C-terminal analysis of degraded basic protein

Amino acids[a]	Relative amounts of aa's in protein	Relative amounts of C-terminal groups			
		0.1 F ascorbic acid		0.01 F ascorbic acid	
		Phosphate buffer	Triethylamine buffer	Phosphate buffer	Triethylamine buffer
Arg[b]	1.00	1.00	1.00	1.00	1.00
Ala	0.78	0.01	0.005	0.03	0
Asp	0.50	0.07	0.75	0	0.41
Flu	0.44	0.05	0.83	0	0.54
Gly	1.39	0.05	0.03	0.09	0
Leu	0.56	0.06	0.22	0	0
Ser	1.06	0.07	0.52	0.04	0.28
Thr	0.30	0.03	0.23	0.01	0.13

[a] All amino acids are listed which were observed on C-terminal analysis to be present in concentrations greater than 10^{-3} that of arginine in any one of the 4 experiments.
[b] Quantitative comparison of the C-terminal analysis of untreated basic protein with the analysis of treated basic protein indicated that only the C-terminal arginine of the protein contributed to the arginine value determined from the degraded protein. Therefore, we have assumed that the arginine concentration in the degraded and undegraded protein solutions are identical.

Results and discussion. The auto-oxidation of ascorbic acid is a complex process which produces many products including hydrogen peroxide and free radicals. The Figure shows the gel electrophoresis of the basic protein after treatment with either hydrogen peroxide or ascorbic acid. The greater the concentration of either the ascorbic acid or the peroxide, the more extensive the degradation of the protein. In these experiments the breakdown due to ascorbic acid appears to be greater than that due to the peroxide.

The gel electrophoresis of the degraded protein does not indicate the nature of the degradation. In order to study the peptide breakage, we performed C-terminal analysis on the degraded material. The basic protein contains a single C-terminal residue, arginine. The Table gives the relative amounts of amino acids recovered from C-terminal analysis of the degraded protein. Also given in the Table is the content of each amino acid contained within the protein relative to arginine, as determined by amino acid sequence of the protein[11].

By comparing the relative amounts of the different amino acids with the undegraded protein with those observed from C-terminal analysis of the degraded protein, one can determine whether or not the degradation by ascorbic acid possesses a sequence specificity. It would appear that there is some specificity of the reaction. Peptide chains with C-terminal aspartic acid, serine, threonine, glutamic acid and leucine were released most easily. The degradation was more extensive in the triethylamine buffer. It is known that in the presence of phosphate, the basic protein aggregates (E. H. EYLAR, personal communication). The aggregation is minimal in the triethylamine buffer; therefore, the aggregation of the basic protein may have an effect on ascorbic acid degradation.

We have shown that ascorbic acid is capable of extensively cleaving the peptide chain within the myelin basic protein. It has been suggested that the protein degradation which has now been demonstrated in aerobic ascorbic acid solutions of catalase[7], transferrin[8,12], and basic protein may be related to the biological function of ascorbic acid[13].

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Effect of Cholesterol Oxidation on (Na^+, K^+) ATPase Activity of Erythrocyte Membranes

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Summary. By incubation of human erythrocyte ghosts with cholesterol oxidase (EC 1.1.3.6) part of the cholesterol of the membrane is replaced by 4-cholesten-3-one. This alteration in the sterol composition is accompanied by an inhibition of the (Na^+, K^+) ATPase of the erythrocyte membrane.

In a previous paper[2] we were able to demonstrate that the alterations in (Na^+, K^+) ATPase activity of erythrocytes from rats fed 20,25-diazacholesterol is due to the partial replacement of cholesterol by its biological precurser, desmosterol. In order to get more insight into the role of cholesterol on (Na^+, K^+) ATPase activity, we tried to alter the cholesterol molecule in the intact membrane.

A very simple method to induce changes in the sterol fraction of erythrocyte ghosts is the oxidation with cholesterol oxidase (cholesterol-O_2 oxidoreductase, EC 1.1.3.6) from Norcardia erythropolis. This enzyme effects the oxidation of cholesterol to 4-cholesten-3-one in the

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presence of oxygen[3]. 20 ml venous blood from healthy blood doners were drawn into heparinized syringes, plasma and red cells were separated and the erythrocyte ghosts were prepared as described earlier[4]. Protein determination was performed according to LOWRY et al.[5]. Erythrocyte ghosts (12 mg protein) were divided in 2 parts, one part was incubated with cholesterol oxidase (12 units) in 10 m*M* *Tris*-HCl-buffer pH 7.0 for various times (6 to 24 h) in a total volume of 6 ml. The other part served as control and was incubated in 10 m*M* *Tris*-HCl alone. After incubation was finished, 3 ml of each suspension were used for the determination of ATPase activity (for details see ref.[4]) and the other 3 ml were analyzed for the sterol composition. Total lipids were removed from the 3 ml of erythrocyte ghost suspension by a 3 times extraction with 20 ml chloroform/methanol (2:1; v/v). The combined extracts were washed 3 times with water. Then the lipid extract was brought to dryness and resuspended in 0.1 ml chloroform/methanol (2:1).

