# ANNUAL REVIEW OF BIOCHEMISTRY

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# PREFACE

Once again it is our pleasurable opportunity to thank the many who have collaborated in preparing this present volume of the *Annual Review of Biochemistry*. All of the manuscripts were received; there were no last-minute withdrawals. For this unanimity in response we are especially grateful.

Some manuscripts were unusually late. Unavoidable difficulties were encountered at the Press. This combination of events led to an unfortunate delay in publication. We are increasingly hopeful that within another year or two prompt publication of the *Review* may again be possible.

The usual difficulties attendant upon the war have again been experienced in preparing this present volume. They need not be recited, for to all of us, authors and readers alike, they are quite familiar. In view of the continuing inaccessibility of some foreign journals we would venture to renew our appeal for reprints, especially from our colleagues abroad; these will be distributed to the authors of forthcoming reviews.

Again we would express our gratitude to Professor H. S. Loring, to our editorial assistants, to our office staff generally, and to the Stanford University Press for their fine co-operation throughout.

H.J.A. C.L.A.S.
D.R.H. J.H.C.S.
J.M.L. H.A.S.

# ERRATA

Volume XII, page 34, line 10 from bottom: *for* carbon suboxide ($C_2O_3$), *read* carbon suboxide ($C_3O_2$).

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# BIOLOGICAL OXIDATIONS AND REDUCTIONS

By D. E. Green and P. K. Stumpf

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## Iron Porphyrin Protein Enzymes

There has been a revival of interest in the study of peroxidase after a lull of some years. Agner (1) working with empyema fluid of tuberculous patients succeeded in isolating the so-called verdoperoxidase of leucocytes in highly purified form. The final product with a pronounced green color contained 0.1 per cent iron and about 1 per cent hemin. It was homogeneous as determined both in the ultracentrifuge and in the Tiselius apparatus.

The catalytic activity of verdoperoxidase per mole of hemin is less than 1/10 of that of peroxidase prepared from horse radish. In addition the two enzymes have different absorption spectra. However, both form compounds with hydrogen peroxide, hydrogen cyanide, sodium azide, and hydroxylamine, and catalyze the oxidation by hydrogen peroxide of much the same types of organic compounds.

Theorell & Åkeson (2) have purified milk peroxidase to the point where it is probably homogeneous. The best preparations contained about 0.076 per cent iron. The absorption spectrum was not identical either with that of verdoperoxidase or peroxidase prepared from horse radish. The catalytic activity, however, was fairly close to that of verdoperoxidase. On reduction with sodium hydrosulfite the brownish green solution turned a pure green color. The α and β absorption bands of the pyridine hemochromogen formed from milk peroxidase did not correspond with those of protohemochromogen, being shifted 5 and 10 Å respectively toward the red end of the spectrum. Compounds with hydrogen cyanide, hydrogen peroxide, and fluoride were also formed by the milk enzyme as shown by changes in absorption spectrum.

Theorell (3) carrying the method of Keilin & Mann for the purification (4) of horse radish peroxidase some steps further has succeeded in isolating the enzyme in homogeneous and crystalline state. The homogeneity was demonstrated in the cataphoresis apparatus and by test of solubility in ammonium sulfate solutions. The hemin content of the final preparation was 1.48 per cent. The molar paramagnetic susceptibility of peroxidase and some of its derivatives were

measured and it was concluded that despite the spectral similarity of peroxidase and ferrihemoglobin noteworthy differences exist in regard to their magnetic properties.

To date four distinct peroxidases have been isolated, viz. (*a*) horse radish peroxidase, (*b*) verdoperoxidase, (*c*) milk peroxidase, and (*d*) cytochrome-*c* peroxidase. The last mentioned isolated from yeast (5) is unique in that it catalyzes the oxidation by hydrogen peroxide of ferro cytochrome-*c* only. The other three peroxidases attack a great variety of substrates including aromatic amines, phenols, diamines, ascorbic acid, and ferrocytochrome-*c*. Sumner & Gjessing (6) have reported variations in the properties of plant peroxidases. Apparently the peroxidases from horse radish and milk weed have different affinities for hydrogen peroxide. These are probably minor differences like those which exist between the hemoglobins of different animal tissues.

