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Nature and Mode of Action of Oxidation Enzymes

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Practically all chemical reactions in living nature are started and directed in their course by enzymes. This being the case, man has of course since time immemorial seen examples of what we now call enzymatic reactions—for example, fermentation and decay. It would thus be possible to trace the history back to the ancient Greeks, or still further for that matter. But this would be rather pointless, for observing a phenomenon is not the same thing as explaining it. It is more correct to say that our knowledge of enzymes is essentially a product of 20th century research.

An enzyme is a sort of catalyzer, and in this connection a reminder of the origin of the concept of catalysis may be appropriate. The concept was put forward by a schoolfellow of mine from Linköping High School. I never had the honor of meeting him, for he was 124 years older than I. He was one of the founders of the Karolinska Institutet and the Swedish Medical Society, Jöns Jacob Berzelius, who in 1835 wrote in his yearbook:

"This is a new force producing chemical activity and belonging as well to inorganic as organic nature, a force which is undoubtedly more widespread than we have hitherto imagined, and whose nature is still concealed from us. When I call it a new force I do not thereby mean to say that it is a capacity independent of the electrochemical relations of matter; on the contrary, I cannot but presume it to be a particular manifestation of these, but as long as we cannot understand their reciprocal connections it will facilitate our researches to regard it for the time being as an independent force, just as it will facilitate our discussion thereof if it be given a name of its own. I shall therefore to use a derivation well-known in chemistry, call it the *catalytic force* of bodies, decomposition through this force *catalysis,* just as with the term *analysis* we describe the separation of the constituent parts of bodies by means of ordinary chemical affinity. The catalytic force appears actually to consist therein that through their mere presence and not through their affinity bodies are able to arouse affinities which at this temperature are slumbering. . . ."

Enzymes are the catalyzers of the biological world, and Berzelius' description of catalytic force is surprisingly farsighted—one is tempted to say prophetic. Especially is one struck by his expressly refusing to believe that other than chemical forces are here in play; no, if one could once understand the mechanism it would doubtless prove that the forces of ordinary chemistry would suffice to explain also these as yet mysterious reactions.

Almost 100 years were to pass before it became clear that Berzelius had been right. The year 1926 was a memorable one. The German chemist Richard Willstätter gave a lecture then before the Deutsche chemische Gesellschaft in which he summarized the experiences gained in his attempts over many years to produce pure enzymes. Through various adsorption methods, he had removed more and more of the impurities in some enzymes; especially had he worked with peroxidase, an enzyme of general occurrence in the vegetable kingdom. Finally, there was so little substance left that, on ordinary analysis for protein, sugar, or iron, for example, the solutions gave negative results. But the "catalytic" enzyme effect was still there. Willstätter drew the conclusion that the enzymes could contain neither protein, carbohydrate, nor iron and that they did not belong to any known class of chemical substances at all, and he was even inclined to believe that the effects of the enzymes derived from a new natural force; this was the view that 90 years earlier Berzelius had dismissed as improbable.

That same year, through the irony of fate, the American researcher J. B. Sumner published a work in which he claimed that he had crystallized in pure form an enzyme, urease, from "jack-beans." The enzyme splits urea into carbon dioxide and ammonia. Sumner had got his crystals in rather considerable quantities with the help of much simpler methods than those applied by Willstätter in purification experiments on other enzymes. Sumner's crystals consisted of colorless protein. In the ensuing years, J. H. Northrop and his collaborators crystallized out three more enzyme preparations, pepsin, trypsin, and chymotrypsin —hydrolytic enzymes that, like urease, split linkages by introducing water.

If these discoveries had been undisputed from the outset, it would probably not have been 20 years before Sumner, together with Northrop and Stanley, received a Nobel prize. But it was not easy to show that the beautiful protein crystals really were the enzymes themselves and not merely an inactive vehicle for the actual enzymes. Both Sumner and Northrop adduced many probable proofs that what they had produced really were pure enzymes, but no absolutely conclusive experiment could be brought forward, and, as a matter of fact, this was at that time probably not possible for the simple reason that their preparations appeared to consist of only colorless protein. At that time, and even today for the rest, the methods of separation and analysis were scarcely sufficiently refined definitely to exclude the occurrence of small quantities of impurities in a protein preparation. From many quarters, accordingly, objections were raised to Sumner's and Northrop's results, and for obvious reasons especially the Willstätter school made itself heard in this connection.