ATPase activity in human erythrocyte ghosts

	ATPase			Stimulation
	(Mg^{2+})	(Mg^{2+}, Na^+, K^+)	(Na^+, K^+)	(%)
Control	1.162	2.094	0.932	80.2
Control + H_2O_2	1.121	2.240	1.119	85.1
Control	1.186	2.210	1.024	86.3
Control + H_2O_2	1.140	2.186	1.046	91.8
Control	1.104	2.046	0.942	85.3
Control + H_2O_2	1.080	2.000	0.920	85.2

ATPase activity (μmoles P_i/mg protein $\times$ h) in human erythrocyte ghosts after 24 h incubation of 6 mg ghost protein in 0.1 *M* H_2O_2 in 10 m*M* *Tris*-HCl-buffer pH 7.0. Total volume 6 ml. In the controls H_2O_2 was omitted.

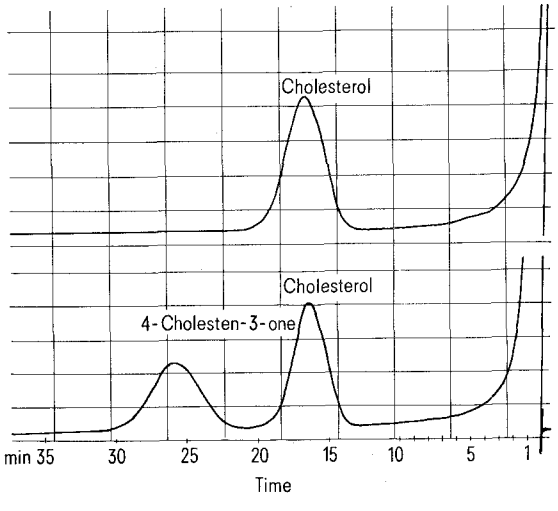

Fig. 1. The effect of incubation with cholesterol oxidase on the sterol fraction of erythrocyte ghosts. The gas-chromatographic procedure was performed in a Varian-Aerograph 1,400 using a 2 ft. column with 3% OV-17 on Chromosorb Q (120 mesh); 250° isotherm; N_2 = 36 ml/min FID. Response factor for 4-cholestene-3-one = 1.30.

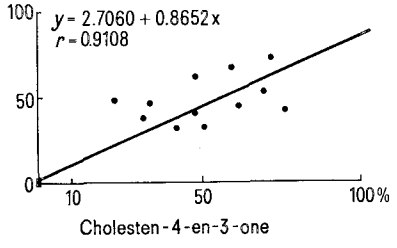

Fig. 2. The inhibitory effect of cholesterol oxidation on (Na^+, K^+) ATPase activity of human erythrocyte ghosts.

The percentage of 4-cholesten-3-one in the total sterol fraction was determined with 2 different methods which gave identical results. A) by quantitative cholesterol determination according to ZAK[6]. 4-cholesten-3-one does not react with the reagent. B) by a gas-liquid chromatographic procedure, the details of which are shown in Figure 1. As can be seen from Figure 1 the incubation of erythrocyte ghosts with cholesterol oxidase leads to a partial replacement of cholesterol by 4-cholesten-3-one in the membrane. The percentage of 4-cholesten-3-one varied from 24 to 76% according to the time of incubation. The total sterol content remained unchanged. In all 12 experiments performed the activity of the basic, (Mg^{2+}) ATPase was not influenced by cholesterol oxidase (1.18 $\pm$ 0.10 μmoles P_i/mg protein $\times$ h), whereas the (Na^+, K^+) ATPase was inhibited by oxidation of cholesterol to 4-cholesten-3-one; the average stimulation by Na^+ and K^+ being 92% for the control erythrocytes and 46% for the treated erythrocytes, the percentage of the latter one dependent on the degree of oxidation.

By plotting the % inhibition of the erythrocyte (Na^+, K^+) ATPase against the % of 4-cholesten-3-one in the membrane, we got the results shown in Figure 2. It can be seen that there is a linear correlation between these 2 parameters (correlation coefficient $r = 0.9108$; $y = 2.7060 + 0.8652 \times$). When about 50% of the cholesterol in the membrane is replaced by 4-cholesten-3-one, the activity of the (Na^+, K^+) ATPase is inhibited by about 50%. As H_2O_2 also occurs in the enzymatic oxidation reaction of cholesterol, we further analyzed the effect of H_2O_2 on the (Na^+, K^+) ATPase activity under the conditions for the oxidation. As can be seen from the Table, there is no change in (Na^+, K^+) ATPase activity after incubation of normal erythrocyte ghosts with an excess of H_2O_2.

By comparing the effect of cholesterol replacement by 4-cholesten-3-one with the results obtained for the replacement of cholesterol by desmosterol[2], we can state that the influence of desmosterol is just the opposite to the 4-cholesten-3-one effect. In the previous paper[2], we assumed that replacement of cholesterol by desmosterol is the cause of increased ATPase activity by altering the fluidity of the lipid moiety in the enzyme environment.

Since a direct involvement of cholesterol in (Na^+, K^+) ATPase activity is favoured only by a few authors[7-9], it is possible that the inhibiting effect of cholesterol oxidation is also due to an altered membrane fluidity but now in the opposite direction. According to our knowledge, up to now no experimental data are available about physical properties of mixed lipid bilayers in which cholesterol was replaced by 4-cholesten-3-one, but BRUCKDORFER et al.[10] were able to demonstrate a slightly increased osmotic fragility of erythrocytes after incubation in 4-cholesten-3-one containing media.

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