PROBLEMS OF MODERN ENZYME CHEMISTRY[1]

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At this time a new approach to the investigation of physiologically active substances seems to be warranted. This group of substances, which comprises hormones and enzymes, but which also includes—in certain respects—the proteins, should not be treated in a purely "static" (descriptive) manner; a more dynamic consideration promises further insight into their constitutional peculiarities. Changes in the mineral constitutents of the crust of the earth, accomplished by processes of solution through geologic periods, find an analogy within mere seconds or fractions thereof in the autolysis of living, dying, and killed cells.

Certain substances in glands or leucocytes display little resemblance to those chemicals of guaranteed constancy of composition and properties which one finds listed in chemical catalogues. Little attention has been paid to the fact that the processes of solution and extraction, preliminary to the study of physiologically active substances, are entirely different from the simple phenomenon of dissolving stable organic compounds. We deal with very reactive substances which must be extracted from the ever-changing array of allied substances, likewise endowed with high reactivity. There always lurks the danger—which indeed often materializes—that denatured or changed derivatives are isolated instead of the original natural compounds.

Enzymes are more suitable for these dynamic tests than hormones, although the latter offer the additional attraction of practical medicinal importance. The biologists measure hor-

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monal activity by ingenious and delicate methods such as the cockscomb test for the male sex hormone devised by my famous Chicago colleagues. The quantitative estimations of enzymes by measurement of reaction velocities have the advantage of speed and accuracy. We control each step in the preparative purification of enzymes by quantitative analysis. They sometimes—indeed frequently—yield incorrect values in the case of enzymes, as their action is influenced by activators and inhibitors. Nevertheless, these measurements are always necessary, and discordant results in this field may become the starting points for remarkable discoveries.

The chemical changes during autolytic processes concern not only the substrates, but the enzymes themselves, and influence their essential characteristics. I wish to report today several observations on this phenomenon in the case of leucocyte amylase. Sometimes chemical changes involve those complexes which account for the protoplasmic linkage and which determine its solubility. Sometimes they have to do with the protective mechanism that prevents the enzyme from diffusion. Generally, enzyme research has been concerned with the study of soluble enzymes, or more properly, with the soluble portion of enzymes. It appears, however, that the fraction of an enzyme which cannot be attacked by simple processes of solution, and which can be demonstrated to be present in organ, tissue, and cell residues, is definitely characterized. Those enzymes which are chemically bound to the protoplasm and which are insoluble because of the chemical constitution of those complexes, we term "desmo"-enzymes. They stand in contrast to the well-known "lyo"-enzymes which in their natural state are soluble in water and glycerol, the latter property being advantageous for the avoidance of autolysis.

We unwittingly entered this realm of desmoenzymes twenty-seven years ago, when comparing the chlorophyll of a variety of plants. Before solving the difficult task of isolating chlorophyll in substance, we noted three points essential for the recognition of chlorophyll, and for the comparison of the innumerable instances of its occurrence: (1) Its content of magnesium, 4.5 per

cent coördinately bound magnesia; highly unstable green carboxylic acids are obtained by the saponification of dissolved chlorophyll, the so-called chlorophyllins, which still contain magnesium and are suitable for its determination. (2) Its content of phytol, the alcohol of formula $C_{20}H_{39}OH$. (3) The main portion of the big molecule, the porphin nucleus of the pigment, that is, of the two chlorophylls *a* and *b*, is characterized by two carboxylic acids of the pyrrole series, the olive green phytochlorin *e* and the red phytohodin *g*. These are formed by subsequent hydrolysis with acids and alkali. Under certain very mild conditions, these two carboxylic acids are the only products; under less favorable conditions mixtures of numerous split-products are generated.

