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Fermentation of sugars and fermentative enzymes

Nobel Lecture, May 23, 1930

It is my object to give a report of my work in connection with *The Fermentation of Sugars and the Enzymes Active in Such Fermentation.* In performing this duty and expressing my sincere and warm thanks to my colleagues for their very valuable assistance, I will try to give an idea of the aims of our work, point out the details of our methods and the results obtained, and clarify their relationship to central problems in biology and to what extent our work can contribute to a solution thereof.

Any scientific problem must be attacked by research into detail; the natural scientist did not win his victories until he left meditation on the great riddles of the world and began a careful study of special problems; our knowledge - of more general associations and of far-reaching laws has grown out of the results of such research. Thus the study of alcoholic fermentation led men like Lavoisier, Pasteur and Liebig beyond the immediate results to experience of the greatest scope.

The fermentation of sugars forms a special reaction complex and each of the reactions co-operating therein constitutes a still more limited field of study, but it is precisely this combination of reaction phases within the cell that is in many respects typical of the processes of life.

We also know that the decomposition of sugar during fermentation is identical with the reactions by which sugar begins to burn during respiration, and there is therefore no organism whose life is not dependent on the sugar reactions under discussion.

What I shall deal with mainly in this lecture relates to the *means* by which the living cell decomposes its sugar and thus produces energy for its life and growth. By these means the reactions of the *living cell* differ from *other* chemical processes.

Within the living organism, the majority of reactions are brought about by special substances already active in minimum quantities, such substances being known as *enzymes* or ferments. Every group of substances, and in fact practically every substance, requires its specific enzyme for its reaction. Only a few enzyme types were known in the early days, such as pepsin in the gastric

juice, which splits proteins, or amylase in saliva and in malt, which converts starch into sugar, but in more recent times the number of enzymes whose existence has been proved or substantiated has risen to over 100.

A study of the kinetics of the enzyme reactions confirmed Berzelius' brilliant intuition to the effect that in respect of their activity the enzymes are analogous with the other, non-enzymatic catalysts. For example, we can split sucrose into glucose and fructose either by means of a strong acid, such as sulphuric acid, or by an enzyme obtained from yeast, namely saccharase. The difference is principally that we require much fewer molecules of enzyme (about 1 millionth) than molecules of sulphuric acid to achieve the same effect, and this is due to the fact that sucrose has a much greater affinity for the enzyme than for sulphuric acid; thus it binds its special enzyme more completely than the acid and holds it within the reaction sphere.

All earlier hypotheses to the effect that the enzymatic activity was associated with a special state of matter have been refuted; enzymes are fundamentally not different from other substances.

Enzyme research is now advancing in two complementary directions: *a purely chemical and a biological.*

With regard to the former, the problem is to unravel the *chemical nature* of the enzymes; we cannot reach any definitive conclusions if we do not know the atom combinations governing the properties of enzymes. The problem in this case therefore is to concentrate these substances, which are enormously diluted in animals and plants, then eliminate the accompanying substances from their solutions, obtain them in the pure state, and finally analyse them and unravel their molecular structure.

At the beginning of this century the work on enzymes had still been predominantly qualitative; it has become necessary for us to introduce exactly defined and rational units for the activity of enzymes if we are to be able to follow the course of purification.

Concentration and purification, which have frequently resulted in isolation of the required substance in biochemistry, come up against particular difficulties as far as enzymes are concerned. Enzyme molecules are large, unstable and are easily destroyed by acids and alkalies; with regard to their sensitivity to elevated temperature, they behave like proteins and are rapidly denatured or inactivated at temperatures above 60°. Thus it is not possible to use sharp chemical agents for their study.

Substances of high activity are obtained under mild conditions, by precipitation with alcohol and acetone, by diffusion and by adsorption methods - the

latter specially devised by Willstätter - and the unremitting work done in this respect both here and by other schools has certainly not been in vain. This preparative method, however, has hitherto had only limited success.

It was obvious to try to break through by *indirect* methods. I shall discuss these briefly for they constituted a quite important part of our research, and I also think that their continued use will give valuable results.