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Yellow Enzyme

When in 1933 I went on a Rockefeller fellowship to Otto Warburg's institute in Berlin, Warburg and Christian had in the previous year produced a yellow-colored preparation of an oxidation enzyme from yeast. It was obviously very impure with respect to the high-molecular constituent parts, which consisted chiefly of rubberlike polysaccharides. The yellow color was of particular interest: it faded away on reduction and returned on oxidation with, for example, oxygen gas, so that it was evident that precisely the yellow pigment had to do with the actual enzymatic process of oxidation-reduction. It was possible to free the yellow pigment from the high-molecular carrier substance, whose nature was still unknown, by treatment with acid methyl alcohol, for example, whereupon the enzyme effect disappeared. Through simultaneous works by Warburg in Berlin, Kuhn in Heidelberg and Karrer in Zürich, the constitution of the yellow pigment (lactoflavin, later riboflavin, vitamin B_2) was determined.

It was here for the first time possible to localize the enzymatic effect to a definite atomic constellation: hydrogen freed from the substrate (hexose monophosphate) is with the aid of a special enzyme system (triphosphopyridine nucleotide-Zwischenferment), whose nature was elucidated somewhat later, placed on the nitrogen atoms of the flavin at positions 1 and 10 (Fig. 1), giving rise to the colorless leukoflavin. This is reoxidized by oxygen gas, hydrogen peroxide being formed, and may afterwards be reduced again, and so forth. This cyclic process then continues until the entire amount of substrate has been deprived of two hydrogen atoms and has been transformed into phosphogluconic acid, and a corresponding amount of hydrogen peroxide has been formed. At the end of the process, the yellow enzyme is still there in unchanged form, and has thus apparently, as Berzelius expressed it, aroused a chemical affinity through its mere presence.

But there now remained some extremely important questions to elucidate: Why was the flavin in free form completely inactive, but active when it was anchored to a high-molecular carrier? And what was this carrier? In order to find this out, I decided to try to purify the yellow enzyme, using for the purpose electrophoretic methods worked out by myself. At this time Tiselius had not yet worked out his technically more perfected electrophoretic methods, but my own proved in any case surprisingly useful.

The polysaccharides, which constituted 80 to 90 percent of the entire weight, were completely removed, together with some inactive colorless proteins. After fractional precipitations with ammonium sulfate, I produced a crystalline preparation which on ultracentrifuging and electrophoresis appeared to be homogeneous. The enzyme was a protein with molecular weight 75,000, and it was strongly yellow-colored by the flavin part. The result of the flavin analysis was 1 mole of flavin per mole of protein. With dialysis against diluted hydrochloric acid at low temperature, the yellow pigment was separated from the protein, which then became colorless. In the enzyme test, the flavin part and the protein separately were inactive, but if the flavin part and the protein were mixed at approximately neutral reaction, the enzyme effect returned, and the original effect came back when one mixed them in the molecular proportions 1/1. That in this connection a combination between the pigment and the protein came about was obvious, moreover, for other reasons: the green-yellow color of the flavin part changed to pure yellow, and its strong yellow fluorescence disappeared when it was linked to the protein.

Developments in the flavin field were at this time (1934), to say the least, hectic. For a while Richard Kuhn believed he was able to resynthesize the yellow ferment by bringing together lactoflavin and my colorless protein component; when his work was published, however, we were already aware in Berlin that this assumption was not correct. The point was that in my electrophoretic experiments lactoflavin behaved as a neutral body, while the pigment part that was separated from the yellow enzyme moved rapidly toward the anode and was thus an acid. An analysis for phosphorus showed 1 mole of phosphorus per mole of flavin, and when after a time (1934) I succeeded in isolating the natural pigment component, this proved to be a lactoflavin phosphoric acid ester, thus a kind of nucleotide, and it was obvious that the phosphoric acid served to link the pigment part to the protein.

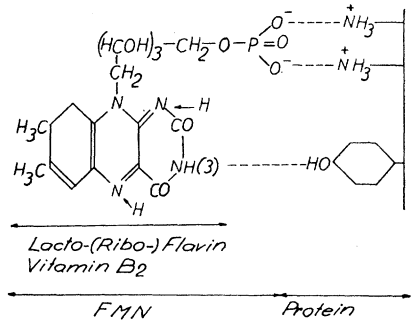

Fig. 1. Mode of linkage between flavin mononucleotide and apoprotein in the "old yellow enzyme," according to H. Theorell and A. P. Nygaard [*Acta Chem. Scand.* 8, 1649 (1954)] and A. P. Nygaard and H. Theorell [*Acta Chem. Scand.* 9, 1587 (1955)].

I will now describe some simple experiments with the yellow enzyme, its colored part, which we now generally refer to as FMN (flavin mononucleotide), and the colorless enzyme protein.

