FARADAY LECTURE

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Problems and Methods in Enzyme Research.

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NEARLY sixty years have elapsed since the death of Faraday. I count it a unique distinction that I may to-day address the Chemical Society in memory of this great pioneer, in the room so closely associated with his labours. I am conscious that it is a special honour to be allowed to join that small band of scientists from various lands—there have so far been twelve—who have spoken in remembrance of Faraday. In these memorial lectures the history of science is reflected, which, in the image of Goethe, may be compared to a great fugue in which the voices of the nations are heard in succession. My reverence for the genius to whom we pay homage is coupled with veneration for the memory of the many other great investigators whom your country has produced. The presence of so many distinguished colleagues in this room augments my sense of the importance of the present occasion, but my thoughts nevertheless travel beyond the living, to the immortals.

My predecessors have mostly selected single chapters of Faraday's lifework as a starting point for a discourse on experimental contributions or theoretical discussions in the realms of physics and chemistry. Faraday's work provides a store of stimulating suggestions, one which is neither exhausted nor likely to become so for a long time to come. Since none of my predecessors has referred to Faraday's experiments "on the power of metals and other solids to induce combination of gaseous bodies," I should like to take this early fundamental investigation on catalysis as my starting point in a discussion of organic catalysts.

This investigation was carried out in the year 1833, at the time, therefore, of E. Mitscherlich's work on the formation of ether from alcohol by contact with sulphuric acid. Faraday and Mitscherlich recognised the frequency of such reactions. Mitscherlich called them reactions through contact, and compared ether formation to the decomposition of hydrogen peroxide, the alcoholic fermentation of sugars, the formation of acetic acid from alcohol, and the decomposition of urea ("Für sich erleiden diese Substanzen keine Veränderung, aber durch den Zusatz einer sehr geringen Menge Ferment, welches dabei die Kontaktsubstanz ist, und bei einer bestimmten Temperatur, findet diese sogleich statt." . . . By themselves these

substances do not undergo any change, but they decompose at once at a given temperature on addition of a very small quantity of a ferment which is the contact substance).

These investigations were discussed by Berzelius in his 15th annual report on the progress of the physical sciences. He collected a number of known cases of inorganic and organic contact actions, and grouped them for the first time under the definition of "Catalysis." Although he saw in catalysis "a new force causing chemical action, present both in organised and in unorganised nature," he was far from rejecting the idea "that it should be considered an action independent of the electro-chemical relationships of matter."

The inorganic contact substances had, in connexion with the earliest observations on catalysis, already been compared with natural ferments, and when, towards the end of the century, W. Ostwald gave new life to research by his definition of catalysis, his suggestion of kinetic measurements led to the stimulation of enzyme research by the methods of physical chemistry. The parallelism between enzymes and artificial inorganic catalysts was particularly emphasised by G. Bredig through his experiments with inorganic ferments, which revealed a manifold and far-reaching agreement between the two kinds of catalysts. Conversely, observations on the behaviour and the nature of enzymes promise new knowledge of the general theory of catalysis. My own experiments and those of my pupils, of which it is my privilege to speak to-day, deal with the adsorption relationships and some other properties of enzymes, more particularly their specificity. With the great example of Faraday before me, I venture to bring forward these modest contributions, encouraged by the deep interest which Faraday evinced in the nature of the relationship between catalyst and substrate. The expectation does not appear altogether unjustified that some new results with enzymes, pointing as they do to a close relationship between the phenomena of adsorption determined by affinity and those of catalytic action, may contribute to a more general knowledge of the selective action of chemical adsorption and of the specificity of catalysts. In their strict specificity, and also in their great potency within a narrow range of reaction conditions, the enzymes are superior to the long-known inorganic contact substances. The modern development of catalysis in chemical industry has emphasised the importance of the problem of improving inorganic catalysts on the pattern of the enzymes so as to make them more selective and at the same time more active. Moreover the activation of enzymes is being imitated with much success. These phenomena of activation and also those of catalyst poisoning, being susceptible of quantitative measurement, have of late led to

remarkable information concerning the nature of catalysts, for example, in the researches of C. N. Hinshelwood, W. G. Palmer, F. H. Constable, and H. S. Taylor.

Faraday examined the action of platinum on a mixture of hydrogen and oxygen. He rejected the idea that a contact substance enters into a chemical union. It is, however, not quite correct, as has often been done, to quote his explanation of the process as being a purely physical one. Faraday spoke of " that attractive force, possessed by many bodies in an eminent degree, and probably belonging to all, by which they are drawn into association more or less close, without at the same time undergoing chemical combination . . . and which occasionally leads, under very favourable circumstances . . . to the combination of bodies simultaneously subjected to this attraction. I am prepared myself to admit . . . both with respect to the attraction of aggregation and of chemical affinity, that the sphere of action of particles extends beyond those other particles with which they are immediately and evidently in union, and in many cases produces effects rising into considerable importance."

In Faraday's view, catalysis and adsorption are related. As unequivocal examples show nowadays, many adsorption phenomena depend on chemical affinity and more especially on the residual affinity of the molecules. The attempt to explain adsorption by means of capillarity breaks down in the case of selective adsorption, as observations on the selective behaviour of adsorbents towards enzymes show.

The behaviour of hydrogen and oxygen towards platinum has ever remained an important problem since the above-mentioned fundamental investigation by Faraday. As I was able to show a few years ago, in conjunction with E. Waldschmidt-Leitz, platinum is capable of transferring hydrogen catalytically only in the continued presence of oxygen. There are various views with regard to this rôle of oxygen in hydrogenation catalysis. Whilst, on the one hand, M. Bodenstein considers the oxygen merely of importance for the renovation, that is, the detoxication, of the platinum surface, R. Kuhn, on the other hand, has developed the view that the oxygen poisons the platinum to some extent, and that without its co-operation only recombined hydrogen molecules would encounter the substrate on the platinum surface. This view makes the oxygen content responsible for the variety of the recombination stages of the hydrogen at the platinum surface. It should, however, be borne in mind that even the course of the reaction in hydrogenation, for instance of naphthalene (according to R. Willstätter and F. Seitz), may vary according to the extent to which the platinum is charged with oxygen. I consider it, therefore, more probable that the

affinity fields of the platinum are so changed by the taking up of oxygen that a new contact substance results, specifically adapted to the hydrogenation. The greater or smaller extent of the oxygen charge would then produce definite peculiarities in the affinity. Platinum charged with oxygen must be ranged among the mixed catalysts, and may be compared with the enzyme–activator complexes. To my mind the experiments indicate that, according to the nature of the contact substance, the naphthalene is variously activated and thereupon hydrogenated to the different stages that are observed. The existence of some relationship between the contact substance and the naphthalene may be inferred from the fact that with platinum *cis*-decahydronaphthalene is exclusively produced; with nickel, on the other hand, the *trans*-isomeride is the chief product of the hydrogenation.