Theorell *et al.* (7) have extended their experiments on the reversible resolution of horse radish peroxidase. The protein moiety was separated from the prosthetic group by precipitation with acetone in dilute solutions of hydrochloric acid. The prosthetic group remained in solution. The split protein combined with protohemin, mesohemin, and deuterohemin, respectively, to form compounds with 100, 53, and 62 per cent of the original activity. Other hemins formed compounds with the specific protein but without catalytic activity. The time for complete combination varied from a few minutes to twenty-four hours. The rate of combination could be followed by the change in spectrum which accompanies the formation of the hemin protein compound. The new peroxidases formed respectively by meso- and deuterohemin had different absorption bands from the natural peroxidase which contains protohemin. The previous review has already dealt with the synthesis of active peroxidases in which metals other than iron are present in combination with protoporphyrin (8). It appears therefore that peroxidase from horse radish is less specific as far as the components of the prosthetic group are concerned than hemoglobin, although both contain the same prosthetic group.

Chance (9, 10) has made a very notable contribution to the kinetics of peroxidase and indeed to the fundamental theory of enzyme kinetics. He has perfected a micro modification of the Hartridge-Roughton flow apparatus for studying the kinetics of rapid enzyme reactions. Enzyme and substrate were mixed rapidly in a suitable flow chamber; the changes in light absorption which accom-

pany chemical reaction were picked up by photocells and the progress of the reaction was measured directly by the photoelectric amplifiers. Mirror oscillograph recordings were made of each kinetic run. He found that horse radish peroxidase forms a compound with hydrogen peroxide with extreme rapidity ($k_1 = 1.2 \times 10^7$ liter mole$^{-1}$ sec.$^{-1}$). This rate is similar to the measured value for the reaction of oxygen with muscle hemoglobin ($1.9 \times 10^7$ liter mole$^{-1}$ sec.$^{-1}$). The equilibrium for the enzyme-substrate combination favors almost complete association ($K = 2 \times 10^{-8}\ M$). Chance's data substantiate the conclusion that a second order combination of enzyme and substrate is followed by a first order decomposition reaction. The rate of breakdown of the intermediate compound of peroxidase and hydrogen peroxide in presence of an acceptor like leuco malachite green is small compared to the rate of synthesis. For a particular concentration of malachite green $k_3$ was found to be 4.2 sec.$^{-1}$. The kinetics of peroxidase indicate that a chain mechanism plays no significant role, if any, in the mode of action of peroxidase. There was some indirect evidence that the acceptor forms a compound with the enzyme-substrate compound. These experiments of Chance provide the first direct experimental proof of the classical Michaelis theory of enzyme action.

The structure and mode of action of catalase are still being actively investigated. Agner (11) has prepared crystalline catalase from horse liver by a new method. His best preparations contained 0.093 per cent iron, 0.8 to 0.9 per cent hemin, and ca. 0.02 per cent copper. Only 75 to 85 per cent of the total iron was bound to hematin. Agner confirmed the presence of bile pigment in the catalase molecule in the ratio of 1 bile pigment to 3 hemins. He is of the opinion that the bile pigment is a natural constituent of the enzyme and that it does not arise by degradation or oxidation of one of the hemins in the catalase molecule during the isolation procedure. Lemberg (12) has championed the theory that the bile pigment of catalase is a degradation product of hemin. His main evidence is that bilirubin is formed by oxidation of hemoglobin in presence of ascorbic acid as catalyst. Perhaps a more cogent line of evidence is the observation of Agner (13) that catalase isolated from erythrocytes does not contain bile pigment and that the catalytic activity of erythrocyte catalase per mole of enzyme is some 50 per cent higher than that of liver catalase.

Theorell & Agner (14) have made paramagnetic susceptibility measurements of horse liver catalase and its derivatives, the results

of which they believe render unlikely the theory of Keilin & Hartree (15) that hydrogen peroxide reduces the hemin iron of catalase from the ferric to the ferrous condition. The present usefulness of paramagnetic measurements can now be put to a decisive test on the issue whether hydrogen peroxide-azide catalase is in the ferrous state as Keilin & Hartree believe or in the ferric state according to the magnetic measurements. In that connection Keilin & Hartree (16) have withdrawn their claim that catalase action does not proceed under anaerobic conditions. One of the supports for the ferrous hypothesis is thereby removed.