Experiment 1. A lantern-slide apparatus is furnished with two cuvette holders and mirrors so that a correctly presented image of the cuvettes may be projected on a white screen. A slide with a solution of yellow enzyme and another with FMN of the same molar concentration are shown simultaneously. The fermenting solution is pure yellow, and the FMN solution green-yellow, owing to the fact that the light-absorption band in the blue of the free FMN is displaced somewhat in the long-wave direction on being linked with the protein component. A reducing agent ($Na_2S_2O_4$) is now added to one cuvette—it does not matter which. The color disappears because of the formation of leukoflavin. Oxygen gas is bubbled through the solution: the color comes back as soon as the excess of reducing agent has been consumed. The experiment demonstrates the reaction cycle of the yellow enzyme: reduction through hydrogen from the substrate side, reoxidation with oxygen gas.

Experiment 2. A flask containing FMN solution so diluted that its yellow color is not discernible to the eye is placed on a lamp giving long-wave ultraviolet light. The solution gives a strong, yellow fluorescence which disappears on reduction and returns on bubbling with oxygen gas.

Experiment 3. Two flasks are placed on the fluorescent lamp. One contains a diluted solution of the free protein in phosphate buffer (*p*H 7), and the other contains phosphate buffer alone. An equal amount of FMN solution is dripped into each flask. In the flask with protein, the fluorescence is at once extinguished, but in the flask with buffer solution alone, it remains. The experiment demonstrates the resynthesis of yellow enzyme, and since the fluorescence is extinguished by the protein, one may draw the conclusion that some group in the protein is in this connection linked to the imino group at position 3 (Fig. 1) of the flavin, which according to Kuhn must be free for the fluorescence to appear.

The significance of these investigations on the yellow enzyme may be summarized as follows.

1) The reversible splitting of the yellow enzyme to apoenzyme and coenzyme in the simple molecular ratio 1/1 proved that we had here to do with a pure enzyme; the experiments would have been incomprehensible if the enzyme itself had been only an impurity.

2) This enzyme was thus demonstrably

a protein. In the sequel, all the enzymes, probably now more than 100, which have been isolated, have proved to be proteins.

3) The first coenzyme, FMN, was isolated and found to be a vitamin phosphoric acid ester. This has since proved to be something occurring widely in nature: the vitamins nicotinic acid amide, thiamine, and pyridoxine form in an analogous way a nucleotidelike coenzyme, which like the nucleic acids themselves combines reversibly with proteins.

During the past 20 years, a large number of flavoproteins with various enzyme effects have been produced. Instead of FMN, many of them contain a dinucleotide, flavinadenine dinucleotide, FAD, which consists of FMN and adenylic acid.

During the last few years, technical advances have made it possible to attempt a chemical definition of the way in which the linkage between coenzyme and enzyme takes place. The now so-called "old" yellow ferment has in this connection once more proved to be an excellent object. We constructed a very sensitive apparatus to record the time interval in changes in the intensity of the fluorescence and were thus able to follow the rapidity with which the fluorescence diminishes when FMN and protein are combined or increases when they are split. Under suitable conditions, the speed of combination is very high.

Thanks to the great sensitivity of the fluorescent method, my Norwegian collaborator Agnar Nygaard and I were able to make accurate determinations of the speed constant simply by working in extremely diluted solutions, where the combination speed is low because an FMN molecule so seldom happens to collide with a protein molecule.

We determined the reaction speeds under different conditions. We varied the degree of acidity, ionic milieu, and temperature, and we treated the protein with a large number of different reagents that affect in a known way different groups in proteins. In this way, we succeeded with a rather high degree of certainty in ascertaining that phosphoric acid in FMN is linked to primary amino groups in the protein and that the imino group in FMN is linked to the phenolic hydroxyl group in a tyrosine residue, whereby the fluorescence is extinguished.

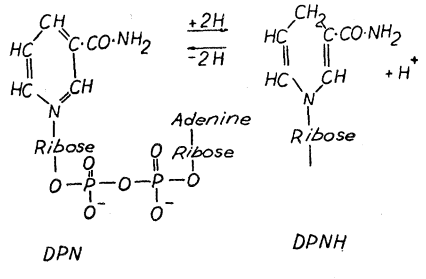

Fig. 2. Oxidation-reduction of diphosphopyridine nucleotide.