As I have already stated, an enzyme is active according to its combination with its substrate by a particular affinity. This combination is produced by certain atom groups, and *inter alia* it is these which have to be clarified.

I will take an example: when proteins are digested, the long chain of amino acids of which the protein consists becomes smaller and smaller, and the final intermediate product containing two such amino acids is known as a dipeptide.

The dipeptides also have their special enzymes, the *dipeptidases.* Like all such substrates, the dipeptides are bound to the enzyme before the latter acts, and the first question is *by whatgroup in the dipeptide* does this take place. It is a general fact that the *decomposition products* arising in an enzyme reaction have an inhibiting effect, and the amino acids are the inhibitors here. We have to ascertain *which group* such a decomposition product must contain to inhibit the reaction, and by Josephson's work concerning a special dipeptidase it could be shown that it is the *amino* group that is involved here.

The study of the circumstances in which this inhibition occurs and in which the enzyme binds the dipeptide has subsequently shown that it is probably a *carbonyl* group in the enzyme that binds the substrate; and thus for the first time a combination between an enzyme and a substrate was traced back to certain atom groups.[1]

Other researchers, particularly Waldschmidt-Leitz and Grassmann, continued with this method of determining active atom groups in an enzyme molecule, firstly for the examination of peptidases from yeast and subsequently for the examination of other protein-splitting enzymes. Two defined enzyme types were discovered, amino-peptidases and carboxyl-peptidases, and confirmation was obtained for a working hypothesis devised by us, the so-called *2-affinity theory.* This means that two different reaction centres cooperate in the splitting of the substrate, namely one which binds the substrate with a measurable specific affinity and one of a shorter life which should be localized at that zone of the substrate where the splitting takes place.

Another way adopted by us to discover something of the chemical nature of the enzyme is as follows:

It was known that certain chemical substances even in very small doses are able to inactivate dissolved enzymes. Phenols, amines, mercury salts and hydrocyanic acid are among them, i.e., substances which even the layman knows as poisons. Their fatal effect on the organism consists precisely in that they inactivate the enzymes necessary to life. Systematic examination has shown that such poisoning of isolated enzymes is of a *purely chemical nature,* i.e., the poison binds the enzyme, which is thus inactivated. By studying this effect quantitatively, we determined enzyme equivalents by known quantities of poison.

Such poisoning can readily be shown with various yeast enzymes; I will take catalase and poison it with hydrocyanic acid (experiment).

The poisoning of the typical complex of alcoholic fermentation with fluoride can be proved and at the same time the co-operation of this enzyme with the activator cozymase can be proved by another experiment: the reaction can be followed in the apparatus in which we conventionally determine the course of fermentation (Myrbäck's microfermentation apparatus). *Demonstration experiment:* three pairs of flasks are filled as follows.

1 and 2 with the enzyme (apozymase) without cozymase,
3 and 4 with the enzyme with cozymase,
5 and 6 with apozymase, cozymase, and fluoride.

The experiment showed evolution of CO_2 only in flasks 3 and 4, namely 5 c.c. per I0 minutes.

The case best examined is the poisoning of the yeast enzyme which splits sucrose into its components, glucose and fructose. The degree of poisoning depends enormously on the acidity of the solution. Myrbäck's test showed that the saccharase is inactivated by silver salts to the same degree as the enzyme reacts with the *free silver base* formed from the silver salt, to form a silver compound, and similar poisoning occurs with certain other toxic metal salts, Hg, Cu, and Pb.

From the quantity of metal salt added and from the degree of poisoning, i.e., the degree to which the enzyme has been inactivated, it is possible, on certain assumptions, to determine how many mg of enzyme correspond to 1 mg of silver. Other measurements by Myrbäck have shown that the poisoning due to phosphotungstic acid, picric acid, etc., depends among other things on the formation of slightly dissociated salts between these acids and a slightly basic group in the enzyme molecule. Other series of tests have given

information concerning which important constituents within the saccharase molecule co-determine the affinity of the enzyme for its substrate.