Haas (17) has made an interesting attempt to render soluble the cytochrome oxidase. He has found that the oxidase occurs in two forms as prepared by the usual method of grinding washed heart tissue in a mechanical mortar. One form is completely sedimented by a force of 10,000 x g while the other form is not appreciably sedimented. Exposure of such a mixed preparation to ultrasonic radiation increases the proportion of oxidase which is not sedimented by 10,000 x g. It appears that such treatment, like prolonged grinding with sand, leads to a progressively smaller particle size. However, the pronounced Tyndall effect reported by Haas for solutions of the oxidase after centrifugation at 10,000 x g militates against the assumption that the enzyme has been separated from its association with particles.

While ferritin, the ferric hydroxide protein of liver, spleen, and other organs, is not strictly germane to a review of biological oxidations, it has enough interesting possibilities to justify some reference to it here (18 to 21). Under appropriate conditions, ferritin can be split into an iron-free protein which has the same crystalline structure as the parent compound. This so called "apoferritin" has now been obtained in a homogeneous state in contrast to the inhomogeneity of the best ferritin preparations. From magnetic susceptibility measurements Michaelis & Granick concluded that ferric hydroxide is present in "micelles which fill the interstices of the structure of ferritin." Experiments with radioactive iron suggest that ferritin is a storage form of iron in the body.

## Flavoprotein Enzymes

With the discovery of glycine oxidase by Ratner *et al.* (22) the number of flavoproteins described up to now has swelled to ten. This enzyme which is widely distributed in liver and kidney of many species catalyzes the oxidation of glycine to glyoxylic acid (CHOCOOH) and ammonia, and of sarcosine to glyoxylic acid and methylamine.

Glycine oxidase as found under normal conditions in liver and kidney is a conjugated flavoprotein containing flavin adenine dinucleotide (FAD). When isolated from lamb, cat, and human kidneys by precipitation with salt within the range of pH 4 to 9 the enzyme does not dissociate appreciably. It is only when the enzyme is exposed to 0.1 *N* hydrochloric acid that dissociation ensues. However, when the enzyme is prepared from pig kidney by the same procedure as used in the preparation of the enzyme from the other kidneys, it no longer behaves as a conjugated flavoprotein but appears to be largely dissociated. Thus after two precipitations with ammonium sulfate no activity was shown except in the presence of added FAD. Analyses showed that this was not a genuine dissociation but that a factor in pig kidney presumably enzymic in nature was responsible for the cleavage of the flavoprotein into its constituent parts. Whether this cleavage was the result of destruction of FAD has yet to be clarified. When adequate precautions were taken to prevent this prosthetic group splitting factor from acting, preparations of the glycine oxidase could be made from pig kidney which failed to show any appreciable dissociation during salt precipitation over the pH range 4 to 9.

Precisely the same considerations were found to apply to the *d*-amino acid oxidase (22). When prepared from pig kidney the enzyme appeared to be a dissociating flavoprotein, whereas the enzyme from lamb kidney was split only under strongly acid conditions. Again when steps were taken to minimize the action of the prosthetic group splitting factor, the preparation from pig kidney showed no tendency to dissociate whatsoever over the pH range 4 to 9.

Unfortunately, practically all the early work on *d*-amino acid oxidase was carried out on preparations from pig kidney. The conclusion that the *d*-amino acid oxidase is almost completely dissociated at neutral pH was based on the analysis of so-called dissociation curves which relate rate of oxidation as a function of added FAD in presence of a fixed amount of the split protein (23, 24). It now appears that these curves were determined largely by rates of combination and therefore could not be used to calculate thermodynamic dissociation constants.

Fischer *et al.* (25) have made more observations on fumaric hydrogenase, the flavoprotein which catalyzes the oxidation of reduced dyes by fumarate. Originally they found the enzyme in crude preparations of the "old yellow enzyme" of Warburg & Christian (26). Thus far they have been unable to separate fumaric hydrogenase from other

flavoproteins in the "old yellow enzyme" preparation. The evidence favors FAD as the prosthetic group of fumaric hydrogenase. Since flavinmonophosphate is the prosthetic group of the Warburg & Christian "old yellow enzyme," there can be no doubt of the non-identity of fumaric hydrogenase and the "old yellow enzyme." In the process of isolating fumaric hydrogenase, the enzyme is partially split and FAD must be added to restore full activity. The identity of fumaric hydrogenase with eight of the known flavoproteins has been excluded by Fischer *et al.* There is a possibility that a highly purified flavoprotein isolated from top bakers' yeast by Green *et al.* in 1941 (*27*) may be identical with fumaric hydrogenase.

