Papers and Originals

Landmarks and Perspectives in Biochemical Research*

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The science of biochemistry has become a magnificent structure of enormous dimensions, diversity, and complexity, with many thousands of people engaged in advancing it, with new avenues opening up all the time, and new information pouring in at such a flood rate that to follow the literature even superficially is a hopeless task.

To-day's occasion seemed to me an excellent opportunity for stocktaking and survey, as well as to do a little crystal-gazing into the future: to see which position we have actually reached, by what means we have got there, and where we are going hence.

The most appropriate way in which this task could be performed was, I decided, to give you a broad panoramic review of the great landmarks of biochemical achievements, and to analyse the most important features of the strategy and tactics by which they were reached. Then, looking from the heights of achievements into the distant horizon of the future, we can, I think, discern certain perspectives of paths to be explored and can gather at least a vague conception of the exploratory kit with which we shall have to equip ourselves in order to penetrate into the unknown regions.

Three Basic Approaches to Biochemical Research

Before starting with my task, allow me to make a few general remarks about biochemical research which I consider important and which, in any case, will serve to illustrate to you clearly my own attitude to this field of scientific activity.

It has been apparent to me for a long time—and became increasingly clear as I prepared this lecture—that successful biochemical research throughout the history of the subject has involved the application of the following three basic lines of approach either singly or in combination:

1. The Recognition of the Indivisibility of Biochemistry

By this is meant that the choice of the biological system for the study of a certain biochemical effect is determined primarily by the criteria as to where this biochemical effect manifests itself most strongly and lends itself most readily to experimentation. Any species, whether mammal, bird, mollusc, crustacea, insect, plant, mould, protozoan, bacterium, or alga, can serve the purpose. Very often the most important factor determining the success of biochemical research has been the choice of the most suitable biological system. In particular, animal biochemistry and chemical microbiology form one inseparable, closely interwoven unit.

2. The Importance of the Biological Approach to Biochemical Research

Many great biochemical discoveries had at their origin a biological observation which subsequently, through the application of suitable biochemical techniques, could be explained in chemical terms. The

sequence of events in these cases was always: biology first, chemistry second—not vice versa. The importance of the biological approach for biochemical research cannot be overemphasized. Close contact and collaboration in a harmonious team between the biochemist and the biologist in the different regions of the biological sciences—and this includes, it should not be forgotten, clinical medicine—has been a characteristic feature of much successful biochemical research in the past and will increasingly become such in the future.

When the chemist in his approach to biochemical problems neglects the biological aspects the work will usually remain dull and of limited general significance; when the biologist in his observations neglects the chemical aspect, the work will remain descriptive and come to a dead end.

Through lack of collaboration between the biologist and the chemist progress has been delayed, sometimes for years. Two examples of many that could be given will serve to illustrate this assertion.

The first, Fleming's penicillin, is known to you all. Fleming reported its existence in 1929, but for lack of collaboration with chemists it lay dormant for almost ten years, until Florey and myself decided in 1938 to undertake a reinvestigation of this substance and, together with our collaborators, discovered in 1940 its unique curative properties in experimental and clinical bacterial infections.

The second example, of perhaps less spectacular practical but certainly of no less fundamental importance, is the discovery of the transforming principle. In 1928 the London bacteriologist F. Griffith published a remarkable observation in the *Journal of Hygiene*: rabbits infected with a mixture of dead smooth virulent pneumococci of type III and living avirulent rough pneumococci of type II unexpectedly succumbed to infections, and bacteriological examination showed that the organism causing death of the animals was the smooth virulent type III strain. There was obviously something in the dead type III pneumococci which had caused the transformation of the avirulent rough type II strain into the virulent smooth type III strain. The morphological and biological properties of these strains were known to be genetically controlled, and for this reason a transformation of one type into another by a chemical substance was a most remarkable and entirely new phenomenon opening up revolutionary perspectives in the sciences of both genetics and biochemistry. Yet again, because of lack of contact between the biologist and the biochemist, nothing was done to follow up, from the chemical point of view, this extraordinarily interesting and striking biological observation and to characterize the principle responsible for the transformation phenomenon, until 16 years later Avery, McCarthy, and McLeod, of the Rockefeller Institute of New York, decided to reinvestigate it and succeeded in isolating the active principle and identifying it as deoxyribonucleic acid, thus demonstrating for the first time the role of deoxyribonucleic acid in transmitting genetical information.