Thus for each enzyme of the saccharase type it is possible to determine an equivalent corresponding to each poisonous metal atom. Somewhat complicated calculations are required to arrive at the said final results from the experimentally determined degrees of poisoning. Without going into these, I will simply state that on this basis we now understand how quantities of one millionth of a gram and less of a poison may be sufficient to paralyse or annihilate an organism; the quantity of the catalysts required to be inactivated in the poisoning of an organ is not greater than the quantity of poison used. There is therefore nothing mystical in the homeopathic doses.

Poisons have also been used in other ways in work on enzymes. As I stated, fermentation from sugar to alcohol and carbon dioxide passes through a considerable number of intermediate stages, amongst which each has its own enzyme. These partial reactions are so interwoven that it is difficult to separate them directly. It has now proved possible to poison the long series of reactions *partially,* so as to separate one or other partial reaction so as to cleanse the others as it were. Something along these lines has been possible with fermentations by the addition of *fluoride.* To clarify this I must give a brief idea of the view previously held concerning the various phases of fermentation.

I must first recall A. Harden's important result, namely that fermentation not only requires a catalyst of enzyme nature but also a more stable substance which was called *cozymase.*

Examination by Myrbäck in 1924 had the result that the fermentation reaction phase requiring the cooperation of the cozymase is a very early one, namely one preceding the combination with phosphoric acid.

According to Harden, the splitting of sugar is associated as follows with the formation of a phosphoric acid ester, known as phosphorylation:

$$\text{I.}\quad 2C_6H_{12}O_6 + 2PO_4HR_2 = 2CO_2 + 2C_2H_5OH + 2H_2O + \\ + C_6H_{10}O_4(PO_4R_2)_2$$

$$\text{II.}\quad C_6H_{10}O_4(PO_4R_2)_2 + 2H_2O = C_6H_{12}O_6 + 2PO_4HR_2$$

Thus a compound, *zymo-diphosphute,* is formed simultaneously with alcohol and carbon dioxide.

After the sugar has undergone conversion by its reaction with phosphate, the actual splitting takes place. The chain of 6 carbon atoms is split in the middle and amongst the possible splitting products, dioxyacetone, glyceraldehyde and methyl glyoxal it is precisely *methylglyoxul* which really does

occur according to C. Neuberg. Fermentation and respiration differ only after this stage. In the case of the former, a number of oxidation-reductions result in alcohol and CO_2 while in the case of respiration the atmospheric oxygen oxidizes that part of the resultant intermediate products which is not involved in the synthesis, to form CO_2.

I will leave the later fermentation stages and intermediate products, amongst which is pyruvic acid, and I will turn to the processes which occur when the sugar supplied to the yeast cell or the blood is involved in metabolism.

In unravelling these very complicated affairs, the thermostable activator *cozymuse,* to which I shall shortly return, has given valuable guidance. The three main forms of biological sugar decomposition, fermentation, respiration and glycolysis, have a common partial reaction of the oxidation-reduction type, and a co-enzyme is active in this.

The character of this reaction is illustrated by the fact that the Cannizzaro reaction of acetaldehyde in liver is dependent on *the same co-enzyme;* this co-enzyme has also proved to be *identical with cozymase.*

This fact led to the conclusion that an oxidation-reduction, or a mutation, combined with energy equalization, is *the fermentation phase requiring the presence of the cozymase.*

In recent years this fact has been valuably supplemented by the very thorough research by R. Nilson. According to Nilsson's result, *that part of the fermentation reaction in which the cozymase co-operates is inactivated by fluoride.* A hexose monophosphoric acid, known as the *Robison* ester, is formed as an intermediate product in this.

From the enzymatic aspect it was important that a phosphorylation could be demonstrated in these circumstances both in yeast and in muscle *without the co-operation* of cozymase and without the simultaneous evolution of CO_2. This proved the existence of an *independent phosphorylating enzyme* (H. von Euler, H. Ohlsén and S. Kullberg, 1911), a phosphatase.