Berzelius relinquished any attempt to explain the processes of catalysis, and emphasised in his discussions with Liebig the danger of explaining incompletely understood phenomena by means of hypothetical assumptions. To-day this reserve is still maintained and approved by many; the absence of an explanation, however, has limited the interest in the phenomena. There is no single method of scientific investigation which can be described as the right one in all circumstances. A hypothesis may vary in value according to the mentality and the temperament of the investigator and according to its utility in suggesting and co-ordinating new observations. A hypothetical explanation of incompletely understood phenomena is often a necessary condition of scientific progress.

An elaboration of an incomplete attempt at explanation by Liebig may perhaps be seen in the hypothesis of C. Nägeli (1879), who explained ferment reactions by the transference to the substrates of " states of motion," that is, of "vibrations of the atoms and particularly of atomic groups." His view seems to me to have been improved by J. Böeseken and to have been further developed, in accordance with modern conceptions, by the consideration of electron orbits instead of atomic vibrations.

The definition of W. Ostwald (1894), which gave a fresh stimulus to research, is as follows : " Katalyse ist die Beschleunigung eines langsam verlaufenden chemischen Vorgangs durch die Gegenwart eines fremden Stoffes " (Catalysis is the acceleration of a slow chemical process brought about by the presence of a foreign substance). Ostwald considered the principle that the catalyst changes the reaction velocity only, to be the key to the interpretation of catalytic phenomena. As he himself stated, the new point of view which was of most value to research was the conception that a catalyst or enzyme cannot bring about a reaction which does not

occur without its co-operation. This conception still holds the field, although it is true that several investigators (J. J. Thomson, H. E. Armstrong) did not agree with it. This principle, which has been deduced from thermodynamical considerations, should not, however, be maintained too dogmatically. At the present day it seems to me to have little value and to hamper progress. Like a dogma, it hinders us in any attempt to find a new explanation of the phenomena of catalysis without intermediate compounds and of the causation of reaction without an influx of energy.

The catalyst may function in degrees of association with the substrate, varying from *fixation* to *approach*. It can, naturally without extraneous energy, bring about a change of constitution in the substrate molecule, and it is only through this constitutional change that reaction may be induced. Here it is not an essential, nor even a general condition, that the molecule should change to a new compound capable of independent existence, as, for instance, to an isomeride such as the so-called γ-glucose (with a changed oxygen bridge) which has been postulated as an intermediate in the action of F. G. Banting and C. H. Best's insulin on glucose. In general the catalyst only requires to bring about a change in the molecule such as was first suggested intuitively by F. Raschig (1906), in advance of exact interpretation. F. Haber (1922) considers the heterogeneous catalysis of gas reactions to be a process "in which the first phase . . . is apparently represented by an electrodynamic distortion of the molecules by the atomic fields at the interface between the solid contact substance and the gas." M. Bodenstein also bases an explanation of the activation of hydrogen by platinum on the deformation of the molecules.

Our new views concerning the structure of atoms and molecules permit us to characterise the hypothesis of the deformation of molecules somewhat more fully as the deformation of their electron orbits. Making ourselves more independent of the hypothetical formulation of discrete orbits, we may, in accordance with E. Schrödinger's wave mechanics, conceive the action of the catalyst on the substrate, through fixation or approach, to be such that the continuous distributions of the electrical charges of the catalyst and the substrate mutually influence each other. A superposition of the fields of charge associated with catalyst and substrate may amount to the same thing as the presence of points of greater condensation of the charge.

The immense store of facts comprehended in " catalysis " contains examples of so diverse a nature that it would be futile to attempt to explain all the phenomena by means of a single hypothesis. The Ostwald school has proved the existence of intermediate reactions

in important cases. The explanation is derived from A. de la Rive's assumption (1846) of a platinum–oxygen compound and from the much older investigation by N. Clément and Ch. B. Désormes of the action of oxides of nitrogen on sulphurous acid. In the case of homogeneous systems this explanation has proved so fruitful that E. Abel declared : " nicht Stoffe, nur Reaktionen katalysieren " (Only reactions may catalyse, substances do not). It is a weakness in Böeseken's views that they do not contemplate this kind of catalytic reaction. Although many catalytic phenomena may be explained without postulating the formation of stoicheiometrically constituted intermediate products, and although a theory of affinity modification without the production of intermediate stable compounds has its uses, such a theory can nevertheless not lay claim to universal validity. In enzyme chemistry there is no theory which appears to be so fruitful and so satisfactory as that assuming the existence of intermediate compounds between catalyst and substrate. Accordingly this is the specifically chemical conception of the primary process of enzyme action, in contradistinction to the physical one, the colloidal–chemical adsorption process suggested by W. M. Bayliss, G. S. Hedin, and J. M. Nelson.

According to Böeseken a catalyst produces a very rapid change (" dislocation ") of the links which it activates by means of a " temporary, very unstable addition to the specific links of the molecules " (" action orientatrice "), and then, without forming a true compound, transfers peculiarities of its own " open system " by induction to the closed system of the substrate (" action dislocante "). By way of explanation it is represented that the catalyst changes the electron orbits of the substrate. According to N. Bohr's view this deformation of an electron orbit would occur through the action of a force, and would therefore involve a change in the energy of the electron. The assumption of an energy change might be avoided in the case of the superposition of two continuous fields of charge. Thus no change in the energy level or in the distributions of charges would occur.

