions were dissolved in 15 ml of a solution at 5 g diphenyl oxazole and .5 g popop in 1 l. toluene and were counted on a Packard Tricarb Liquid Scintillation Spectrometer operating at 80% efficiency.

Quantitative determination of ascorbic acid in the S_5 fractions was carried out using a modification of the method of Roe *et al.* (8).

RESULTS

Table I shows the radioactivity of the carbon dioxide collected from incubations of the S_4 fractions of scorbutic and control guinea pig brains. It can be seen that there is very little activity in the scorbutic brain preparation as compared to the control. The presence of $5.8 \times 10^{-5} \text{ M}$ ascorbic acid in the scorbutic incubation brought the activity to normal, whereas it had little effect on the control homogenate.

In Table II are shown the comparisons of different combinations of the P_5 and S_5 fractions from control and scorbutic guinea pigs. As might be expected, the combined fractions from the control brains were the most active. Almost as active is the scorbutic P_5 and control S_5 combination whereas those combinations with scorbutic S_5 fractions had relatively

low activity.

Analysis of the ascorbic acid concentrations in the S_5 fractions revealed 20 $\mu\text{g/ml}$ for the controls, 4 $\mu\text{g/ml}$ for the 10 day scorbutic animal and 10 $\mu\text{g/ml}$ for the 14 day scorbutic animal.

DISCUSSION

Although the above experiments do not constitute incontrovertible evidence that ascorbic acid is the natural cofactor for alpha oxidation, it is strongly indicative. It might be argued that ascorbic acid has been shown to be active in the in vitro alpha oxidation system and that it has been known for some time that the brain, in common with certain other tissues, normally has a relatively high ascorbic acid concentration. However, it seems to be more than coincidental that in preparations from the brains of scorbutic guinea pigs, alpha oxidation is reduced and that it is restored maximally by ascorbic acid concentrations in the same range as is normally found in the brain. That the failure of alpha oxidation in the scorbutic brain is due simply to a lack of ascorbic acid is evident from the fact that the P_5 , or microsomal, fraction is active and that the loss is in the S_5 fraction, in which the ascorbic acid is found (5). In confirmation of this idea is the finding that the ascorbic acid content of the S_5 from the 10-day scorbutic guinea pig was 20% of normal while that from the 14-day scorbutic animal was 50% of normal. It is interesting that the decrease in alpha oxidation activity of the S_4 fractions is roughly proportional to these values. The implications of these results are not yet clear, but it seems evident that alpha oxidation represents yet another oxidase reaction in which ascorbic acid is a cofactor. Present experiments designed to elucidate the mechanism of the in vitro reaction may shed light on the function of the in vivo reaction as well.

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REFERENCES

1. Mead, J. F. and G. M. Levis, J. Biol. Chem. 238, 1634-1636 (1963).
2. Hajra, A. K. and N. S. Radin, J. Lipid Res. 4, 448-453 (1963).
3. Levis, G. M. and J. F. Mead, J. Biol. Chem. 239, 77-80 (1964).
4. MacDonald, R. C. and J. F. Mead, Lipids 3, 275-283 (1968).
5. Hare, Roberta and J. F. Mead (unpublished experiments).
6. Davies, E., A. K. Hajra, S. S. Parman, N. S. Radin and J. F. Mead, J. Lipid Res. 7, 270-276 (1966).
7. Levis, G. M., Biochim. Biophys. Acta 99, 194-197 (1965).
8. Roe, J. H. and C. A. Kuether, J. Biol. Chem. 147, 399-407 (1943).

TABLE I

Comparison of Alpha Oxidation in the 10,000xg Supernatant Fractions
(S₄) from Scorbutic and Control Guinea Pig Brains

Fraction	Ascorbic acid (6×10^{-5} M)	Duration of Experiment	
		10 days	14 days
Radioactivity (cpm) of CO ₂			
S ₄ (Cont)	+	-	129
	-	70	95
S ₄ (Scorb)	+	91	122
	-	20	38

Each vial contained S₄ (1.5 ml), phosphate buffer (2.4×10^{-2} M), bicarbonate, (3.4×10^{-2} M), ferrous ammonium sulfate (6×10^{-5} M), substrate (0.2 ml 5.4×10^{-6} M), and 0.1 ml of ascorbic acid (6×10^{-5} M) or water for a final volume of 3 ml. Incubations were for one hour at 37° C followed by addition of 0.2 ml hyamine solution in the cup and 0.2 ml, 9N H₂SO₄ to the homogenate and 1.5 hours further incubation at room temperature.

TABLE II

Comparisons of Alpha Oxidation in Combinations of the 110,000xg Pellet (P₅) and Supernatant (S₅) Fractions from Scorbutic and Control Guinea Pig Brains

Combinations	Duration of Experiment	
	10 days	14 days
Radioactivity (cpm) of CO ₂		
P ₅ (Cont), S ₅ (cont)	93	81
" " , S ₅ (scorb)	46	46
P ₅ (Scorb), S ₅ (cont)	85	69
" " S ₅ (scorb)	25	41

Incubations were as described for Table I except that 1 ml each of P₅ and S₅ were substituted for 1.5 ml of S₄.