The search for antibacterial agents in molds has resulted in the isolation by Raistrick *et al.* and others (28 to 31) of a flavoprotein which has been identified with glucose oxidase. The final product obtained by Raistrick *et al.* appears to be homogeneous. Strains of *Penicillium notatum* W. secrete this enzyme into the growth medium. The prosthetic group of the enzyme is probably FAD (28). The enzyme catalyzes the aerobic oxidation of glucose to gluconic acid with the production of hydrogen peroxide. It is in fact the production of hydrogen peroxide which is at the root of the antibacterial action of the flavoprotein system (28). The addition of glucose to the enzyme bleaches the yellow color instantaneously. The reduced form of the enzyme in turn is oxidized by molecular oxygen. The enzyme thus undergoes a catalytic cycle of reduction by the substrate followed by oxidation of its leuco form in air with production of hydrogen peroxide.

Since the antibacterial action of glucose oxidase was presumed by Raistrick *et al.* to be due to hydrogen peroxide production it followed that other flavoprotein systems should have identical action. This was tested and found to be so in the case of milk xanthine oxidase (32, 33). Other flavoproteins will be more difficult to test since they are usually contaminated with catalase which nullifies the antibacterial effect.

## Glycolysis and Fermentation

Cori and his co-workers have rounded off their researches on muscle phosphorylase with four elegant papers (34 to 37) on its preparation, properties, and kinetics. Under physiological conditions the enzyme is a firmly conjugated adenylic acid-protein compound which cannot be resolved by mild purification procedures such as salt pre-

cipitation. However, skeletal muscle contains an enzyme which splits off adenylic acid from phosphorylase. It is thus possible to obtain by enzymic means a split form of phosphorylase which is inactive unless supplemented with adenylic acid. Crystalline trypsin at pH 6 has the same effect as the muscle-splitting enzyme in resolving phosphorylase.

Phosphorylase from skeletal muscle has been obtained in highly purified and crystalline state though the best preparations are not completely homogeneous. According to Oncley (38) the molecular weight is probably about 340,000 to 400,000 assuming a value of 0.74 for partial specific volume. The amount of phosphorylase in skeletal muscle is rather high (40 to 80 mg. per 100 gm.) and is roughly half that of zymohexase.

It has been known for some time that the presence of certain polysaccharides like glycogen and amylopectin is necessary to catalyze the enzymic condensation of glucose-1-phosphate to polysaccharide. In other words, polysaccharide cannot be formed unless a highly branched polysaccharide is already present. Cori *et al.* interpret this effect as follows: The catalytic polysaccharides are in effect nuclei which grow by deposition of glucose units. For the sake of simplicity, we may regard the catalytic polysaccharides as consisting of a central core with large numbers of spurs coming off, each spur averaging from 6 to 18 glucose units in length. The glucose unit at the end of each spur condenses with glucose-1-phosphate by 1 : 4 linkage, and the process is continued until each spur, according to Hassid *et al.* (39), attains a straight chain length of some 200 glucose units. The net effect is the conversion of a highly branched catalytic polysaccharide into an essentially unbranched non-catalytic amylose-type polysaccharide. The iodine color serves as an indicator of the degree of branching. A pure blue color such as is given by amylose means zero or very slight branching. The greater the degree of branching the more the iodine color tends towards brown or red. Whereas phosphorylase from skeletal muscle yields a polysaccharide which stains iodine blue, the corresponding enzymes from liver and heart yield a glycogen-like product which stains iodine brown or brownish red. Cori *et al.* regard the synthesis of highly branched glycogen from glucose-1-phosphate as the result of the collaboration of two enzymes one of which catalyzes condensations of glucose-1-phosphate with terminal glucose by 1 : 4 linkage while the other presumably catalyzes condensation by 1 : 6 linkage.

Under standard conditions each molecule of phosphorylase con-

denses $4 \times 10^4$ molecules of glucose-1-phosphate per minute. Relatively high concentrations of a reducing agent like cysteine and a polysaccharide like glycogen must be present for maximum activity to be attained. When phosphorylase is split into its component parts adenosinemonophosphate but not adenosinediphosphate or adenosinetriphosphate restores activity. Inosinemonophosphate has a weak effect in high concentrations only.