Another important finding was that fluoride prevents the reaction: hexose monophosphoric acid $\rightarrow$ hexose diphosphoric acid.

Finally, I should mention the result that glucose in its isolated and stable form behaves in all biological decompositions differently from the form in which this hexose immediately arises out of the higher carbohydrates, glycogen and starch, by means of which animal and plant organisms store their sugar reserves; in other words : the form of sugar which is formed enzymatitally from glycogen and from zymophosphates, behaves as *blood sugar.*

Time does not allow me here to go into the medically interesting question

of the effect of insulin on the blood sugar balance and the enzymatic changes occurring in diabetes mellitus.

Returning to my main subject, I will go back to the previously mentioned Harden equations. This formulation, which has played such a great part in modern fermentation chemistry, says nothing about the type of the assumed association between the two reactions. By a modification of Harden's formulae (H. von Euler and K. Myrbäck, 1924), the connection between the two reaction stages becomes clear.

Our first formulation was that one molecule of zymohexose undergoes the reaction:

$$C_6H_{12}O_6 + PO_4HR_2 = C_3H_6O_3 + C_3H_5O_2(PO_4R_2) + H_2O$$
$$2C_3H_5O_2(PO_4R_2) = C_6H_{10}O_4(PO_4R_2)_2$$

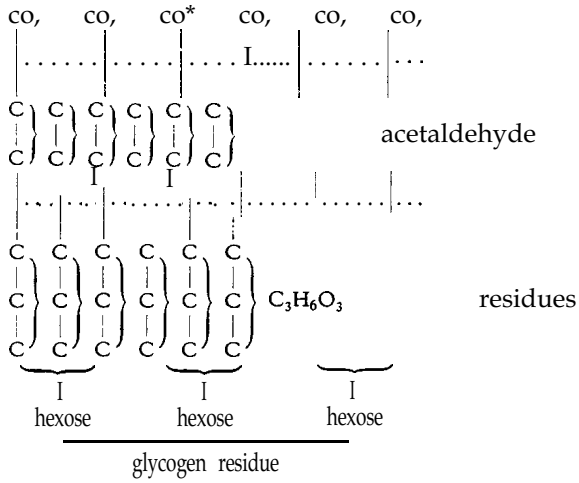
This formulation shows that the molecule which undergoes fermentation is split into two partial molecules each having three C atoms, *one of which is richer in energy and the other poorer in energy than half the substrate,* and that the zymophosphate synthesis is obtained at the expense of the fermenting hexose half which is poorer in energy. It is of course a further improvement to adopt Meyerhof's assumption of monophosphate instead of glucose as starting-material (cf. H. von Euler and K. Myrbäck, 1929[2]). The main thing, however, is the principle that phosphorylation introduces an energy dismutation within the hexose residue.

I mention this contribution because it touches on a general and biologically not unimportant question: it has long been clear that many organisms recover the greater part of their energy from anaerobic or aerobic sugar decomposition which, as is well known, generates heat. However, how the energy developed by reaction can be transferred to other processes within a cell has rarely been discussed by physiologists, as Kluyver recently pointed out. It is however clear that that part of the fermentation or respiration energy which really is developed in the form of heat within the cell is of secondary importance to the organism; the main function is played by that part which is transferred to other forms of chemical energy forms within the cell; only that part can be utilized for the synthesis and hence for reproduction, growth, and regeneration.

Generally, an energy-rich decomposition product occurring in the energy-yielding reaction must be involved in the energy-absorbing reaction.