The mild treatment of chlorophyll with acid results in the formation of a difficultly soluble olive-colored wax, phaeophytin, in which the ester group is intact. It contains 33 per cent of the alcohol phytol. It is the magnesium complex which is obviously destroyed by this acid treatment, while left intact by treatment with alkali, just contrary to the case of the phytol ester linkage. Chlorophyll from more than two hundred plants of different classes and geographic origin showed complete uniformity. However, this result was only obtained by way of a peculiar detour; originally great variations in the phytol percentages were found with different plants, for instance, instead of 33 per cent only 17, or 6, or 2, or 1 per cent. The plants with chlorophyll of apparently low phytol content served as material for a startling discovery. They furnished "crystallized" chlorophyll, a compound of marvelous beauty, difficultly soluble dark green crystals with 6 per cent magnesia. Russian botanists had discovered them in microscopic sections. When we chemists penetrated to this point, the nature of crystallized chlorophyll was still veiled in darkness. Was it a natural product or an artificial one? This puzzle was solved by examination of the process of solution. When chlorophyll is rapidly withdrawn from the leaves and the solution at once separated from the leaf residue, then the phytol content is always normal, one-third. But if the alcoholic solution of the pigment remains in prolonged contact with the leaves, the phytol percentage drops more or less, with some plants nearly to

zero. In such cases, phytol-free insoluble crystallized chlorophyll is formed instead of the waxy phytol-containing variety. The phytyl group has been replaced by ethyl, on one single carboxyl group of the chlorophyll. This reaction, an alcoholysis, is brought about by the enzyme chlorophyllase, which accompanies chlorophyll in leaves in variable quantities. Chlorophyllase in aqueous medium causes hydrolysis to free carboxylic acids, the chlorophyllides; even the partial synthesis of chlorophyll from these acids plus phytol was accomplished by means of this enzyme. Chlorophyllase, according to our present terminology, is a desmoenzyme; it remains in the leaf residue after treatment with all solvents. Have these leaf residues, the chaff so to speak, well-defined enzymatic character? They certainly have; they react only with the ester chlorophyll, and a more recent investigation showed that the desmoenzyme is inactive after thorough washing. Addition of calcium ion restores its activity, a distinctive characteristic.

Changes like that suffered by chlorophyll through the action of solvents on the leaf substance, also threaten other plant constituents during extraction, especially alkaloids with ester groups and glucosides. This principle guided my former pupil, A. Stoll, who was my assistant during the study of chlorophyllase and who is now director of a chemical factory in Switzerland. In an excellent research on digitalis glucosides, he succeeded in isolating three new glucosides—digilanid A, B, and C—by the most delicate exclusion of enzymatic hydrolysis during extraction from *digitalis lanata*. They are tetraglucosides and contain three molecules each of digitoxose and one molecule of glucose, besides an acetyl group. They are the hitherto unknown parent substances of all the various digitalis glucosides, digitoxin, gitoxin, gitalin, and the corresponding aglucones digitoxigenin, gitoxigenin, and digoxigenin. The original glucosides are accompanied by specific glucosidases causing partial cleavage of the sugar. The known digitalis substances are secondary products; the new ones are the natural parent substances.

The observation of the insolubility of chlorophyllase induced Waldschmidt-Leitz and myself to attempt the purification of

castor-bean lipase as an example of an insoluble enzyme. This desmoenzyme is very unstable towards water or glycerol unless it is protected by oil. It is especially labile in dried and defatted condition. This lipase can be obtained in higher concentration by shaking its emulsions in oil with aqueous reagents, e.g., dilute alkali, which removes proteins.

Other methods for the systematic increase of enzyme concentration apply to soluble enzymes, especially the methods of specific adsorption, developed for this purpose. The investigation of various lyo- and desmo-enzymes led to the first, although primitive, conception of the structure of enzymes. We visualized this structure as a specific active group with a high-molecular colloidal carrier. We shall examine today whether this conception proved fruitful and whether it is already possible to make any statements as to the properties of the specific groups and molecules on the one hand and the importance and influence of the colloidal carrier on the other hand.

Strong hydrochloric acid, or even better, pepsin in acid medium causes a remarkable change in the nature of castor-bean lipase. After such treatment, it coincides in its properties with the lipase of the germinating seed. The lipase of the (dormant) bean, spermato-lipase, and the lipase of the germinating seed, blasto-lipase, behave like two entirely different enzymes. The one acts in acid medium, the other at neutrality. The second one is far superior for synthetic reactions. The characteristic transformation of castor-bean lipase must be interpreted as partial proteolysis of the colloidal carrier.