We still do not quite understand how through its linkage to the coenzyme the enzyme-protein "activates" the latter to a rapid absorption and the giving off of hydrogen. But some things we do know. The oxidation-reduction potential of the enzyme is in any case of great importance, and it is determined by a simple relation to the dissociation constants for the oxidized and for the reduced coenzyme-enzyme complex. The dissociation constants are in their turn functions of the velocity constants for the combination between coenzyme and enzyme and for the reverse process, and these velocity constants we have been able to determine both in the yellow ferment and in a number of enzyme systems. Without going into any details, I may mention that the linkage of coenzyme to enzyme was found to have surprisingly big effects on the potential of the former. In a number of cases, the effect is strongly dependent on variations in the salt content. Here we may suspect physiologically significant relationships.

Alcohol Dehydrogenases

I may now mention something concerning our work on the alcohol dehydrogenases, although I will be relatively brief. These enzyme systems, like the flavin proteides, consist of colorless protein in reversible combination with a coenzyme, which in the present case is the diphosphopyridine nucleotide, earlier generally referred to as cozymase, now as DPN, which has been elucidated in works by Hans von Euler, Karl Myrbäck, Ragnar Nilsson and others in Stockholm, and by Otto Warburg and Walter Christian in Berlin. It contains as active atomic group nicotinic acid amide, which analogously with the flavin in the yellow enzyme functions by absorbing hydrogen from one direction and giving it off in another. It contains, further, DPN ribose, phosphoric acid, and adenine (Fig. 2).

Alcohol dehydrogenases occur in both the animal and the vegetable kingdom—for example, in liver, in yeast, and in peas. They are colorless proteins which together with DPN may either oxidize alcohol to aldehyde, as occurs chiefly in the liver, or conversely reduce aldehyde to alcohol, as occurs in yeast.

The yeast enzyme was crystallized by Negelein and Wulff (1936) in Warburg's institute, the liver enzyme (from horse liver) by Bonnichsen and Wassen at our institute in Stockholm in 1948.

These two enzymes have come to play a certain general role in biochemistry on account of the fact that it has been possible to investigate their kinetics more accurately than is the case with other enzyme systems. The liver enzyme especially we have on repeated occasions studied with particular thoroughness since especially favorable experimental conditions here present themselves. For all reactions with the DPN system, it is possible to follow the reaction

$$DPN^+ + 2H \rightleftharpoons DPNH + H^+$$

spectrophotometrically, since DPNH has an absorption band in the more long-wave ultraviolet region, at 340 millimicrons, and thousands of such experiments have been performed all over the world. A couple of years ago, moreover, we began to apply our fluorescence method, which is based on the fact that DPNH but not DPN fluoresces, even if considerably more weakly than the flavins. With regard to the liver enzyme, there is a further effect, which proved extremely useful for certain spectrophotometric determinations of reaction speeds; together with Bonnichsen, I found in 1950 that the 340-millimicron band of the reduced coenzyme was displaced, on combination with liver alcohol dehydrogenase, to 325 millimicrons, and together with Britton Chance, we were thus able with the help of his extremely refined rapid spectrophotometric methods to determine the speed constant for this very rapid reaction.

I shall not go into further details, but simply point out that extremely complicated reactions result from the fact that we are here dealing with a three-body problem containing the enzyme protein, the coenzyme, and the substrate, where, furthermore, both the coenzyme and the substrate occur in both oxidized and reduced forms.

A more or less complete system may be described thus: only after nine steps does the enzyme become free to begin a new cycle; the net result is that the alcohol has given two of its hydrogen atoms to the coenzyme. Even this simplified schema means that one must determine 18 speed constants, two for each partreaction, which is of course a formidable task. We have succeeded, however, in determining some of them for the yeast enzyme.

The kinetics of the liver enzyme is quite other than that of the yeast enzyme. Here, almost literally, we have to do with a simpler reaction process that can be expressed with only three equations and six velocity constants.

We have here been able to determine all the six constants at different degrees of acidity and with different salt contents; hence that the reaction speeds of this enzyme system are probably at present the best known of all (Theorell, Bonnichsen, Chance, Nygaard).

The difference between the yeast and

liver enzymes indicated here explains why the yeast enzyme produces alcohol from aldehyde, while the liver enzyme afterwards does the contrary.

It is curious that the same coenzyme which in the yeast makes alcohol by attaching hydrogen to aldehyde also occurs in the liver to remove, on the advent of the alcohol—for example, through the taking of food—the same hydrogen again, so that the alcohol becomes aldehyde again, which is then oxidized further.

When we had studied the kinetics of the alcohol dehydrogenases it was a simple matter to use these to determine alcohol quantitatively—for example, in blood samples. This so called "ADH-method" is about as accurate as Widmark's method, but it is more sensitive and above all practically specific for ethyl alcohol. It has now been legally introduced in forensic chemical practice in Sweden and in West Germany.