One of the most general questions on the subject of catalysis is concerned with the chemical nature of the catalysts. The point has hitherto been somewhat neglected, but the above-mentioned observation on hydrogenation catalysis by platinum suggests that our knowledge of the chemical composition, even of simple inorganic catalysts, is still very incomplete. There are many non-specific catalytic actions, especially cases of hydrolysis which are catalysed by acids and alkalis. Thus the non-specific catalytic activity of hydrogen ions may bring about the fission of proteins and their degradation products, of carbohydrates, whether composed of many

or only a few monose-units, and of the fats and other esters. The need for selective and highly active inorganic catalysts has in the last seventeen years led to remarkable results in directing the course of reactions by means of mixed catalysts. These results, possessing great practical value as well as theoretical interest, are largely due to the investigations of A. Mittasch and others in the laboratory of the Badische Anilin- und Soda-Fabrik.

A fundamental conclusion reached by Mittasch is "that the catalytic action of iron becomes both more intense and more permanent on the admixture of numerous metals and oxides." In the catalytic oxidation of ammonia, ferric oxide to which bismuth oxide has been added gives, according to Mittasch, quite as good results as an efficient platinum catalyst. Yet another example among many: pure zinc oxide acts on a mixture of carbon monoxide and hydrogen under pressure in the direction of methanol formation; if a small quantity of iron is added, the reaction is diverted towards the formation of hydrocarbons. The oxidation of carbon monoxide by means of air at the ordinary temperature was made possible by the use of certain mixtures of the oxides of the heavy metals, for example, those of manganese, copper, cobalt, and silver (hopcalite), in the experiments of A. B. Lamb, W. C. Bray, and J. W. C. Frazer. These mixed catalysts have close analogues among the enzyme systems; trypsin differs from trypsinkinase, and papain from papain–hydrogen cyanide, not only in potency, but also in specificity.

It is not enough to assume that the admixture to the simple catalysts merely increases the frequency with which the catalytically active atoms project from the lattice. This effect is produced by only a small category of added substances, the carriers. It is rather that catalysts composed of two or more substances are so clearly characterised by specific actions, or deflection of the reaction, that the status of new substances must be ascribed to them. Hitherto it has been generally accepted as a principle in chemistry that the chemical properties of the constituents are lost in compounds, but are preserved in mixtures. This principle has ceased to be valid since more delicate chemical means have become available for the detection and measurement of chemical affinities. Observations on platinum containing oxygen, on the activated catalysts or mixed catalysts of Mittasch and his co-workers, as well as on activated enzymes, have convinced me of the validity of the statement that *mixtures may have the nature of new chemical compounds*. The similarity between mixtures and compounds may be explained by considering that the electrostatic and the electromagnetic fields of force of the individual components of an intimate

mixture may exert a mutual influence on each other. Thus a new kind of affinity field would result. The same explanation as that already brought forward for the action of a catalyst on the substrate, be it a deformation of electron orbits or a superposition of fields of charge, may be adduced. Thus it becomes intelligible that, by mixing two or more components, individual catalysts may result having an enzyme-like specificity and potency comparable with that of homogeneous compounds; whereas the comparatively simply constituted fields of affinity of the elementary atoms do not suffice for this purpose. The very narrowest range of specificity is a peculiar property of the organic catalysts of high molecular weight produced by the living cell. There appears to be a deep gulf between the inorganic catalysts and the enzymes, the chemical constitution of which is still quite obscure. In reality this gulf has already been bridged by accurately defined organic compounds which act like enzymes.

Chlorophyll may be considered one of the organic catalysts; I have refrained from calling it an enzyme, as this would have been a mere empty phrase. It is now, however, becoming useful to recognise connecting links between enzymes on the one hand and catalysts, accurately defined chemically, on the other. Colloidal chlorophyll forms a dissociating additive complex with carbon dioxide. In conjunction with A. Stoll I have suggested as an explanation of photosynthesis that the adsorbed radiation produces a molecular rearrangement of the carbon dioxide. An isomeric peroxide form of the latter would then decompose into oxygen and hydrated carbon.

Similarly oxyhæmoglobin partakes of the nature of enzymes. Recently I have shown, in conjunction with A. Pollinger, that the oxyhæmoglobins of various animal species act exactly like peroxydases of an unequal potency. Under similar conditions 1 mg. of the oxyhæmoglobin from horse blood, ox blood, and pig's blood furnishes respectively 0·152, 0·114, and 0·093 mg. of purpurogallin in the oxidation of pyrogallol. The oxyhæmoglobins do indeed correspond to peroxidative enzymes of low potency, as they are about 10,000—30,000 times weaker than our best preparations of vegetable peroxydases. We attribute the difference in potency of the individual hæmoglobins to the fact that the same specifically active (prosthetic) group is influenced in its action by association with the globin molecules, which in the various varieties of blood differ in their constitution. The peroxidative reaction of the iron-containing pigment group which is united to various globin complexes is therefore subject to modification, so that the hydrogen peroxide adsorbed by the individual oxyhæmoglobins is activated in a varying degree.

Hence differences in the constitution of the colloidal carrier of the active group result in differences in the potency of this group. This may show how the differentiation of the enzymes, the so-called species specificity, may be determined by variations in the colloidal complex.

The dependence of catalytic activity on chemical constitution has recently been further investigated in the Munich laboratory by R. Kuhn and L. Brann in quantitative experiments with hæmochrome; they used as examples enzyme-like catalysts with accurately defined differences in their chemical constitution. If hæmoglobin is broken down to hæmin, the peroxydase action is enfeebled and the new form of organically bound iron shows a considerable catalase activity which was previously lacking. According to a letter from Prof. R. Kuhn, the activity of hæmin, functioning as catalase, may, under the best conditions, be related to that of G. Bredig's colloidal platinum as 1 : 1·5, and to that of the best preparations of liver catalase, purified by S. Hennichs's adsorption process, as 1 : 1000. In mesohæmin, which is richer by two atoms of hydrogen, the dependence on the p_H of the peroxidative action is considerably changed (optimum 6·5, instead of 5) and the catalase action is lacking. We see, therefore, that the catalytic action is subject to considerable variation without change in the iron content and without the possibility of attributing the variation to changes in the degree of dispersion.