Buchanan *et al.* (40) have made some studies with radioactive carbon on glycogen synthesis from acetic, propionic, and butyric acids. They found glycogen formation from the 3 and 4 carbon fatty acids but not from the 2 carbon fatty acid. Since they showed previously that pyruvic acid gives rise to glycogen (41) it now appears that both the 3 and 4 carbon fatty acids eventually give rise to pyruvic acid.

Doudoroff (42, 43) has obtained a cell free enzyme from *Pseudomonas saccharophilia* which catalyzes the phosphorylysis of sucrose to glucose-1-phosphate and fructose. There is some evidence that the reaction is reversible since sucrose can be formed when starting with mixtures of glucose-1-phosphate and fructose. No hexose catalyst is required for sucrose synthesis. The enzyme is specific for sucrose being without effect on trehalose, maltose, and glucose. Neither fructose-1-phosphate nor fructose diphosphate can replace fructose in the condensation reaction. Kagan *et al.* (44) have made similar observations on *Leuconastor mesenteroides*. Under the conditions of Kagan's experiments, fructose formed by phosphorylysis of sucrose disappears in some side reaction.

Warburg & Christian (45) have prepared zymohexase from rat muscle in highly purified and crystalline state but obtained no information about its prosthetic group or active groups. They claim that their enzyme from rat muscle is twice as active as the homogeneous enzyme isolated in 1940 from rabbit muscle by Herbert *et al.* (46). Since the methods and conditions for estimating activity as well as the sources of the enzyme were different in the two cases, the significance of the comparison is dubious.

Meyerhof & Junowicz-Kocholaty (47) have gone to considerable pains to determine whether *d*-3-glyceraldehyde phosphate actually combines with inorganic phosphate before oxidation takes place. Since the equilibrium reactions in which *d*-3-glyceraldehyde phosphate is involved were not affected by the concentration of inorganic phosphate, the authors were of the opinion that phosphate formed a loose physical addition product rather than a definite chemical compound.

Kalckar (48) has isolated in highly purified state myokinase, an enzyme found in skeletal muscle which catalyzes the reversible dismutation of adenosinediphosphate to adenosinemonophosphate and adenosinetriphosphate. Approximately 60 per cent of adenosinediphosphate is converted into the two products of dismutation at equilibrium. The enzyme can be heated in boiling 0.1 *N* hydrochloric acid and precipitated with trichloroacetic acid without loss of activity. It is apparently not identical with insulin. It is found in large amounts in skeletal muscle, in traces in heart and brain, and not at all in liver and kidney.

Kubowitz & Ott (49) have added another to the long list of glycolytic enzymes which they are preparing in highly purified state. They have now isolated the lactic acid dehydrogenating enzyme of rat sarcoma in the form of a crystalline mercury protein complex which can be converted into an active form by dialysis against hydrogen cyanide or cysteine. Straub in 1940 (50) was the first to report the preparation of lactic dehydrogenase in a homogeneous and crystalline state. He has also brought the malic dehydrogenase of pig heart to a similar high degree of purity (51). Both these enzymes require the presence of coenzyme I for activity. The activity per mg. dry weight is practically identical for the two enzymes ($Q_{O_2}$ = ca 60,000).

In 1939 Engelhardt & Lyubimova (52) reported that the adenosinetriphosphate activity of muscle was almost entirely associated with the myosin fraction and they suggested that the enzyme might be identical with myosin itself. D. M. Needham has confirmed their observation that myosin preparations split off one phosphate group from adenosine triphosphate but have little or no action on adenosinediphosphate (53). She has also shown that myosin while very active as adenosinetriphosphate has no phosphatase action on α-glycerophosphate or hexosediphosphate, nor can it catalyze the transfer of phosphate from adenosinetriphosphate to fructose-6-phosphate. Bailey (54) has studied the activation of myosin adenosinetriphosphatase by divalent metals, particularly calcium, magnesium, and manganese, and he has found that the myosin enzyme is unique among phosphatases in the way it is affected by divalent cations. He regards all the available evidence as consistent with the assumption that myosin and adenosinetriphosphatase are identical and suggests "that stimulation of muscle is connected with the availability of calcium ions to the myosin adenosinetriphosphatase fibrillar surface."

Ziff (55) has studied the action of pharmacological agents on myo-