Research limited to investigations on the chemical structure of cell constituents, irrespective of whether these are of low or high molecular weight, once it becomes too remote from the living cell, ceases to be functional biochemistry, and becomes a different,

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though of course not less important, field of science: natural-products chemistry. Knowledge of the structure of a compound and its biologically active centres is often a useful help, but not necessarily essential—and, in any case, never the only or even the main premise for the advancement of our understanding of the metabolic reactions in the living cell, which is the primary task of functional biochemistry. What I said about the relationship between biology and biochemistry applies also to natural-products chemistry and biochemistry: close collaboration of the biochemist with the natural-products chemist is the surest way to success.

3. The Development and Application of New Techniques

The courageous development and application of both new analytical and preparative techniques, using the whole range of physico-chemical, physical, and engineering methods which progress in the respective fields of science, has made available irrespective of the complexity and cost of the apparatus requested, and with the only criterion of whether or not they will bring nearer the solution of a particular important biochemical problem.

The realization of the immense complexity of cell organization, on the one hand, and on the other hand the recognition of the greatness of the impact which almost every major biochemical discovery has had on human health and welfare, and, in consequence, of our stakes in the advance of biochemical knowledge, justifies the use of almost any technique which will bring us nearer our goal of unravelling yet another of nature's great biochemical secrets. Good facilities for development of new instrumentation and preparative techniques must therefore form an essential feature of a modern biochemical research laboratory.

Ten Major Landmarks in Biochemical Research

I shall now proceed to illustrate the validity of these theses which, for better or worse, are at the basis of the whole structure and organization of my new Institute, with some concrete examples of important landmarks in biochemical research.

The whole course of development of functional biochemistry can, I believe, be mapped by ten major landmarks. These are:

1. The Pasteur era in which the endowment of micro-organisms with well-defined specific biochemical activities was discovered.
2. The discovery by Buchner that a cell-free yeast-press juice retained the fermenting capacity of the living cell.
3. The discovery by Harden and Young that Buchner's yeast-press juice needed a cofactor of low molecular weight for its activity, in addition to high molecular proteins.
4. The recognition of the biochemical and medical significance of low molecular coenzymes.
5. The discovery of new cofactors through nutritional and clinical studies on man, animals, and micro-organisms, and of enzyme systems in cell-free extracts.
6. The discovery of the significance of cofactors for enzymic reactions.
7. The study of enzymic reactions leading to the recognition of metabolic pathways: (a) in the pre-isotope era, (b) by methods involving heavy and radioactive isotope markers, (c) by **genetical methods**.
8. The discovery of the hormones and their significance as regulators of metabolic reactions.
9. The discovery of the role of nucleic acids in protein synthesis.
10. The discovery of the antibiotics and their significance as inhibitors of metabolic reactions.

These ten landmarks could be a good synopsis of a comprehensive two-year course of biochemistry such as we intend to organize in the new Department, but in this inaugural lecture I must, of course, limit myself to a few brief comments.

1. The Pasteur Era

First of all a few words about the Pasteur era, which marks the beginning of modern functional biochemistry. The main purpose of my remarks is to convey to you something of my own feelings of boundless admiration for the immense contributions towards the development of functional biochemistry which we owe to Pasteur, one of the greatest scientific geniuses of all time, and the school he created.

For those who frown from lofty heights on contacts between the universities and industry as irreconcilable with and debasing the great ideals of pure scientific research, it may be salutary as well as instructive to remember that the impulse which led Pasteur to the line of research from which later a whole new science—or perhaps it would be more correct to say several new sciences—was destined to emerge and which had vast theoretical and practical repercussions, came from an industrial consultant job which he was asked to undertake while he was Professor of Chemistry in Lille, for an industry with as hedonistic an aim as that of wine production. Pasteur was a great French patriot to whom the economic fortunes and prosperity of his country were at least as important as his scientific research. The wine industry was of great economic importance for France. For this reason he felt that he could not refuse a request from the wine producers of his region to take an interest in the wine fermentation process, then considered to be a purely chemical process, and to make suggestions, if possible, on any steps which could be taken to avoid the occurrence of irregularities and aberrations in this process which they encountered at unpredictable intervals and under ill-defined circumstances, and which cost them a great deal of trouble and heavy financial losses.