Gradations in colloidal dispersion cannot provide an explanation of the highly selective and specific affinities of enzymes. A few years ago it still seemed doubtful whether enzymic actions depend on peculiar conditions in the dispersion of any known substances, or whether they depend on definite organic compounds of unknown constitution. Our views on this fundamental question have been clarified more especially by the three following sets of observations:

(1) In some cases chemical analysis had caused enzyme action to be ascribed to known organic substances or types. The enzymes which had served for the analyses were, however, very impure; they contained only a few units per cent. or even a fraction of a unit per cent. of the purified enzymes available to-day. According to A. Fodor, the saccharase of yeast was supposed to be substantially identical with a carbohydrate yeast-gum, but now the enzyme has been wholly freed from yeast-gum without change of activity. More frequently and with more reason relationships have been assumed between enzymes and proteins. Thus E. Fischer, in his Faraday lecture, deduced from observations then available, indeed with a certain degree of probability, that the enzymes "are derived from proteins and possess a protein-like character." In a variety of cases it has, however, been possible to purify enzymes to

such an extent that the protein reactions disappear completely (lipase, peroxydase, saccharase).

(2) It results from recent observations by H. Kraut and myself that simple inorganic colloids, showing peculiarities which were likewise thought to be attributable to dispersion, actually owe their specific properties to the individual chemical structure of their molecules. The gels of alumina, ferric oxide, stannic acid, etc., do not consist of metallic oxides with various quantities of adsorbed water, but there are numerous metallic hydroxides with various amounts of water in chemical combination.

(3) In making pure preparations of enzymes from the living cell, they are subjected to great changes in dispersion in the passage from the cells into solution, then into adsorbates, and again into solution; mixtures of enzymes could be separated into their components. Yet in many such cases the enzymic action was preserved almost quantitatively.

The conception of the enzymes as peculiar organic compounds suggests the problem of isolating them in a state of purity. The task of increasing the concentration of the enzymes has progressed so far that they may be more accurately characterised. For we can now distinguish between the properties of the enzymes themselves and the influence of accompanying substances, some of which form with the enzymes naturally occurring, physiological complexes, whilst others become accidentally associated with them during the isolation from the cells. In order to increase the concentrations of the enzymes, which are very low *in situ* or in the crude extracts, it is essential to measure each step in the isolation and purification so far as may be possible by quantitative estimations of the relative activities of the preparations. The literature of the simplest inorganic contact substances seems as yet to be poor in such observations. When, for instance, a metallic catalyst is obtained by reduction of its oxide, it is customary to be satisfied with the conversion of an unknown small fraction of the atoms of the crystal structure into the condition in which they are available for catalysis; that is, into a condition of high energy, of smallest saturation by the other atoms of the crystal lattice. The ratio between the catalytically active atoms and the total number in the case of iron catalysts was determined last year by J. A. Almquist in experiments on poisoning by oxygen. The preparation of an inorganic catalyst should aim at increasing the efficiency of unit weight as far as possible. In the isolation of an enzyme we aim at diminishing as far as possible the mass of catalyst necessary for a given action under definite conditions.

The very labile and very soluble enzymes must be separated from

many times their weight of accompanying substances such as proteins, carbohydrates, and salts, to which they are apparently united by adsorption, and this separation must be effected without the use of the ordinary chemical means, such as conversion into salts or other derivatives. The general method which has been adopted in the investigations of recent years consists in the application of the processes of adsorption by so-called surface-active substances, such as alumina, kaolin, lead phosphate, tristearin, etc. Adsorption is made so selective that not only are the enzymes largely freed from foreign admixtures, but many are also separated from the accessory substances or activators, with which they form complexes.

The application of adsorption processes to the purification of enzymes goes back far into the past. It does not, however, seem to have been well known, or it has been considered doubtful or improbable, that the enzyme could actually be freed from the adsorbates by a process of elution. The small amount of active affinity by which the enzymes are bound in the adsorbates may in general be overcome by very gentle chemical means, for example, by very dilute solutions of alkalis or alkaline phosphates. In this case we do indeed often observe, for instance with lipase, that the elution of an enzyme which takes place quite easily and in good yield in the early adsorption processes becomes more and more difficult as purification proceeds, giving smaller and smaller yields until finally these means fail to decompose the adsorbates. At lower degrees of purity the adsorbents generally also bind certain substances which accompany the enzymes as adsorption complexes; these substances are able to co-operate in bringing about elution as well as adsorption, and sometimes this is the only mechanism for effecting these processes (co-adsorbents and co-eluents).

The oldest reference to the adsorption of enzymes which I have been able to find is the isolation of pepsin by A. Vogel (Munich, 1842). Pepsin was precipitated together with proteins by means of lead acetate, and again passed into solution when the precipitate was treated with hydrogen sulphide. One of the oldest references of this kind is the investigation by E. Brücke (1861), who was able to bind pepsin " mechanically to small solid bodies " such as calcium phosphate, sulphur, or cholesterol and to free it again from the adsorbates. Even in those days, particularly in the following year, A. Danilewsky and J. Cohnheim attempted in Kühne's laboratory to separate enzymes, more particularly the components of the pancreatic mixture, by means of adsorption processes. Differentiation of this kind was met with much later by S. G. Hedin, who found that two proteases present in the spleen behaved differently during adsorption by kieselguhr.

The adsorptive method, which had almost fallen into oblivion, was revived some twenty years ago in an investigation of L. Michaelis and M. Ehrenreich. In using adsorbents of such a kind that they have under all conditions a definitely electropositive or electronegative charge, it appeared as if the opposite charge on the enzymes, whether basic or acid in nature, determined their adsorption. Thus saccharase was believed to be an acid and trypsin to be amphoteric. It has been found, however, that these results are not valid for the enzymes themselves, but only for arbitrary aggregates of foreign substances which happen to be present in the impure solutions. Thus saccharase, which was not supposed to be adsorbed by electronegative kaolin, is readily adsorbed by this substance after the first few stages of purification, or even at once from autolysates of yeast prepared by an improved method. In many cases the true adsorption behaviour of an enzyme only appears clearly in the course of its purification. Pancreatic amylase, for example, may be adsorbed by alumina under certain conditions, as long as it is in the crude state. The purification of the enzyme can be effected by preliminary adsorption, that is, by removal of a portion through fractional adsorption, but its behaviour then changes so that it is no longer appreciably adsorbed under any conditions by alumina gels of excellent adsorptive power. The adsorption of enzymes is determined by chemical peculiarities which cannot be predicted.

