completely utilized, II could not be found. This evidence suggests that the reaction to form IV proceeds through II.

In previous papers from these laboratories,[1,2,3,10] 6 and 11 hydroxylation and reduction of the double bond in the A ring by microörganisms have been described. The present communication describes the formation of 4-androstene-3,17-dione from C_{21} steroids. It is interesting to note that these metabolic end products from steroidal substrates are similar to those produced by higher vertebrates.[11,12,13]

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HYDROLYTIC POLYMERIZATION OF ZIRCONIUM(IV)[1]

Sir:

Granér and Sillén[2] suggested that in the hydrolysis of Bi(III) a continuous series of particles is formed, all in equilibrium with each other and ranging in size from monomers to "infinitely" large polymers, the exact distribution depending on acidity and concentration. Recently Connick and Reas[3], in an attempt to interpret solvent extraction data on Zr(IV), advanced the same hypothesis of continuous polymerization and equilibrium between the species and postulated the existence of high molecular weight particles in acidic solutions of Zr(IV). Other workers[4,5,6] drew the conclusion that only low molecular weight polymers are formed in strongly acidic media. High molecular weight polymers, not in equilibrium with the more "normal" species, are apparently formed under considerably drastic conditions (*e.g.*, lower acidity or after boiling).[7]

Since the assumption that high molecular weight polymers are in equilibrium with low molecular weight polymers and monomers appears rather improbable and since measurement of the acidity of $ZrCl_4$ solutions[8] made it unlikely that an infinite series of polymers exists at high acidities, equilibrium ultracentrifugations of Zr(IV) were carried out in chloride and perchlorate solutions.[9] The data were recently augmented and reanalyzed by a modification of the method of Lamm[10] which was suggested to us by Professor George Scatchard, details of which will be published separately. In this computation the charge of the polymer units was considered, and estimates of this charge were obtained by ultracentrifugations under a variety of conditions. Centrifugation of 0.05 *M* Zr(IV) solutions in 1 *M* HCl–1 *M* MCl (where M was Li, Na and Cs) revealed the existence of only one principal species of Zr(IV) with an apparent degree of polymerization of 3.0 and charge $Z' < 1$ per monomer unit. At considerably higher and lower acidities (3 *M* HCl and 0.1 *M* HCl–1.9 *M* NaCl) mixtures were found with apparent degree of polymerization varying between *ca.* 2 to 2.6 (3 *M* HCl) and 4 to 5.4 (0.1 *M* HCl).

Similar low degrees of polymerization were found in ultracentrifugations in perchlorate solutions (1 *M* $HClO_4$–1 *M* $NaClO_4$). In this medium the zirconium particles appeared to carry a considerable charge and hence preliminary estimation of the degree of polymerization is somewhat more uncertain than for the chloride solutions. The most probable degree of polymerization for 0.05 and 0.12 *M* Zr(IV) solutions in this medium was 3, with an outside possibility that it may be as high as 4.5. There was no indication of an increase in degree of polymerization with concentration. These results may be compared with the (weight average) degrees of polymerization (N_w) estimated by Connick and Reas.[3] These authors report $N_w = 18$ for a considerably more dilute solution (0.03 *M* Zr(IV)–1 *M* $HClO_4$–1 *M* $LiClO_4$) and a value of N_w between 10 and 300 at a higher acidity (0.17 Zr(IV)–2*M* $HClO_4$).

The results of the ultracentrifugation experiments described here thus indicate that Zr(IV) in strongly acidic solutions (M $H^+ > 0.1$) does not show continuous polymerization with high molecular weight products but rather forms only low molecular weight polymers with trimers apparently predominating at acidities near 1 *M*.

(1) This document is based on work performed for the Atomic Energy Commission at the Oak Ridge National Laboratory: "Hydrolytic Behavior of Metal Ions. II," previous paper, THIS JOURNAL, **72**, 3901 (1950).