As you know, Pasteur discovered that the fermentation process was not purely chemical in nature, but was a biological phenomenon, caused by the action of micro-organisms; the regular fermentations were found to be brought about by certain types of yeast, the abnormal "pathological" ones by other yeasts or bacteria. These observations were then extended from the diseases of wine and beer to diseases of animals and man, and led to the revolutionary results in public health, medicine, surgery, and agriculture with which we are all familiar. Pasteur and his school were thus the first to demonstrate that different species of microbes were endowed with specific biochemical activities. They were shown to be capable of producing lower and higher alcohols, lower and higher fatty acids, such as acetic, propionic, and butyric acid, hydroxy acids such as lactic and malic acid, toxins responsible for the pathogenic action of bacteria in man and animals, antigens producing specific antibodies (observations on which the science of immunology, both in the biological and in the chemical aspects, is based). They were shown to display antagonistic properties, one species against another (which later were found to be due to the production of antimicrobial substances now known as antibiotics). Some species of micro-organisms were found to be capable of living and exerting their chemical function in the absence of molecular oxygen, *la vie sans oxygène*, a startling discovery at that time, and of great fundamental importance for biochemical research. The discoveries of Pasteur and his school were extended by the great soil microbiologists of the end of the last and the beginning of this century, among them particularly prominent the great Russian soil microbiologists Winogradsky and Omeljanski and the Dutchman Beijerinck. We owe to the latter the immensely important fundamental discovery of the process of nitrogen fixation by the root nodule bacteria of leguminous plants, to the former the discovery of the nitrifying bacteria which oxidize ammonia to nitrite and nitrate, and of the fact that these and other soil organisms are capable of chemo-autotrophic existence—that is, that they can synthesize their cell material in the absence of light entirely from inorganic substances, using CO₂ as their carbon source.

Altogether, it may be said that the discovery of the biochemical activities of micro-organisms by Pasteur and his

school, supplemented by those of the soil microbiologists, who followed in their trail, has provided a reservoir of new facts and phenomena so rich and vast that even to-day biochemists in many laboratories still draw freely and extensively on it as one of the most important sources of research subjects.

2. Buchner's Discovery of the Fermenting Capacity of a Cell-free Yeast-press Juice

The next important landmark in biochemical research is Buchner's discovery in 1896, one year after Pasteur's death, that a juice obtained from compressed yeast ground with sand and subjected to the action of a hydraulic press, in which no living cells were present, had retained the capacity of the living yeast cell to perform all the reactions of alcoholic fermentations. The first consequence of this discovery was that Pasteur's theory *pas de fermentation sans vie*, which he defended tenaciously and successfully over 25 years against fierce opposition, had the fate of all theories: a limited lifetime; it survived Pasteur by exactly one year. But the great importance of Buchner's discovery for biochemistry lies in the fact that it rendered complex metabolic systems accessible to chemical experimental study; for this reason it had very great repercussions. It marks the beginning of the modern biochemistry of intermediate metabolism, enzyme chemistry, electron transport, and energy transfer. It has kept biochemists busy for almost 80 years and still continues to do so. Naturally Buchner's success with yeast-press juice of obtaining a complex metabolic system in fully functioning state in the absence of any living cells led to the extension of this method to other cell kinds, mammalian, plant, and microbial. The result has been that a whole array of enzyme systems have thus become accessible for study, covering a gamut of increasing complexity, with the simple hydrolytic enzymes, the simple dehydrogenases and oxidases at one end of the scale, and on the other the very complex particulate multi-enzyme systems, bringing about complex reactions such as oxidative phosphorylation, fat and steroid synthesis, purine and porphyrin synthesis, protein synthesis, nitrogen fixation, and photo-synthesis. In fact, the rate-determining factor for progress in several important fields of intermediate metabolism has been to find cell-free extracts retaining the metabolic function under study.

The various particulate multi-enzyme systems can be separated from the soluble enzymes and fractionated by differential centrifugation in density gradients. In this way morphologically and biochemically well-characterized subcellular fractions have been obtained, such as nuclei, cell membranes, mitochondria, and microsomes; for the separation of the latter high gravitational fields, reached by ultracentrifugation, are required.

The subcellular fractions exhibit a definite pattern of enzyme action. Thus, the expressive concept of the "cytoskeleton" advanced by Peters in the 1920's to indicate that the cell interior is not just a homogeneous solution of enzymes, but has a definite structural organization, has found consolidation by irrefutable experimental evidence.

The preparation of cell-free metabolically active extracts is one of the typical specifically biochemical operations, and requires great skill in the choice of the right organisms and the right conditions as well as luck. Bacteria have proved a particularly important source for complex enzyme systems. These are much more difficult to break up than yeast cells, and the hydraulic press method was found to be inadequate for this purpose. Several other tools for breaking up this kind of cell had therefore to be developed, including treatment with ultrasonic waves, treatment by high pressure and sudden release, squeezing through small apertures in special presses under high pressure, such as the Hughes and the Edebo press. This problem of breaking up bacterial cells belongs to the area of

biochemical engineering with which our new Department will be concerned, and has only partially been solved. It is still only possible to obtain relatively small quantities of bacterial cell extracts, and the design of continuous method for this purpose which would enable people to obtain many litres of such extracts is one of the important tasks of biochemical engineering for the future.