Whilst amylase escapes adsorption, peroxydase provides a case of an enzyme being adsorbed not, indeed, from aqueous solution, but from one containing alcohol. The acidity of the solution commonly has an influence on the adsorption. Papain is very weakly adsorbed from an acid solution, better from a neutral one, still better from a weakly alkaline one, and in this case also much more readily from a solution containing alcohol; it is eluted from the adsorbent by weak acid. Experiments show that these adsorption processes are not at all governed by the capillarity of the substances whose surfaces are strongly developed, nor is adsorption in any way determined by such gross differences as the acid and basic nature of the enzyme and of the adsorbents. Among the various aluminium hydroxides the meta-compound (AlO_2H), which does not appreciably react either with concentrated hydrochloric acid or with sodium hydroxide, is distinguished by the most selective adsorptive action.

The success of the preparative application depends on whether it is possible to make the adsorption sufficiently selective. The smaller the quantity of adsorbent which is required, the higher is in general the degree of purity in the adsorbate. It was therefore necessary to follow the adsorption by quantitative measurements which determined the number of enzyme units taken up by 1 gram

of an active adsorbent (adsorption value). In our first investigation of saccharase we obtained an adsorption value of 0·15 for alumina by improving the autolysates of yeast, by preparing suitable kinds of alumina, and by the choice of conditions favourable to adsorption. Later this value was increased twelve-hundred fold, to 200, by the proteolytic degradation of substances accompanying the enzymes, by using suitable conditions of dilution and acidity, by repetition and by fractionation. Thus the enzyme from 14 kilos. of fresh brewers' yeast was adsorbed by 1 gram of aluminium oxide and the adsorbate weighed 2·5 grams.

A more accurate insight into the course of adsorption is furnished by the adsorption isotherm according to H. Freundlich, which expresses the relationship between the quantities of enzyme in the adsorbate and in the residual solution. Since in the case under review we are not concerned with homogeneous substances, but with complicated mixtures, the adsorbed quantities depend very greatly on the initial concentrations. In the case of saccharase the use of very dilute solutions brings about a great improvement in the selective action and in the purity of preparations. Adsorption curves also show in which direction in a given case fractionation should be applied, whether the degree of purity will be improved by removal of the first or of the last fraction. Thus fractionations were controlled by the quantitative studies of H. Kraut on the adsorption behaviour of mixtures. The object was to obtain normal adsorption isotherms such as are given by homogeneous substances, and this has been attained in the case of certain enzyme preparations (saccharase). Once this is attained, the same adsorption process does not, of course, bring about any further improvement. Yet the preparations so obtained were found to be far from homogeneous. The observed adsorption curves are not those of the enzymes themselves, but of enzyme-containing complexes. The adsorption method reaches a limit of utility with certain aggregates in which the enzymes and substances most nearly related to them are firmly joined by adsorption to accompanying substances of different kinds. The proof that the constantly adsorbed enzymes are only apparently single substances follows from qualitative and quantitative analysis. If a given constituent can be tested for by colour reactions or by precipitation, it must be possible either to accumulate it or to get rid of it completely. For instance, my first investigation (with A. Stoll) on vegetable peroxydase led to preparations of which sugar groups formed a considerable part. They showed an important iron content (0·5%) which seemed to be approximately proportional to the peroxidative action. Starting from this condition, the degree of purity could be raised about

fivefold by adsorption methods : meanwhile the carbohydrate constituents were eliminated and the iron content fell to one-seventh (0·06%). Hence iron does not enter into the constitution of this oxidative ferment. At the degree of purity ultimately attained, a new property of the enzyme appeared, which could not be recognised at an earlier stage : the enzyme had a reddish-brown colour resembling that of porphyrins.

Phosphorus seemed to be an essential constituent of saccharase, for its quantity amounted to 0·16—0·19% for saccharase of saccharase value 0·1—0·15 (H. von Euler and O. Svanberg). A fractionation was then carried out by means of adsorption on lead precipitates in such a way that the enzyme was isolated from the fractions containing least phosphorus. While the saccharase value rose to 2 and further to 5, the phosphorus content fell to 0·02 and even to 0·006%. The change in the ratio phosphorus : activity shows that this constituent is of no significance.

There is no sure method available for freeing the enzymes from the protein derivatives. However, if a certain enzyme in isolated cases could be freed quantitatively from accompanying proteins, this might be considered a sufficient demonstration that this enzyme neither is a protein nor requires protein for its activity. Nevertheless the tenacity with which proteins cling to enzymes has threatened again and again to impose the conclusion that the enzymes are of a protein character. Progress in the technique of methods of separation has been achieved mainly with yeast saccharase. Autolysates of yeast and the enzyme preparations made from them always gave the strong Millon reaction of tyrosine peptides, about as strongly as egg albumin. But the protein fission products responsible for this reaction only accompany the enzymes in the course of the proteolytic degradation to which the yeast plasma is subjected after destruction of the cell. It was therefore only necessary to carry out a cautious autolysis of the yeast at neutrality and to avoid the proteolytic processes in the isolation of the enzyme, in order to free saccharase completely from the substance giving the Millon reaction. On the other hand, the enzyme preparations which have been obtained in this gentle fashion are characterised by a striking tryptophan content which was discovered by H. von Euler and K. Josephson, who attributed to it a real importance as regards the composition and the function of saccharase. Actually it has been found impossible to free saccharase entirely from tryptophan by any systematic process of adsorption, but it was nevertheless possible in single cases. And my purest preparations of this enzyme show only about $\frac{1}{4}$ to $\frac{1}{8}$ of the tryptophan content found in the preceding research (saccharase values 8·4, 11·9, and 9·7 with 0·12, 0·16, and

0·18 mg. of tryptophan per unit). The crude material, the autolysate of yeast, can, however, be obtained in such a way that after dialysis it contains much less of this amino-acid than was considered to be characteristic of the pure enzyme. The quantity of tryptophan for different saccharase units is 150 mg. in the case of ordinary yeast and 70 mg. in the autolysates formerly employed. By increasing the enzyme content of the yeast itself and by improvements in the autolysis, the quantity of tryptophan corresponding to one unit was lowered to 0·23 mg. in the case of an autolysate after prolonged dialysis. Saccharase could therefore be freed almost completely from various chemically recognisable substances of rather high molecular weight, such as carbohydrates, phosphorus compounds, and protein substances, and this without loss of activity, even without loss of stability.