Hemin Proteides

Even before going to Berlin in 1933, I had become interested in a close relative of hemoglobin, the myochrome or, as it was afterwards called, the myoglobin discovered spectroscopically by K. A. H. Mörner in 1897, and had crystallized it in the pure form in 1932.

After my return home, I set about purifying another hemin proteide, cytochrome *c*, one of the "histohematins" or "myohematins" observed by the Irishman MacMunn in his home-made spectroscope at the end of the 1880's. MacMunn's "hematins," after a period of obscure existence in brevier type in larger textbooks, had been brought out into the light again by David Keilin in Cambridge in 1925. In 1936 we obtained the cytochrome approximately 80-percent pure, and in 1939 at all events very close to 100-percent pure.

It is a beautiful, red, iron-porphyrin-containing protein that functions as a link in the chain of the cell-respiration enzymes, the iron atom now taking up and now giving off an electron, and the iron thus alternating valency between the trivalent ferric and the divalent ferrous stages. It is a very pleasant substance to work with, not merely because it is lovely to look at, but also because it is uncommonly stable and durable. From 100 kilograms of heart meat of horse one can produce 3 to 4 grams of pure cytochrome *c*. The molecule weighs about 12,000 and contains one mole of iron porphyrin per mole.

Experiment 4. Two cuvettes each contain a solution of ferricytochrome *c*. The color is blood red. To one are added some grains of sodium hydrosulfite: the color is changed to violet-red (ferrocytochrome). Oxygen is then bubbled

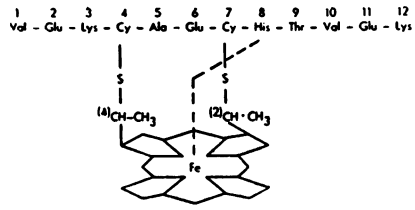

Fig. 3. The peptide chain 1 to 12 (*Val* is the amino acid, valine; *Glu* is glutamine; *Lys* is lysine; and so forth) is linked to the hemin by means of two cystein-S-bridges and a histidine-iron linkage.

through the ferrocytochrome-solution: no visible change occurs. The ferrocytochrome thus cannot be oxidized by oxygen. A small amount of cytochrome oxidase is now added: the ferricytochrome color returns.

From this experiment we can draw the conclusion that reduced cytochrome *c* cannot react with molecular oxygen. In a chain of oxidation enzymes, it will thus not be able to get next to the oxygen. The incapacity of cytochrome to react with oxygen was a striking fact that required an explanation. Another peculiarity was the extremely firm linkage between the red hemin pigment and the protein part; in contradistinction to the majority of other hemin proteides, the pigment cannot be split off by the addition of acetone acidified with hydrochloric acid. Further, there was a displacement of the light-absorption bands which indicated that the two unsaturated vinyl groups in the hematin of the cytochrome occurring in ordinary protohemin were saturated. In 1938 we succeeded in showing that the porphyrin part of the cytochrome was linked to the protein by means of two sulfur bridges from cystein residues in the protein of the porphyrin in such a way that the vinyl groups were saturated and were converted to α-thioethyl groups. The firmness of the linkage and the displacement of the spectral bands were herewith explained. This was the first time that it had been possible to show the nature of chemical linkages between a "prosthetic" group (in this case, iron porphyrin) and the protein part in an enzyme. Karl Gustav Paul has since found an elegant method whereby the sulfur bridges may be split with silver salts, and he has with organic chemical methods conclusively confirmed the constitution in this respect.

The light-absorption bands of the cytochrome showed that it is a hemochromogen, which means that, as a rule, two nitrogen-containing groups are linked to the iron, in addition to the four pyrrol-nitrogen atoms in the porphyrin. From magnetic measurements that I made at Linus Pauling's laboratory in Pasadena, and from amino acid analyses, titration curves, and spectrophotometry together with Å. Åkeson, it emerged (1941) that the nitrogen-containing, hemochromogen-forming groups in cytochrome *c* were histidine residues, or, to be more specific, their imidazole groups.

