amine was added in order to bind protons liberated during metal chelation. It was found that the metal chelates of the flavin radicals possess an intense absorption in the near infrared with maximum in the region 810 to 840 mμ (Fig. 1.) in the case of valence-stable transition metal ions Zn^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+} and Mn^{2+}. It has been ascertained that this absorption band is due to the complex between the metal ion and the flavin free radicals by establishing for a diamagnetic metal ion (Zn^{2+}) that there is a linear relationship between the ESR and light absorptions (Fig. 2).

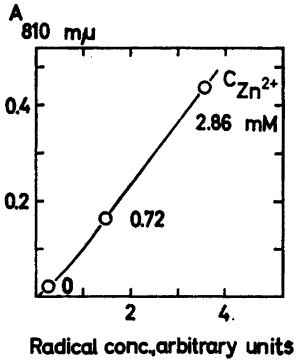

Fig. 2. Comparison of light absorption at 810 mμ and the integrated ESR absorption for different concentrations of added Zn^{2+} ions. Conditions as in Fig. 1.

The observed sharp absorption band of the radical chelated is easily distinguished from the broader bands of the dimolecular flavin complexes.[2,3]

The absorption band has not been observed with reducing metal ions (Fe^{2+}, Cu^{+}, MoO^{3+}),[4] nor has it been observed in partially reduced xanthine oxidase,[5] a metalloflavoprotein containing Fe and Mo.

Acknowledgements. Technical assistance by Miss Marie Ahlén is acknowledged. The work was supported by a PHS research grant AM-05895 from the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service and a travel grant from *Statens Naturvetenskapliga Forskningsråd.*

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Received July 10, 1964.

Formation of Stable Free Radicals in Alkaline Solutions of Some Monosaccharides

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With the technique of electron spin resonance (ESR) it has been found that free radicals are produced in a comparatively high yield when heating solutions of monosaccharides in a strongly alkaline medium. Radicals were obtained from D-glucose, D-fructose, D-mannose, D-galactose, D-glucuronic acid, D-glucosamine, L-fucose, and D-xylose. Most of the experiments were performed with 6 N KOH in H_2O (non-degassed), and with a molar concentration of the substances between 0.5 and 1.0. After heating on a boiling water bath for 1 to 1½ min the reaction mixtures were chilled to room temperature and filled into the sample tubes. The results obtained with D-glucose are shown in Fig. 1. Immediately after filling into the sample tube, the ESR-spectrum exhibited a complicated hyperfine structure (Fig. 1a), very probably due to the presence of more than one radical species. This pattern changed with time, and after about 20 min the spectrum was dominated by a triplet with a splitting constant of 0.81 gauss, and an intensity ratio of 1:2:1, indicating an interaction of the unpaired electron with two equivalent protons (Fig. 1b). The radical species giving rise to the triplet was rather stable, and the spectrum persisted without any decrease of the intensity for more than a week when the reaction mixture was kept in a stoppered sample tube.

Most of the substances considered exhibited radical spectra similar to those de-

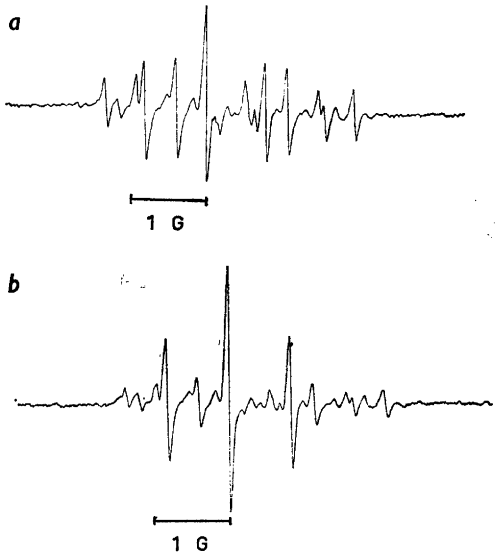

Fig. 1. ESR-spectrum of the radicals formed when heating a 1 M solution of D-glucose in 6 N KOH: *a*. Immediately after filling the sample tube; *b* After 20 min.