Apart from the enzymatic couplings which we take as a basis for the phosphorylation process, we find *another coupled reaction* within carbohydrate

metabolism biochemistry, and this is linked up with a problem no less important to enzyme research, namely the relationship between anaerobic and aerobic sugar metabolism. Meyerhof has shown that the relationship between anaerobic and oxidative decomposition is the same in muscle and in yeast. On the assumption that sugar decomposition in the animal organism and in yeast takes place by way of glycogen, and on the assumption of an introductory dismutation between two different sugar residues, we have acquired an idea of the fate of sugar during oxidation[3]. The decomposition does not take place as was previously assumed in such a manner that a sugar molecule is oxidized to 6 CO_2 - this would be burning of little use to the organism - but in such manner that each hexose molecule produces one molecule CO_2 one molecule having 2 C-atoms, and the residue having 3 C-atoms. Thus in the decomposition of 6 molecules of zymohexose 6 CO_2 are formed, and from 6 $C_3H_6O_3$ residues there are again formed 3 molecules of a hexose which come into the carbohydrate reserve, and also 6 molecules of acetaldehyde disappear which provide the C-material and the energy for the cell syntheses. The following diagrammatic representation does not take into account the by-reactions owing to which the $CO_2/C_6H_{12}O_6$ ratio frequently differs considerably from the value 1.



It remains for me to mention briefly our chemical researches in connection with *cozymase.*

The reason for our doing so much work on the purification and determination of the constitution of this substance is that cozymase is one of the most

widespread and biologically most important activators within the plant and animal world.

It is only in a few microorganisms that no cozymase has been found. Its presence in lactic-acid bacteria, as found by A. Virtanen, is particularly interesting. In a recent examination, Dr. Ernst Sym and Assistant Professor Ragnar Nilsson have found that the majority of tissues and organs with high carbohydrate metabolism are particularly rich in cozymase, e.g., in addition to the working muscle, the retina and cerebral grey matter.

The difficulty in isolating cozymase is above all due to its considerable dilution. Yeast has proved the most suitable starting material despite the fact that in the most favourable cases 1 kg of yeast did not yield more than 2 cg.

By a long series of purification processes a preparation was obtained with a maximum activity - expressed in rational units - of ACo = 85,000, the starting material being characterized by ACo = 200. It was possible to convert this preparation into salts - salts of the alkaloids and of the alkaline-earth metals were used for the isolation - and cozymase can be recovered therefrom with practically unchanged activity[4]. The composition of the most highly purified product corresponds approximately to a so-called nucleotide, for it contains a sugar residue, a purine residue and phosphoric acid, and everything points to a close relationship with a substance occurring in muscle in small quantities, *adenylic acid.* In a preparation with an activity ACo= 70,000 recently prepared by Associate Professor K. Myrbäck, the ratio of adenine: reducing sugar group : phosphoric acid was found to be very close to 1. The relative molecule concentrations were, in facts:

adenine 0.208
reducing sugar 0.204
PO_4 0.212

The relationship between adenylic acid and cozymase offers special interest since G. Embden in new interesting work has shown that the ammonia liberated in the muscle originates - at least largely - from adenylic acid. Attempts to prove a connection between the state of the muscle and the co-enzyme content have not given clear result yet[6], but are to be repeated with special reference to the oxygen supply whereby cozymase possibly acts as a link between the energy-producing glycolysis and the energy-absorbing synthesis.

The coenzymes, of which cozymase is the most important representative, constitute one type of the large group of *biocatalysts* which include not only enzymes but also certain hormones, growth factors, and vitamins. Of no less interest is the fact that the chemical results hitherto available point to a rela-

tionship between cozymase and many other factors essential for life, viz., the vitamins B_1 and B_2 which have been thoroughly studied particularly in England by Miss Harriette Chick and R. A. Peters, and in America by Levene, and also Wildier's "bios" and finally our factor Z.

Another group of biocatalysts distributed throughout all organisms and even in yeast fungi is the group which effects the actual oxidation, hence the final stage of respiration (C.A. MacMunn; D. Keilin; O. Warburg). Substances very closely related to haemin, the characteristic blood-pigment constituent, are found in aerobically living cells of the plant and animal world. The porphyrin residue, combined with the iron atom in these substances, belongs to a group to a knowledge of which a very progressive contribution has been made in recent years by Hans Fischer. It is interesting that an enzyme which undoubtedly participates in the respiration process, viz. *catalase,* also contains a haemin residue[7].