Recently we have got a bit farther. Tuppy and Bodo in Vienna began last year with Sanger's method to elucidate

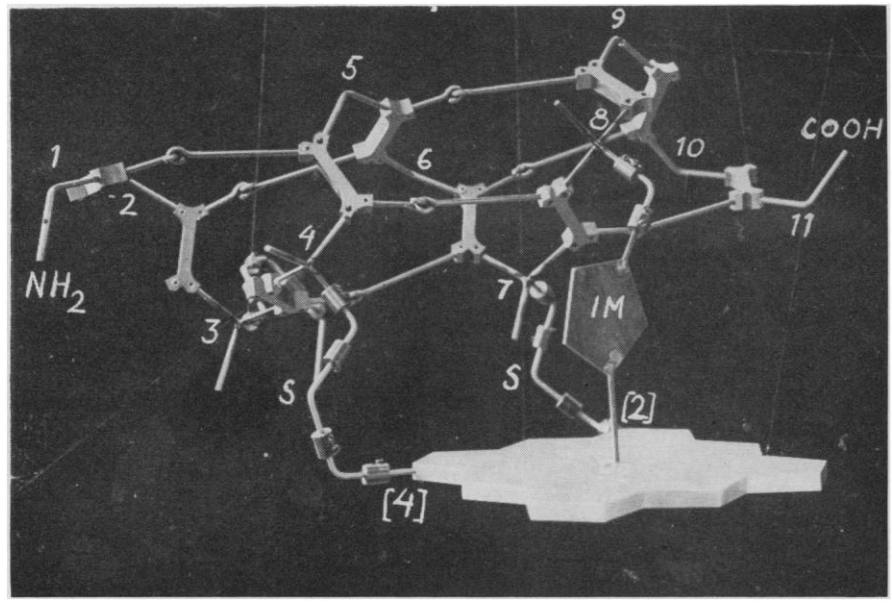

Fig. 4. Steric model of the hemopeptide remaining after pepsin digestion of cytochrome *c*, constructed of metal parts representing the atomic distances and valency directions. In the figure, the peptide chain is disposed by means of "hydrogen linkages" as a left-twisting α-spiral, seen at right angles to the longitudinal axis. Both sulfur bridges (*S*) and the imidazole group (*IM*) of the histidine then fit their correct linkage positions, and the peptide chain becomes parallel to the hemin plate (the light, polygonal metal plate at the bottom of the picture).

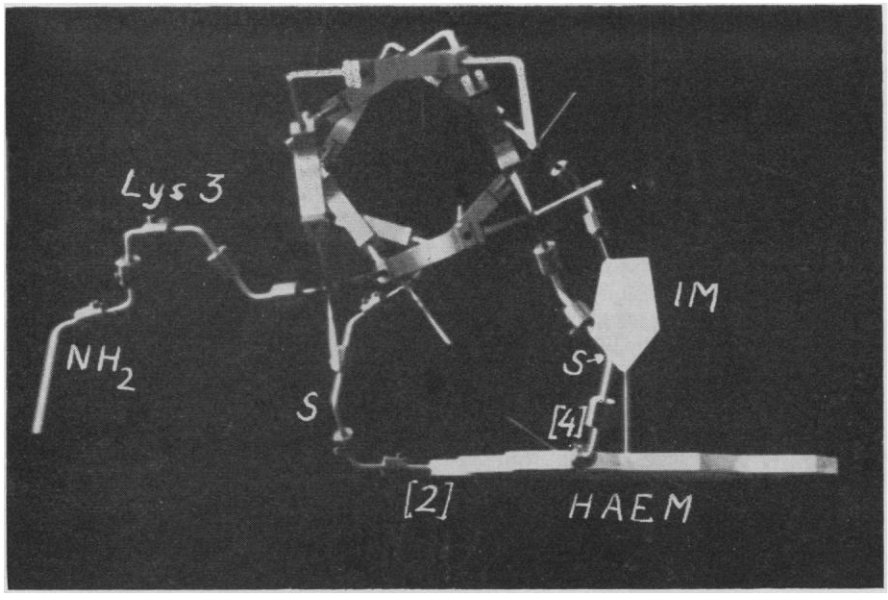

Fig. 5. The model in the longitudinal direction of the α-spiral. For the sake of surveyability, the lateral chains have been fitted only to the amino acids lysine, 3, cysteine, 4 and 7, and histidine, 8.

the amino acid sequence in the hemin-containing peptide fragment that one obtains with the proteolytic breakdown of cytochrome *c*, and they succeeded in determining the amino acids nearest the hemin. The experiments were continued and supplemented by Tuppy, Paleus, and Ehrenberg at our institute in Stockholm with the result shown in Fig. 3.

When in 1954 Linus Pauling delivered his Nobel lecture in Stockholm, he showed a new kind of model for the study of the steric configuration of peptide chains, which as we know may form spirals or folded leaves of various kinds. It struck me then that it would be extremely interesting to study the question which of these possibilities might be compatible with the sulfur bridges to the hemin part and with the linkage of nitrogen-containing groups to the iron. Pauling was kind enough to make me a present of his peptide-model pieces.