A few fundamental facts have been ascertained regarding the specific active groups of enzymes. The way had been paved primarily by the high degree of enzyme concentration achieved through extensive purification; but accurate observations and improvements in the (preliminary) procedures, leading to solution, contributed a great deal to these results. A good illustration for this methodical development is furnished by the case of yeast saccharase. Previously solution of this enzyme was effected, for instance, by quantitative autolysis of yeast, whereupon the concentration of the dissolved enzyme should corre-

spond with that in the yeast cell. Or, yeast was treated with water in the presence of a preservative, under the assumption that the enzyme could diffuse from within the cell. Only a minute percentage, and an unknown one besides, passed into solution, and even that without significant increase of purity. In reality, saccharase cannot be withdrawn from an intact cell, but poisoning of the yeast cell is the sine qua non for solution of the enzyme. Once autolysis, a multitude of enzymatic hydrolyses, is initiated, then there is one definite singular reaction, the liberation of the enzyme saccharase, which can be favored selectively among the other autolytic processes. Thus, one can accomplish complete liberation of the saccharase, together with as little as one-twentieth of the inert contents of the yeast cell. An investigation carried out by Dr. C. D. Lowry in my laboratory afforded additional aid. By a special fermentation process, yeast could be enriched in saccharase fifteen to twenty times. A combination of these experiences yielded enzyme solutions which, to begin with, were three hundred times superior in purity to ordinary yeast. Systematic development of adsorption methods permitted further considerable increases of enzymatic purity in the case of saccharase as well as in that of peroxidase or pancreas amylase.

The method of selective adsorption by specific adsorbing agents was also fruitfully developed in another direction, namely, for the separation of enzymes from each other. The first example was the group of pancreatic enzymes, amylase, lipase, and trypsin. Another step in the progress was the separation of such closely related enzymes as saccharase and maltase. The last application of greater importance was the resolution of animal and vegetable proteases into their numerous components by Waldschmidt-Leitz and by Grassmann, and the segregation of β-glucopolysaccharidase, specific for cellulose and cellodextrins, from β-glucoöligosaccharidase, capable of hydrolyzing the lower molecular carbohydrates originating from cellulose. This separation by means of aluminum metahydroxide has been published this summer by Grassmann and Zechmeister.

Since saccharase becomes soluble only through a definite en-

zymatic process, one might assume that it is tied to the protoplasm. This view seemed to be supported by the following experiment. Autolytic reactions, essential for the solution of saccharase, can be eliminated by treatment with warm ethyl acetate, which destroyes the enzyme responsible for the liberation of saccharase. Then the yeast cell may be emptied by the action of pepsin or trypsin with complete recovery of the saccharase originally present. Yet this saccharase remains in the cell residues, from which afterwards it may be dissolved by the action of plant amylase or plant proteinases. Was the enzyme linked to the protoplasm? The answer is no, it was only protected and fixed; so to speak tucked away by the cell membrane which, according to the two possibilities of attack, must consist of protein linked to carbohydrates. For, according to more recent experiments, yeast may be finely triturated with liquid air and treated with anhydrous glycerol, strictly eliminating all autolytic processes. Then saccharase passes into solution, after complete, but purely mechanical, destruction of the cell membrane. We propose to call an enzyme of this type, protected from solution by mechanical, not by chemical means, an "endoenzyme."

This protection of endoenzymes against diffusion is in accord with the properties and the behavior of the yeast cell. A comparison with other cells and with glandular tissues reveals gradations of solubility and stability of what might be called anchorage; little attention has been paid to these facts up to the present time, and it has been customary to prepare enzyme solutions with aqueous media or glycerol, without any regard to the possibility of autolytic reactions, while the share of enzyme remaining in tissue, gland, and organ residues was neglected. However, if the much investigated, long known enzyme pepsin was advantageously prepared by five days' digestion with an excess of 0.5 per cent hydrochloric acid at 37°C. according to C. A. Pekelharing, this is certainly not the dissolving of a soluble enzyme, but, for the most part, the liberation of an insoluble, plasma-linked pepsin. If we avoid autoproteolysis by treatment of stomach mucosa with dilute hydrochloric acid at 0°C., desmopepsin is left in the tissue

residue and can only be rendered soluble by various proteolytic procedures.