In addition to this *chemical* method, enzyme research is also advancing in another direction towards biology. The first problem here is the relationship of the enzyme to plasma and to the living cell.

Twenty-three years ago E. Buchner was awarded the Nobel Prize in Chemistry for the discovery that fermentation occurs in a practically cell-free "Presssaft"*. The question frequently raised since then is to what extent the enzyme complex present in Buchner's extract participates in the activity of the living cell. In more recent work in this direction use has been made mainly of so-called dried yeast.

It was principally important to determine as exactly as possible a difference between such a dried-yeast and fresh-yeast cells, particularly with reference to its power of fermentation and to its reproduction potency. In an examination carried out together with Professor Chr. Barthel[8], it was found that after treatment with alcohol and ether a dried yeast contains less than 0.1% cells capable of reproduction, but had practically the same fermentation power as the fresh yeast; the fermentation enzymes were therefore retained in the dry cells freed of lipoids but a catalyst participating in the reproduction process was destroyed. The fact that such a dry yeast - in opposition to the fresh yeast - is completely insensitive to toluene can hardly be explained otherwise than by the assumption that the enzyme system in the living cell is combined with the protoplasm and dependent on the structure thereof. In connection with these structural changes there arises a problem of even more general

* Juice extracted by Buchner from yeast cells by compression with a hydraulic press.

scope, viz., the existence of transitions between enzymes and living matter.

In addition to certain facts found in the study of dry yeast, the properties of bacteriophages and particularly of plant virus as it occurs in certain chlorophyll diseases, point to the existence of enzyme-like substances, *enzymoids,* which are distinguished from the actual enzymes by their power of reproduction.

In many fields ofbiology which were hitherto treated mainly descriptively, new and exact questions can be put by enzymochemical thought processes and methods.

As a first example I will take the *science of adaptation.*

When the individual adapts himself to changes in nutritional conditions, changes in temperature, and so on, the first measure of the organism is to develop the enzyme system necessary and appropriate to the new conditions. Under normal conditions - as we have been able to show in the case of yeast - this is surprisingly constant, but under certain culture conditions enzymes, for example saccharase, may develop to ten times the normal amount.

I think that the *sciences of immunity* and *heredity* are in particular the fields in which we may expect new discoveries by the application of enzyme chemistry.

With regard to immunity research, the analogies found between the enzyme activator-substrate system on the one hand and the complement-amboceptor-substrate system on the other hand are all the more obvious. Since purification and characterization of the enzymes and activators have provided the preconditions to more detailed study of enzymatic processes, there is even reason to begin the study of antigens and antibodies by purification and concentration.

Heretofore it has not been possible to bring into contact with chemical facts the extremely interesting results of genetics, which have led to a very exact but purely formal determination of hereditary units (genes). Although many years of work in many fields will be required to obtain a link between genetics and biochemistry, no geneticist will have any doubts concerning the need for such work. Our researches are aimed at finding the lines of development of chemical substances in mutants subject to Mendel's law. Independently of current hypotheses (R. Goldschmidt *inter alia),* a search should be made into the origin of such lines of development in substances of an enzymoid nature.

Investigation of the enzymes and biocatalysts means a step forward to an understanding of Life on the chemical and physical basis. But although an accurate knowledge of the chemical processes of life is a precondition to an understanding of life phenomena, and although the laws discovered for in-

animate matter must apply fully and strictly to living matter as well, I nevertheless think that it is rash to assert that our laws concerning transmutation of matter and the conversion of energy are sufficient to describe the life phenomena.

The more we are able to follow and see through the chemical and physical phenomena whereby organisms maintain their individual life in the face of external influences and preserve the race by reproduction, the more we can hope to advance to the knowledge of the great principle which together with the laws of conservation of matter and energy, and of the decrease in free energy, govern organic life. The knowledge of this principle and its clear formulation is one of the highest scientific purposes of natural science, biology and biochemistry in the first instance.

I must express my sincere and respectful thanks to the Royal Academy of Sciences of Sweden for the extended possibilities afforded to myself by the Nobel Prize to continue my biochemical research.

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