Anders Ehrenberg and I then made a hemin model on the same scale as the peptide pieces and constructed models of hemin peptides with every conceivable variant of hydrogen linkage (Figs. 4, 5). It proved that many variants could be definitely excluded on steric grounds, and others were improbable for other reasons. Of the at least 20 original alternatives, finally only one remained—a left-twisting α-spiral with the cysteine residue No. 4 linked to the porphyrin side chain in the 4-position, and cysteine No. 7 to the side chain in the 2-position. The imidazole residue fitted exactly to linkage with the iron atom. The peptide spiral becomes parallel to the plane of the hemin plate.

I think it may be said that it was of considerable interest to have Pauling's and Corey's most important spiral confirmed by purely chemical methods, which in our case of course was possible thanks to the unique circumstance that we had a short peptide linked at no fewer than three places to a rigid structure, the hemin. After we had sent this work to the printer, there arrived from Arndt and Riley in England a roentgen-crystallographic confirmation that cytochrome *c* contains left-twisting α-spirals.

Through calculations on the basis of the known specific weight of the cytochrome, we now consider it extremely probable that the hemin plate in cytochrome *c* is surrounded by peptide spirals on all sides in such a way that the hemin iron is entirely screened off from contact with oxygen gas; here is the explanation of our experiment in which we were unable to oxidize reduced cytochrome *c* with oxygen gas. The oxygen simply cannot get at the iron atom. There is, on the other hand, a possibility for electrons to pass in and out in the iron atom via the imidazole groups (Fig. 6).

One-sixth of the entire steric structure of the cytochrome molecule is herewith elucidated, and we glimpse further possibilities of gradually elucidating the rest. It strikes me as interesting that even at this stage the special mode of reacting of the cytochrome is beginning to be understood from what we know of its chemical constitution.

Peroxidases and Catalases

Peroxidases and catalases, according to what we know, are hemin proteides, thus close relatives of hemoglobin and cytochromes. Their way of acting was observed as early as the 19th century—the catalases at the beginning, and the peroxidases in the middle. It was almost inevitable that the first to produce hydrogen peroxide, the Frenchman Thénard, should also discover the catalase effect. As soon as hydrogen peroxide is brought into contact with the majority of native biological materials, the hydrogen peroxide is broken down with development of gas to oxygen and water. That the catalases contain hemin as an active group was shown as probable by Zeile and Hellström at Hans von Euler's institute in 1930, and the first catalase, from cow's liver, was crystallized by Sumner and Dounce in 1937.

Experiment 5. To 3 percent H_2O_2 in phosphate buffer at *p*H 7 is added 0.2 milligram of catalase. A vehement formation of gas is observed. When the bubbling has abated, the contents are poured out and fresh H_2O_2 and buffer are added. The bubbling nevertheless continues in consequence of minimal amounts of remanent catalase.

The peroxidases are of general occurrence in the vegetable kingdom. When in 1941 we crystallized a peroxidase for the first time, from horse radish, it proved, contrary to all Willstätter's assumptions, to contain protein as well as carbohydrate and hemin, and thus, iron. The reason Willstätter was unable to show the presence of any of these in his diluted solutions, which were nonetheless still active, was simply that Willstätter had happened upon a quite unusually strongly active enzyme. One-thousandth of a milligram in 1 liter of solution still gives a distinct effect. The peroxidases do not break down hydrogen peroxide directly, but

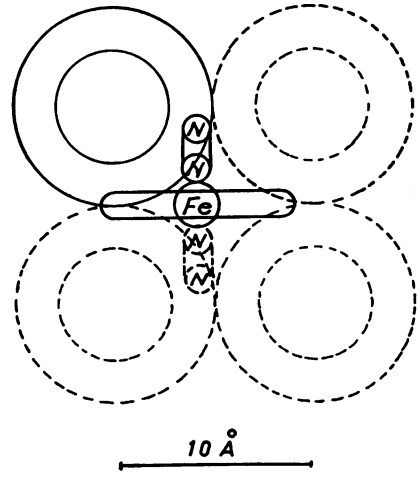

Fig. 6. Hypothetical section through a cytochrome *c*-molecule. The solid lines show the hemopeptide. The dashed lines show the parts of the natural molecule split off with pepsin. The region between the outer and inner circles is taken up by the side chains of the amino acids. The four peptide chains surround the iron atom, making it inaccessible to oxygen gas.

"activate" it to oxidize quite a number of hydroquinones, anilines, or even nitrite.

Experiment 6. To H_2O_2 in acetate buffer, *p*H 4.6, is added a trace of peroxidase: no effect. Benzidine is now added: blue coloring results.

Peroxidases occur here and there in animal material—for example, in leucocytes (verdoperoxidase, isolated in pure form by Kjell Agner in 1941) and in milk (lactoperoxidase, isolated by myself and Åkeson a year or so later).