Analogously, a great portion of trypsin is found ready for secretion as a lyoenzyme in the pancreas gland; but another part is bound as desmotrypsin in the inactive state and forms an insoluble complex together with a precursor of enterokinase, its natural activator. These conditions are highly significant for the decision of the question whether certain enzymes are simply identical with certain proteins.

Histozyme, a kidney enzyme, which decomposes hippuric acid and conjugated bile acids, occurs to a greater extent as lyo- or as desmo-enzyme according to animal species. In dog kidney it is easily soluble, in horse kidney insoluble; hog kidney contains partially soluble, partially insoluble, histozyme.

Deeper insight is offered by the study of the white blood corpuscles, which have no secretory function. They are extremely prone to autolysis, but if this is avoided by momentary desiccation, then leucocytes contain, for instance, trypsin almost completely plasma-bound, as proven by careful treatment with anhydrous glycerol.

The desmoenzymes investigated are not uniform as a rule. For example, desmoamylase of leucocytes consists of one fraction, which is soluble in alkali phosphate and other fairly neutral electrolytes. From the cell residues, a second fraction can only be rendered soluble by proteolysis, e.g., by papain. A third fraction, absolutely resistant against proteinase, is left over. The comparison of these fractions with each other and with the lyoenzyme shows that the colloidal carrier proper of the enzyme, the indispensable component of its molecule, may in turn be associated with other high-molecular substances, which determine its solubility and resistance against attempts at isolation. The enzyme may occur in the form of solid solutions or adsorption compounds with proteins; its colloidal carrier may also be chemically combined with proteins and other substances of high molecular weight.

The significance of the protein content of enzymes has become the subject of a fruitful discussion between some American

colleagues and ourselves. While our own efforts in the case of saccharase, amylase, and other enzymes aimed at the gradual and, if possible, complete liberation of enzymes from protein, these American colleagues proceeded exactly in the opposite direction. They succeeded in the isolation of proteins endowed with enzymatic activity. The isolation of urease by Sumner, of pepsin by Northrop, and of trypsin by Northrop and Kunitz in the form of crystallized proteins are brilliant discoveries and important preparative successes. But it remained doubtful whether the protein content of these enzymes really forms an indispensable constituent and whether indeed "the proteolytic activity is a property of the protein molecule itself." Dyckerhoff and Tewes in Munich and Waldschmidt-Leitz and Kofranyi in Prague were able to replace the protein, which carried pepsin activity, by vegetable protein. According to these authors, the enzyme of a pepsin solution can be completely adsorbed on certain seed globulins, while the original protein component remains completely in the mother liquor without peptic activity. Thus the crystallized protein should not be considered identical with the enzyme; it cannot even be acknowledged as its indispensable colloidal carrier.

The existence and significance of the true colloidal and molecular carrier of the active group can be proven, however, by delicate procedures, which cause well-defined changes in the properties of an enzyme, similar to those mentioned in the case of castor-bean lipase and to those encountered with the peroxidase action of oxyhemoglobins. Unpublished work on leucocyte amylase which Miss Rhodewald has carried out with me, exemplifies this possibility. It does not deal with the differentiation of the various fractions of desmoamylase, mentioned before, but with transformations of lyo- (and desmo-) amylase, changes of sharply defined properties without loss of enzymatic activity. Such characteristic properties are as follows: (a) Amylase may, or may not, be inhibited by glycerol. (b) It may be independent of the presence of phosphate, that is, completely active with or without phosphate ion, or it may be inactive without the addition of phosphate.

These two characteristics permit the existence of four amylases, with the properties a+ b+, a− b+, a+ b−, and a− b−. We have learned to prepare these four amylases, one only as a by-product, from leucocytes, or rather to prepare four lyoamylases and the four corresponding desmoamylases or indeed groups of desmoamylases. They all belong, according to E. Ohlsson's classification of dextrinogenic and saccharogenic amylases, to the former group and to the alpha-amylases of R. Kuhn.

One should not object to the complicated nature of these results. The observations are accurate, and cannot be thrust aside. There are further instances where analogous observations will serve the characterization of enzymes and the study of their primary changes.