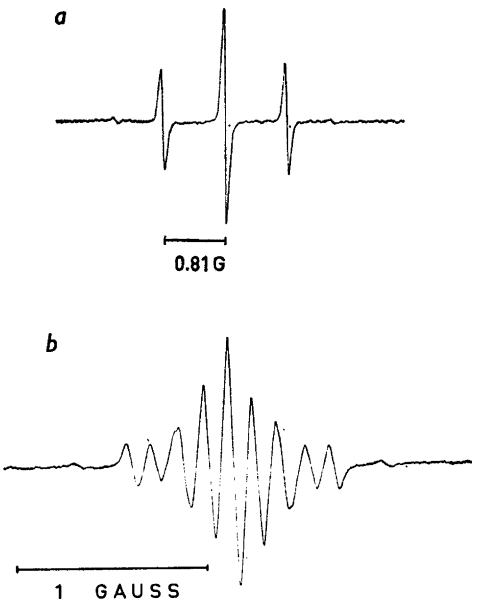

Fig. 2. ESR-spectrum of the radicals formed at room temperature in a 1 M solution of 1,3-dihydroxyacetone dissolved in *a*. 6 N KOH in H_2O; *b*. 6 N KOH in D_2O.

scribed for D-glucose. In all cases the triplet splitting dominated sooner or later, even if the initial transient pattern was different, as observed especially with D-glucosamine. No attempts were made to analyze these initial structures. D-Glucuronic acid and D-xylose exhibited the triplet splitting from the beginning with very little contamination of other structures.

In order to find the molecular structure of the radicals giving rise to the triplet splitting, some substances were studied which are known to be formed by the action of strong alkali on carbohydrates.[1,2] 1,3-Dihydroxyacetone (Fig. 3a) and DL-glyceraldehyde (Fig. 3b) were found to give rise to free radicals exhibiting a triplet splitting identical with that described above, other structures being absent (Fig. 2a). The radicals were formed at room temperature and could be observed about 5 min after the substances had been dissolved in 6 N KOH. The appearance of the radicals and the signal intensity were very much enhanced by bubbling oxygen through the solutions.

When 1,3-dihydroxyacetone was dissolved in 6 N potassium hydroxide prepared with D_2O, it was found that the two equivalent protons giving rise to the characteristic triplet (Fig. 2a) were partially exchanged for deuterium nuclei as shown in Fig. 2b. This spectrum exhibits a central five-line structure with an intensity ratio close to 1:2:3:2:1, and a hyperfine coupling constant of 0.12 gauss, in satisfactory agreement with the expected ratio $a_H:a_D = 6.51$[3] (found: 0.81:0.12 = 6.75), indicating an interaction of the unpaired electron with two equivalent deuterium nuclei. On each side of the five-line spectrum there is a triplet with a splitting constant of 0.12 gauss and an intensity ratio close to 1:1:1. The inner line in each triplet is almost completely overlapped by the outermost line belonging to the central five-line structure. The coupling constant of the doublet splitting giving rise to the two triplets is 0.81 gauss, indicating that these structures are due to the combined interaction of one proton and one deuterium nucleus in equivalent positions. The two lines of low intensity

symmetrically situated outside the five-line structure and the two triplets, correspond to the outermost lines of the triplet splitting due to the original proton-proton interaction.

An identical triplet (1:2:1) with a splitting constant of 0.81 gauss was further obtained from hydroxymethylglyoxal [4] (Fig. 3c) (= hydroxy pyruvic aldehyde) and from methylglyoxal [5] (Fig. 3d) = pyruvic aldehyde) at room temperature, and in a relatively low yield after heating from 1-hydroxyacetone [6] and from 1-bromoacetone.

No radicals could be observed from triose reductone [7] (Fig. 3e) when dissolved in

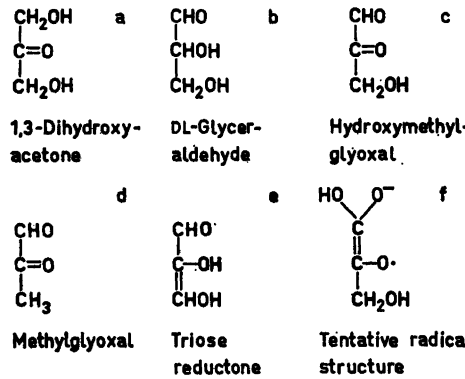

Fig. 3.

alkaline solutions of varying strengths, including 6 N KOH, neither at room temperature, nor after heating. Piette *et al.*[8] have described free radicals derived from triose reductone in the peroxidase catalyzed oxidation of this substance with H_2O_2 at pH 4.8. These radicals, which were rather unstable, exhibited an ESR-spectrum consisting of a triplet with an intensity ratio of 1:2:1. However, the splitting constant was 3.6 gauss, *i.e.* more than four times the value obtained for the radicals here considered. In view of the different splitting constants and the failure to observed any radicals from triose reductone in strongly alkaline media, the radicals described by Piette *et al.* must differ from those here described. Any conclusive evidence for the structure of the latter radicals seems difficult to give. They are evidently formed by oxidation in a strongly alkaline medium. When formed directly from a three-carbon substance such as 1,3-dihydroxyacetone or hydroxymethyl-glyoxal, it is very probabel that the two equivalent protons giving rise to the triplet are those of the methylene group. A tentative structure for the radicals is shown in Fig. 3f. The formation of the characteristic triplet also from methylglyoxal, 1-hydroxyacetone and 1-bromoacetone does not appear consistent with the presence of a hydroxymethylene group. It may be possible, however, that the methyl group of these substances is enolized and that a hydroxymethylene group may be formed by addition of water to the double bond.