In the living cells, both catalases and peroxidases function in the same way by using the hydrogen peroxide arising in connection with the reaction of oxygen with a number of autoxidable substances, such as flavins, ascorbic acid, and so forth, to oxidize otherwise difficultly combustible substrates. Especially interesting is Agner's observation that the verdoperoxidase in the leucocytes + H_2O_2, can detoxify, for example, tetanus and diphtheria toxins, which would of course explain one of the main functions of the leucocytes.

Catalases and peroxidases both give, with H_2O_2, first greenish complex compounds and then reddish ones. The study of these has presented immensely intricate problems, where we have had to use combinations of spectrophotometric and now very refined magnetic methods to try to elucidate the mechanism. Our American colleague Britton Chance, who in 1946–1948 worked at the Nobel Institute, has successfully carried investigations still further in this field.

Conclusion

What is the final goal of enzyme research? The first stage is to investigate the entire steric constitution of all enzymes—a nice little job! So far, we know only the most easily accessible sixth part of the smallest enzyme molecule, cytochrome *c*.

In the second stage, it is a matter of deciding how the enzymes are arranged in the cell structures. This implies, as a matter of fact, the filling of the yawning gulf between biochemistry and morphology.

Osmotic Pressure

Francis P. Chinard and Theodore Enns

Two recent articles, by Hildebrand (*1*) and Babbitt (*2*), have made evident some of the differences of opinion concerning the meaning of osmotic pressure. As is indicated by Hildebrand, the concept of osmotic pressure is of considerable importance in biological and physiological problems in which membranes are involved. Many physiologists have considered osmotic pressure to be the pressure exerted by solute molecules on a membrane permeable to the solvent but not to the solute. However, most, if not all, physical chemists and thermodynamicists consider osmotic pressure to be the pressure difference that must be imposed by the analyst across a membrane or barrier in order to establish equilibrium with respect to a substance that can cross the barrier. To some of the former group, osmotic pressure appears to be an intrinsic property of solute molecules. To the latter group, the determination of osmotic pressure is simply a convenient means of quantifying the effect of one constituent of a system on the properties of another constituent, just as determinations of freezing-point depression, boiling-point elevation, and vapor-pressure lowering are also measures of this effect.

We consider that the continued emphasis, in the physiological literature and occasionally in some of the more physicochemical contributions, on osmotic pressure as a property solely of solute molecules is unfortunate and misleading. We believe it is desirable, therefore, to set forth an elementary statement of the meaning of osmotic pressure (*3*). The following exposition presupposes some knowledge of the basic terms used. However, it may be noted here that the end-result as applied to physiological problems gives emphasis to the fact that the properties of *water* are affected by solutes. Consideration of the problem of capillary permeability by this approach has led to a reexamination of some fundamental physiological concepts (*4*).

To simplify the discussion, use is made of the concept of chemical potentials, which was introduced by Willard Gibbs (*5*). An elementary exposition of the concept will be found in W. Mansfield Clark's textbook (*6*). One of us (F.P.C.) has attempted a brief qualitative description (*7*).

Development

Consider a system comprised of two liquid phases, *A* and *B*, separated by a rigid membrane or barrier. Phase *A* contains only one constituent, denoted by the subscript 1, to which the barrier is permeable; phase *B* contains constituent 1 and another constituent, 2, to which the barrier is not permeable. Constituents 1 and 2 are soluble in each other. The pressure, *P*, is initially the same in the two phases. We wish to obtain a measure of the effects of constituent 2 on the properties of constituent 1. This effect will be expressed in terms of the chemical potential of constituent 1, in other words, in terms of the Gibbs free energy per mole of constituent 1. Under the initial conditions of equality of pressure in the two phases, equilibrium does not obtain with respect to constituent 1; experimentally there will be found net passage of constituent 1 from phase *A* to phase *B*; the chemical potential of constituent 1 in phase *A*, μ'_1, is greater than the chemical potential of constituent 1 in phase B, μ''_1.

The chemical potential of a given substance may be defined by the relationship

$$\mu = \mu^0 + RT \ln a \qquad (1)$$

where *R* is the gas constant, *T* is the absolute temperature, ln denotes natural logarithm, *a* is activity and μ^0 is a constant at any given set of values for *T* and *P*.

Under the initial conditions, the chemical potential of constituent 1 in phase *A* is

$$\mu'_1 = \mu^0 + RT \ln a'_1 \qquad (2)$$

and in phase *B* it is

$$\mu''_1 = \mu^0 + RT \ln a''_1 \qquad (3)$$

The difference between the chemical po-

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