By the adsorption processes the saccharase is concentrated 3000—4000 fold as compared with dry brewers' yeast. The enzymic concentration of the purest preparations corresponds to saccharase values of 11—12 obtained from the yeast autolysates of a saccharase value equal to $\frac{1}{150}$. After this degree of purification had been reached, it seemed desirable to increase the purity of the yeast itself and of its autolysates, and to test whether a greater increase of the enzymic concentration could thereby be achieved. It is possible to make yeast richer in enzymes. H. von Euler and also J. Meisenheimer had increased the saccharase content of yeast considerably by prolonged intensive fermentation. Their results were soon greatly improved upon by conducting the fermentation with very low concentrations of sugar so that the saccharase content was increased ten to fifteen times, while other enzymes, such as maltase, trypsin, and the members of the zymase complexes, suffered only a slight increase or none at all. The importance of this process therefore consists in increasing the ratio of the saccharase to those other enzymes from which it may be separated only with the greatest difficulty. Improvements were also made in the method of dissolving the enzyme, and in this connexion it was necessary to reach a better understanding of the processes which take place on the passage of an enzyme from the yeast cell into aqueous solution. The cell begins to give enzyme to the water only after its death. The liberation of a sucroclastic enzyme is itself an enzymic process and indeed a definite stage in the entire enzymic degradation of protoplasm. The liberation of the saccharase and of the maltase must therefore be separated as far as feasible from the whole process of yeast autolysis. It was possible to make these two enzymes pass quantitatively into aqueous solution in the course of a single day, accompanied by not more than one-tenth or even only one-twentieth

of the substance of the yeast. The crude solutions attain saccharase values of about 1, and the dialysed solutions values of about 2·5; whereas a few years ago the purest known preparations corresponded approximately to the value 0·3. By means of the adsorption method the autolysates, obtained with the greatest possible precaution, now readily yield the enzyme, but not in a greater state of purity than the earlier autolysates, which were 100—375 times weaker. The enzymic concentrations are on the whole even a little lower, but the stability of the enzyme is greater. Here, indeed, the natural enzyme complexes seem to have been isolated by gentle means. In energetic autolysis, followed by a proteolytic process, the mass of these complexes becomes less and they become less stable. It seems that we must consider an enzyme to be composed of a specifically active group and a colloidal carrier. To this, other substances of high molecular weight cling in various ways. The colloidal carrier seems to vary somewhat in its nature, but to be necessary for the stability of the active group.

In the whole course of enzyme isolation the adsorption method has so far only served to separate the enzymes from substances which are not closely related to them. In every case the components that are most tenaciously retained are those substances which are most closely related to the enzyme in colloidal and chemical properties, for instance, its transformation products, which differ from it only by the absence of the active specific group. In this connexion we attribute importance to a process of fractional adsorption, that is, adsorption on a very finely divided precipitate, for instance, of lead phosphate, produced in the enzyme solution itself. The highest degree of enzymic purity yet attained has been reached by a fractional formation of such an adsorptive precipitate. By this means it has also become possible for the first time to separate an enzyme from the products of its inactivation, when a loss of activity has occurred through keeping or warming a solution; the more easily adsorbable portions contain the inactive, and the more difficultly adsorbable ones the active, fraction of the enzyme.

The most important and theoretically interesting problems of the adsorption method are presented by (1) the complexes of enzymes with their activators and their retarders, and (2) the natural mixtures of enzymes.

The pancreatic mixture of lipase, trypsin, and amylase offers the first example of the separation of enzymes by means of adsorption. Lipase is most readily adsorbed; it can be removed from the mixture by means of alumina and eluted from the adsorbate by means of weakly alkaline phosphate solution. Amylase and trypsin remain in solution. It should be borne in mind, however, that this

process requires the use of a particular kind of alumina (γ-alumina). Another kind (β-alumina) readily adsorbs the trypsin as well. Pancreatic amylase is the least adsorbable, in a pure condition scarcely at all by ordinary means. For solutions which are still impure, kaolin is suitable for the complete adsorption of trypsin, leaving amylase behind. This investigation was continued by E. Waldschmidt-Leitz in my laboratory, and he succeeded in completely resolving the proteolytic systems of the pancreas and the intestine. In consequence of the detailed experiments of W. M. Bayliss and E. H. Starling, trypsin was supposed to be secreted by the gland in the form of an inactive precursor (trypsinogen) and changed into trypsin itself by a second enzyme, enterokinase, which accompanies it. Enterokinase is, however, no enzyme, but a specific activator of trypsin. This kinase may be separated from trypsin by adsorption with alumina, even after the latter has been activated. What was considered to be trypsinogen is a true enzyme which even in the absence of its kinase has a specific action on peptones, histones, and certain protamines. Activation by enterokinase increases the activity and extends it to more resistant substances such as fibrin, gelatin, casein, etc. Trypsin and the trypsin–enterokinase complexes prove to be two proteases differing in specificity and complementary to each other. The best means of separating trypsin and enterokinase depends, according to E. Waldschmidt-Leitz and K. Linderström-Lang, on the adsorption of the activator by a precipitate of casein produced in the solution; the trypsin remains behind quantitatively. Since trypsin free from its activator and also enterokinase are both obtainable in a state of purity, the enzyme and the kinase of various animals may be compared and combined. E. Waldschmidt-Leitz found that the trypsin of one species is activated by the kinases of other animals in quite unequal degrees; for instance, the trypsin of the pig is activated much more strongly by the kinase of the cat than by the corresponding kinase of the pig.

Apart from kinase, pancreatic protease consists of two components, the second being erepsin, discovered by O. Cohnheim in the intestinal mucous membrane. Of the two proteolytic enzymes, only erepsin is readily adsorbed by alumina (γ), according to E. Waldschmidt-Leitz. On repetition of the adsorption several times, the separation becomes complete. In the same way we can separate, by means of adsorption on alumina, the two proteases of yeast, but in this case the tryptic component is the more readily adsorbable. It is only when the enzymes of a mixture have been thus prepared in a homogeneous state that it is possible to determine their specificity. Erepsin does not act on the higher proteins, but exclusively

on the simpler peptides—a point which was hitherto doubtful. The homogeneous proteolytic enzymes will in future research be the most suitable agents for the fractional enzymatic hydrolysis of proteins, in order to determine analytically the various stages in their degradation, and for preparative purposes.