The formation of radicals under the prevailing conditions from hydroxymethylglyoxal but not from the closely related substance triose reductone seems to be consistent with the observation made by Hesse *et al.*[4] that these compounds are not the two forms of a keto-enol equilibrium, but are distinct isomers with very little tendency to transform into each other, even in a strongly alkaline medium.

Other possible radical structures may involve substances produced by an aldol condensation of two three-carbon compounds, or the radicals may be formed directly from hexoses or pentoses. In such a case the equivalent protons giving rise to the triplet may be hydrogen atoms situated in α-position on each side of the double bond in an ene-diol configuration.

The ESR-spectra were obtained with a Varian 100 kc spectrometer and a 6″ magnet. The flat aqueous solution cells were used in all experiments. Splitting constants were obtained by comparison with those of the *p*-benzosemiquinone radical.

Acknowledgements. The author is indebted to professor G. W. Kenner for stimulating discussions on the structure of the observed radicals.

This work was supported by grants from *The Swedish Natural Science Research Council, Wilhelm och Martina Lundgrens Vetenskapsfond* and *Stiftelsen Svensk Näringsforskning.*

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Received July 13, 1964.

Amino Acid Analysis of Crystalline Chicken Heart Cytochrome c

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Species specificity [1] has earlier been shown to occur in the amino acid sequence of a polypeptide from chicken heart cytochrome c. This preparation of cytochrome c, however, was not crystallized and therefore it was decided to make another preparation according to a recently developed method [2] and to compare its amino acid content with the already known primary structure of horse heart cytochrome c.[3,4]

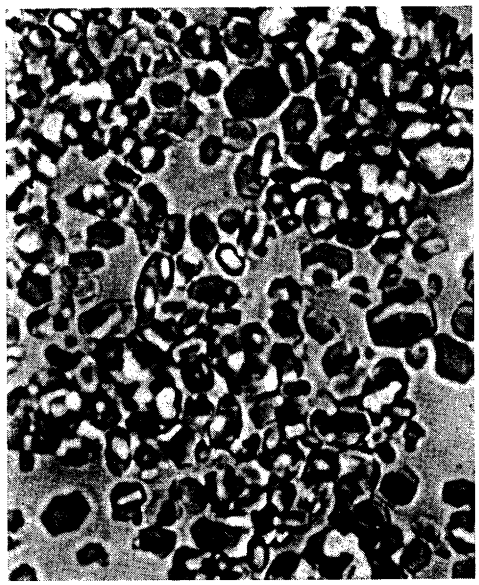

Fig. 1. Crystalline chicken cytochrome c (× 1.750.) Crystal size 3.6 × 4.4 μ. Reduced form.

Table 1.

Amino acid residue	Number per molecule Time of hydrolysis 20 h	70 h	Presumed number of amino acid residues per molecule of cytochrome c	Horse cytochrome c[3]
Aspartic acid	7.0	8.5	(9)	8
Serine	2.7	1.6	(3)	0
Threonine	5.7	5.4	(6)	10
Glutamic acid	7.8	10.0	(10)	12
Glycine	9.9	12.5	(13)	12
α-Alanine	4.1	4.9	(5)	6
Valine	2.5	3.0	(3)	3
Proline	2.1	3.2	(3)	4
Isoleucine	5.4	6.8	(7)	6
Leucine	4.8	5.7	(6)	6
Tyrosine + monochlorotyrosine	2.9		(3)	4
Lysine	14.5	17.5	(18)	19
Histidine	2.5	3.0	(3)	3
Arginine	1.6	1.8	(2)	2
Phenylalanine	3.1	3.8	(4)	4
Methionine	0.9		(1)	2
Cysteine	1.2		(2)	2
Tryptophane			(1)	1

Materials and methods. 3 kg of chicken hearts stored at −15°C were used for preparing cytochrome c. (The author expresses his thanks to AB Findus, Bjuv, for the gift of chicken hearts.) They were thawed just before use and then prepared according to Paléus.[2] The crystals (twice precipitated) are shown in Fig. 1. The yield was 94 mg.

Analytical results. The preparation described was dialyzed until it was salt free, and passed through a column of Lewatite MIH. The iron and sulfur contents were 0.45 % and 0.70 %, respectively. Amino acid analyses were performed according to Moore *et al.*[5] (Table 1). 2.393 mg and 2.053 mg were hydrolyzed with 0.5 ml 5.7 N HCl in a sealed evacuated tube for 20 and 70 h, respectively, at 110°C.

Discussion. Based on the iron analysis chicken heart cytochrome c has a minimal