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DPNH-CYTOCHROME REDUCTASE, A FERRO-FLAVOPROTEIN[1]

Sir:

Within the past year several flavoprotein enzymes have been shown to contain heavy metals as part of their prosthetic groups. Copper was identified as a constituent of butyryl CoA dehydrogen-

(1) Paper IV in a series entitled Studies on Diphosphopyridine Nucleotide-Cytochrome c Reductase. For paper III see L. P. Vernon, H. R. Mahler and N. K. Sarkar, *J. Biol. Chem.*, **199**, 598 (1952).

ase[2] and molybdenum of xanthine oxidase,[3] and nitrate reductase.[4] In view of these observations, the strong inhibition of DPNH-cytochrome reductase by such metal complexing agents as pyrophosphate[1] and 8-hydroxyquinoline[5] has led us to investigate the possible presence of a metal ion as part of the prosthetic group of the enzyme which had been identified previously as a flavoprotein of somewhat unusual properties.[6]

The presence of iron in the enzyme can be unambiguously demonstrated as follows: the enzyme is isolated and purified in the usual manner, the protein is removed by centrifugation after precipitation with 5% (w./v., final concentration) trichloroacetic acid, and the supernatant solution is analyzed for iron by a micro-adaptation of the *o*-phenanthroline method.[7] Four preparations showing specific activities (units per mg. protein) of 18.0, 34.0, 78.0 and 190 (corresponding to estimated enzymatic purities of 9, 17, 40 and 95%) had an iron content of 0.250, 0.453, 1.05 and 2.67 μg. Fe per mg. protein. Thus the ratio enzyme units per μg. Fe is constant and equal to 73 ± 2. A similar constancy is observed when the flavin:iron ratios are compared; *e.g.*, our two best preparations contained 6.9 and 11.7 mμmoles flavin per mg. (as a flavin adenine dinucleotide[8]), respectively, and had an iron content of 28.0 and 47.8 mμmoles per mg. The ratio iron:flavin equals 4.1 ± 0.1. Calculation of the molecular weight, assuming four gram atoms of iron per mole of enzyme, leads to a value of 80,000, in good agreement with previous determinations based on sedimentation constant and flavin content.[6]

Samples of the enzyme show varying distributions of the metal between its two valence states (Table I). The substrate, DPNH, is capable of reducing all the iron to the ferrous form (experiments 1b, 2b and 3b), while ferricytochrome c, even in considerable excess, does not convert all the iron initially present in the ferrous form to the ferric state (experiments 3c and d). It is apparent, however, that not only the flavin[6] but the iron as well is capable of being reduced by the substrate and oxidized by the acceptor.

TABLE I

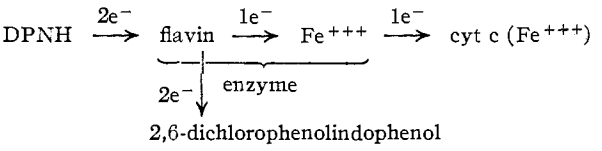
VALENCE STATE OF IRON UNDER DIFFERENT CONDITIONS

Experiment	Conditions	Fe^{++},[a] %	Fe^{+++},[a] %
1a	Enzyme 2 R_5[b] (2.8 γ Fe in 2.8 mg. protein), no additions	0.0	100
b	Same, incubated anerobically with 0.1 μ mole DPNH	92	8
2a	Enzyme 4 R_5 (1.7 γ Fe in 0.63 mg.), no additions	60	40
b	Same, with DPNH	95	5
3a	Enzyme 4 R_4 (2.5 γ Fe in 1.6 mg.)	88	12
b	Same, with DPNH	100	0.0
c	Same, with 200 γ cytochrome c	44	56
d	Same, with 1000 γ cytochrome c	48	52

[a] Fe^{++} estimated without added reducing agent; hydroxylamine hydrochloride is then added and total iron determined. Fe^{+++} is the difference total $-Fe^{++}$. [b] The code designations of various enzyme fractions are described in ref. 6.

Prolonged incubation of the enzyme with *o*-phenanthroline and DPNH or dialysis against 8-hydroxyquinoline leads to a pronounced lowering of the iron content, and of the enzymatic activity, as measured by cytochrome c reduction. When 2,6-dichlorophenolindophenol is substituted for cytochrome c, however (diaphorase reaction[1,6]), little or no inhibition is observed. Full enzymatic activity can be restored by addition of ferric (but not ferrous) iron at a concentration of 5×10^{-4} *M*.

The observations just presented are in accord with the following working hypothesis which is similar to the one previously proposed for butyryl CoA dehydrogenase[2]



Diaphorase,[9] the flavoprotein which catalyzes the reduction of dyes but not of cytochrome c by DPNH, contains less than one-tenth the concentration of iron per mg protein found in a sample of cytochrome reductase of comparable purity, although the flavin content of the two enzymes is similar. Diaphorase may therefore constitute a transformed cytochrome reductase, where the removal of iron has led to certain changes in both structure and function of the enzyme molecule.

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