Whilst the first-mentioned example was concerned with the three pancreatic enzymes, differing greatly in specificity and in properties, the last-mentioned applications of the method extend to similar closely related enzymes in which only small differences in adsorptive behaviour would be expected. The adsorption could therefore be made sufficiently selective to render possible the separation of enzymes acting on closely related substrates. This property, which cannot be explained at present, is also the only one which makes possible the separation of the enzymes that split sucrose and maltose respectively. The relation between the adsorptive actions of various alumina gels on solutions of a given enzyme, for instance, of saccharase, does not hold equally for any other enzyme, and is, for instance, quite different in the case of maltase. Thus there are varieties of alumina (β- and the meta-hydroxide) which are capable of taking up maltase comparatively readily from yeast autolysates, but saccharase only sparingly. In this way it is possible, even by a single application of the process, to obtain adsorbates, and from them again, by means of secondary alkali phosphate, solutions of enzymatically homogeneous maltase; at the same time the solutions of the saccharase are completely freed from maltase with little loss. The selective adsorption may be replaced by a process of selective elution from the adsorbates. By means of γ-alumina saccharase may be adsorbed together with maltase. Weakly alkaline or neutral phosphate solution then liberates both enzymes from the adsorbates, but a primary phosphate liberates saccharase completely and almost exclusively. The greater part of the maltase remains behind in a homogeneous condition and can be isolated by means of a secondary phosphate.

The adsorption of enzymes and their activators by various adsorbents is due to the qualitatively different affinities of single groups of atoms, and must be conceived to be similar to the action of an enzyme on its substrate. This dissimilar behaviour, utilised, for example, in the separation of saccharase from maltase, may, I believe, be attributed to differences in the chemical constitution of these enzymes and indeed to differences in their specific active groups.

In many cases there are indications as to which atomic groups of an enzyme are responsible for its union to an adsorbent. There are adsorbates of saccharase on alumina which under certain conditions hydrolyse sucrose just as rapidly as the saccharase contained in them

would in free solution. On the other hand, the enzymatic activity of pancreatic lipase is to a large extent destroyed in its adsorbates on tristearin or on cholesterol. This suggests a participation of the specifically active group in the adsorption process. The lipase may be liberated again in an active condition from its almost inactive adsorbates. According to recent observations of E. Waldschmidt-Leitz and K. Linderström-Lang, enterokinase is inactive in the alumina adsorbate; its trypsin-binding group is occupied. On the other hand, the alumina adsorbates of trypsin and of trypsin-kinase are quite active.

The most delicate gradations in adsorptive power are determined by the peculiar chemical differentiation of the gels used, particularly of alumina, and by their preparation in a homogeneous state. Until quite recently the view prevailed that the gels of alumina precipitated from salts consist solely of aluminium oxide with adsorbed water and that their ageing results in a diminution of dispersivity, accompanied by a diminution of their adsorptive power. It is found, however, that the power of adsorbing saccharase increases rather than diminishes with the ageing of an alumina precipitate. The greater age of the gels brings about, not changes in dispersivity, but changes in chemical constitution. Under certain conditions the first aluminium hydroxide (α) may be isolated as an unstable gel of the formula $Al(OH)_3$. It changes in the course of a few hours into a second modification (β) and then very slowly into a third stable gel (γ), likewise of the composition $Al(OH)_3$. By heating it with ammonia to 250° there results yet another gelatinous aluminium hydroxide, the molecule of which contains less water; it corresponds to the formula AlO_2H. The chemical differences between these colloidal precipitates are very great, but there is no simple relationship between their basic and acid properties and their adsorptive powers. Inorganic chemistry will find a large field for the investigation of structure among such gelatinous hydroxides.

One of the results which we owe to the adsorption method is a knowledge of the enzymes in a higher state of purity. Most of the properties which had been ascribed to enzymes depended on their degree of purity and may be simulated, or deceptively altered, by accompanying substances, or at least influenced by them. The behaviour of an enzyme is thus influenced partly by quite foreign substances, accidentally associated with it in the course of the isolation, and partly by companions of physiological importance which co-operate with the enzyme when it occurs naturally. The influence of the accompanying substances extends to the stability of the enzymes, to the temperature of their destruction, to the temperature of their optimal activity, to their behaviour on

adsorption, to their behaviour towards activators, inhibitors, and poisons, and even to the dependence of the reaction velocity on the hydrogen-ion concentration of the solution and on the concentration of the substrate.

With regard to both the control of the quantities of enzyme in preparative work and also a knowledge of the specificity of the enzymes, it is very important to remember that the comparison, by means of the reaction constant, of quantities of various enzyme preparations, of different origin and of different degrees of purity, may not be trustworthy. The relationship between the relative quantities of enzyme and the corresponding velocities may differ in various preparations of one and the same enzyme. And even when this relationship is constant, it is still uncertain whether equal velocities correspond to equal absolute quantities of enzyme in various solutions. In this respect some information is given by the dependence of the reaction velocity on the conditions of experiment. Since the fundamental investigations of S. P. L. Sörensen and of L. Michaelis and P. Rona, the dependence of the reaction velocity on the hydrogen-ion concentration of the enzyme solution has been recognised and taken into account. The measurement is usually carried out at the optimal p_H; a comparison is made between equal fractions (equal percentages) of the reaction velocities, maximal in relation to p_H. But even when there is a correspondence in the dependence on the p_H, the dependence of qualitatively similar enzyme preparations on the concentration of the substrate may still vary greatly as was shown in investigations made by R. Kuhn in my laboratory. At equal concentrations of the substrate, arbitrarily varying fractions of the maximal reaction velocity may be found. This varying dependence on p_s may be caused by inhibitors of the enzyme, accompanying substances which bind the specifically active group of the enzyme. The reaction velocities extrapolated for infinitely great substrate concentration then give a true measure of the quantities of enzyme. For this reason we propose reduced units of mass, for instance, for saccharases of different origin. The comparison of the velocities of fission at corresponding concentrations of the enzyme–substrate compound has made it possible to recognise more clearly the specificity of sugar- and glucoside-splitting ferments. Thus it has been ascertained that the hydrolysis of sucrose and of raffinose must be attributed to the same enzyme, saccharase (according to R. Kuhn); the hydrolysis of maltose and the various α-glucosides to the same maltase; and the hydrolysis of the various β-glucosides to the same β-glucosidase of emulsin. It may also happen that the accompanying substances do not attach themselves to the active group, but to other parts of the enzyme molecule, parts

which are not directly concerned with the binding of the substrate. In many such cases the influence of qualitatively and quantitatively varying companions may be overcome by arbitrary additions, that is, by compensatory activation or by compensatory retardation, so that the quantities of the enzyme may be deduced with sufficient accuracy from the reaction velocities (estimation of the lipases).