A few examples will be given here for the individual leucocyte amylases. Fresh leucocytes yield amylase solutions with glycerol which are inactive without phosphate, not inhibited by glycerol, and very unstable in aqueous solution. This amylase is generated during the rapid killing of the leucocytes in glycerol from another amylase of opposite properties (−a, −b from +a +b). Leucocytes suddenly dehydrated by an excess of acetone contain the primary amylase, which requires no phosphate for activation. Hence, with glycerol a solution may be prepared without any manifest amylase action, but which nevertheless contains amylase, inhibited by glycerol, and independent of phosphate. Its presence can be proven by dialysis of the glycerol, and it is active with or without phosphate. The dialyzed solution, however, does not contain the original, but a changed amylase. Its property of being inhibited by glycerol has disappeared; addition of glycerol is now without effect: −a +b. In some instances a great difference of effect was observed with and without phosphate owing to the presence of a fourth lyoamylase +a −b. The transitions of these amylases into one another are so easily accomplished that the physiological difference between batches of leucocytes seems sufficient to yield amylase −a +b and sometimes even −a −b instead of the primary +a +b.

The residues of rapidly dried leucocytes, after treatment with glycerol, contain the desmoamylase +a +b, independent of

phosphate and inhibited by glycerol. It is more stable towards water than the corresponding lyoenzyme. Leucocytes treated in fresh moist condition with glycerol contain desmoamylase, not inhibited by glycerol and only active in presence of phosphate (−a −b). It is labile and loses its activity within a few hours in aqueous solution. The characteristic properties mentioned will suffice to analyze mixtures quantitatively.

The described changes seem to occur on two functional groups, not identical with, but of decisive influence on the specific active group of the enzyme.

It takes place presumably on the molecular carrier, whether the latter is still anchored in the protoplasm (desmo) or dissolved (lyo). While these experiments are merely a first attempt at subjecting the entire enzyme molecule to chemical interpretation, there are a few cases where the specific active group, postulated by our theory, has been chemically defined. Respiration ferments have been recognized by O. Warburg and by D. Keilin as iron porphyrin complexes with unknown colloidal carriers. Furthermore, Zeile and Hellström succeeded in the case of catalase, and Kuhn, Hand, and Florkin in that of peroxidase, after far-reaching purification by adsorption methods, to adduce evidence that these enzymes owe their activity to iron porphyrin complexes.

However, not only the highly active protein crystals of our American colleagues, but even purified catalase and peroxidase are far from the point where one might consider them as pure preparations. Peroxidase, as characterized spectroscopically by Kuhn, is still diluted by a thousandfold excess of foreign material according to its content of active iron. Thus the computable reactivity of an enzyme molecule reaches a value near the theoretically possible limit, namely, occurrence of reaction at every collision of enzyme and substrate molecule. This computation is of course based on the presumption that each effective collision generates a stoichiometric amount of reaction product.

Is this assumption cogent? Newer theories of chain reactions in the auto-oxidation of inorganic substances are based on the remarkable observations of H. L. J. Bäckström and have been developed by J. Franck and F. Heber. This principle is suitable,

as my friend Haber and I have stated, for the explanation of numerous organic and biological processes, such as oxidations, dehydrogenations, and dismutations. We assume the formation of monovalent radicals, whose function is the generation of polymolecular reaction chains. Accordingly, reactions like the cleavage of hydrogen atoms do not proceed in pairs, but the first step consists in the cleavage of a single hydrogen atom. A first radical is formed, for instance, CH_3CHOH—. This radical, together with oxygen, generates a second radical —OH, and this in turn with alcohol the first radical and so on.

By this short reference to reaction chains and chain reactions, as applied to enzymes, we have abandoned the solid ground of facts and have embarked on the rocking ship of hypothesis.

Whether we deal with such tentative explanations, or with the controversial protein nature of enzymes, I feel that it is not important for the scientist whether his own theory proves the right one in the end. Our experiments are not carried out to decide whether we are right, not to prove that we are right, but to gain new knowledge. It is for knowledge's sake that we plow and sow. It is not inglorious at all to have erred in theories and hypotheses. Our hypotheses are intended for the present rather than for the future. They are indispensable for us in the explanation of the secured facts, to enliven and to mobilize them and above all, to blaze a trail into unknown regions towards new discoveries.

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