The dependence of enzymic activity on p_H and p_S may vary according to the degree of purity of the enzyme. An example of the variability of the p_H optimum is afforded by the lipase of the stomach. In the human stomach, lipase, in contradistinction to pancreatic lipase, has the optimum action in the acid region, at p_H 5—6; the same applies to the stomach of the dog. The activity–p_H curve and the position of the optimum are constant for the unpurified lipase of any species, but this constancy is not a property of the enzyme itself. If this lipase is purified by an adsorption process with kaolin, an accompanying substance, which inhibits in alkaline solution, is removed, and apparently also a substance which activates in acid solution. After purification, human gastric lipase has the optimal activity in alkaline solution at $p_H = 8$.

The only property of the enzymes which is independent of the purity is, as far as we know, their qualitative specificity, their adaptation to a definite set of reactions determined by constitution and configuration. As the history of the proteases shows, this specificity remained indeterminable in certain cases, and was indeed determined incorrectly where the enzymes occurred in a non-homogeneous condition, as, for instance, erepsin together with trypsin, and as long as it was impossible to test them in a homogeneous condition. This specificity relating to chemical structure seems to be confined within narrow limits among the carbohydrates, but seems to be much less pronounced among the lipases and esterases. The lipatic enzymes of the pancreas, the liver, the stomach, etc., are adapted to the hydrolysis of higher and lower glycerides and of higher and lower esters of monohydric alcohols. The observations made hitherto have not necessitated the assumption that these enzymes are mixtures of the lipases proper with esterases. It seems rather that good esterases, such as that of the liver, are at the same time feeble lipases and that, conversely, good lipases, such as that of the pancreas, are esterases of low potency.

The determination of the specificity to chemical structure reveals the greatest degree of differentiation among the carbohydrases. It is even possible to ascertain the particular atomic group of the substrate molecule towards which the enzymic activity is directed. The enzymes which hydrolyse a compound sugar may attack the molecule in various places. This idea of assuming variously con-

stituted intermediate reaction products in the enzymic fissions of the same substrate was first put forward by E. F. Armstrong (1904) in the case of the lactases of bitter almonds and of Kefir yeast. He found that the former is not appreciably inhibited by galactose, but strongly by glucose, whilst the latter is only influenced by galactose. The diversity in the points of attack and in the course of the reaction in the hydrolysis of the same compound sugar by hydrolytic enzymes was decisively shown in the investigations of saccharase by R. Kuhn and H. Münch (1923—26). Some saccharose-splitting enzymes react with the glucose half of this disaccharide (saccharase from *Aspergillus oryzæ :* gluco-saccharase inhibited by α-glucose); there are others, however, which exclusively attack the fructose portion of the molecule (saccharase from the usual laboratory yeasts : fructo-saccharase, inhibited by fructose). This sharp division of the various saccharases from animal organs, from fungi, and from higher plants into gluco- and fructo-saccharases seemed unjustifiable when H. von Euler and K. Josephson found that saccharase from Stockholm bottom yeast is also measurably inhibited by α-glucose. It could be shown, however, that the frequently occurring inhibition by α-glucose has nothing to do with the relation of the saccharase to saccharose, that the saccharase of the investigated yeasts possesses no affinity for α-glucose. The yeast saccharases in question hydrolyse all known gluco-derivatives of sucrose in which the fructose group, which determines the reaction with the enzyme, is intact, for instance, raffinose of the formula galactose<glucose<>fructose. On the other hand, R. Kuhn and G. E. von Grundherr found that melezitose of the formula glucose<fructose<>glucose is not attacked at all by yeast, but is hydrolysed by taka enzyme (gluco-saccharase).

A special case of specificity due to chemical structure is that caused by configuration. According to E. Fischer, this influence seems to be less pronounced among lipatic enzymes than among those that hydrolyse glucosides. The first example of stereochemical selection by a lipase occurs in an investigation by H. D. Dakin (1903—1905), who observed unequal rates of hydrolysis of the enantiomorphs of racemic esters of the mandelic acid group by means of an enzyme from liver. Such a preference for one enantiomorph enables us to distinguish all lipatic enzymes from one another. When I compared the action of a number of esterases, *viz.*, pancreatic, gastric, hepatic lipase, and taka-enzyme, on racemic esters of substituted phenylacetic acids, I found them all different in their selective action. Considering, however, the close association of lipases with activating companions, one is led to suspect that this specificity varies with the degree of purity. Hence this example is especially suitable for testing the question whether accompanying substances

are responsible for the optical specificity or whether the lipases preserve their stereochemical peculiarities after purification. In an unpublished investigation (in conjunction with E. Bamann and J. Waldschmidt-Graser) it was found possible to increase the degree of purity of lipases considerably and to remove the associated substances which influence the reaction velocity. Gastric lipase (of the pig) was concentrated to 800—1000 times the original enzymic concentration, and thus freed completely from mucin-like admixtures. The stereochemical specificity of the purified lipatic enzyme remained, however, and still remains, qualitatively unchanged. It may, therefore, with a somewhat greater degree of probability than heretofore, be attributed to the enzyme itself.

Our views concerning the relations between enzymes or other catalysts and their substrates, between enzymes or other catalysts and their promoters and retarders, between enzymes and their adsorbents, depend on the assumption of affinities which cannot be formulated in the same way as the affinities in the constitutional formulæ and reaction equations of simpler organic compounds. The wide field of molecular affinities is not yet suitable for exact representation, nor has it yet been opened up to it. The lack of a strict formulation should not prevent us, however, from penetrating into this region of molecular forces and from collecting facts concerning the capacity of molecules to unite with one another. In view of the imperfection of our explanations, we may find support in the view of Faraday in his paper on the conservation of force (1858): "If in such strivings, we . . . see but imperfectly, still we should endeavour to see, for even an obscure and distorted vision is better than none. Let us, if we can, discover a new thing *in any shape*: the true appearance and character will be easily developed afterwards."