3. Harden and Young's Discovery of Cozymase

The next important landmark in biochemical research on which I must comment is the discovery by Harden and Young in 1905 that Buchner's yeast extract lost its fermenting power after dialysis and that this power could be restored by the addition of a very small amount of boiled yeast extract. This demonstrates that the fermentation system consisted not only of high molecular heat-labile enzymes as was believed until that time, but also of a low molecular heat-stable cofactor which was termed cozymase. The importance of this discovery beyond the limited field of fermentation chemistry became clear when it was demonstrated by Meyerhof that the same factor was necessary for muscle glycolysis which he showed to occur in a muscle-press juice prepared more or less following Buchner's method. The fact that the same factor was required as an essential component of basic metabolic processes in a micro-organism, such as yeast, and in an animal tissue, was one of the first demonstrations of the close similarity of the elementary metabolic processes in living matter.

The isolation and purification of the fermentation cofactor with which the name of Hans von Euler is prominently connected and the elucidation of its structure took over 20 years, and necessitated the working up of many hundreds of kilograms of yeast. It is the first example of what has become and still remains one of the main pillars of biochemical research, the recognition and study of trace factors forming essential components of metabolic systems.

4. The Function of Cozymase in Cellular Oxidation. The Respiratory Chain. Methodology

The full immense significance of the yeast cofactor became apparent only gradually over the course of years through two lines of research which at first seemed unconnected. On the one hand, it was soon demonstrated that the two enzymes responsible for the terminal steps in the reaction sequence of alcoholic fermentation and glycolysis—that is, the enzymes reducing acetaldehyde to alcohol and pyruvic acid to lactate—required the presence of cozymase and were inactive in its absence. Thus the yeast fermentation cofactor was shown to be involved in oxido-reduction reactions.

The mechanism of biological oxidation, as the source of energy for all the manifestations of life, has rightly formed the centre of attention of biochemical research right from its very beginnings. An army of biochemists has been occupied for many years with this fascinating complex problem, but the man who has contributed more than anyone else towards unravelling it was Otto Warburg, one of the greatest giants of biochemical research and, without any doubt, the greatest living biochemist—he has just completed 80 years—in full creative activity. Warburg conceived the idea that biological oxidation was a process catalysed by heavy metals, in the way that the oxidation of some organic substances was known to be metal catalysed, and that in particular iron, and in some cases copper, played a central role in this process.

I have already indicated that half the success in biochemical research is to find a suitable biological system. Warburg, who combined in one person the talents of an outstanding biologist with those of a brilliant chemist, has demonstrated a particular genius in this respect in all his work. The cell system which

he selected for his first studies on cell oxidation was the sea-urchin egg, which after fertilization has a fairly high rate of respiration and because of its homogeneity and small size lends itself readily for experimental investigations. Later he extended his studies on cellular respiration to animal cells, where he introduced the technique of tissue slices, the use of the isolated rat diaphragm and the Ehrlich ascites cancer cells, and to yeast and bacterial cells. In all these systems he demonstrated that the process of respiration proceeded by catalytic activation of oxygen through an autoxidizable iron-containing enzyme to which he gave the name "respiratory enzyme." The iron, he postulated, was present in this enzyme in the form of a porphyrin complex of the haematin type.

The process of oxido-reduction was, according to Warburg's concepts, brought about by continuous valency changes of the iron, which yielded an electron to the oxygen by being oxidized from the divalent to the trivalent stage, and subsequently accepted an electron from the substance, being thus again reduced to the divalent stage; the oxygen in this process was thus reduced to hydrogen peroxide, which in turn was hydrolysed to water and molecular oxygen by the enzyme catalase which also was shown to contain an iron porphyrin complex as prosthetic group.

Warburg's concept of iron-catalysed oxygen activation as the basis of cellular respiration was supported by superb experimental evidence and formulated in a series of papers with a simplicity, clarity, and logical strength which remind one strongly of Pasteur's writings. It received further strong support from the isolation, purification, and extensive study of a cellular haem pigment, which had been discovered many years earlier, in 1886, by McMunn and termed histohaematin, but whose observations had been forgotten. This haem pigment, renamed cytochrome c by Keilin, was shown by this investigator to change its spectrum reversibly on reduction and oxidation. The structure of the haem part of cytochrome c as well as the complete structure of the peptide chain to which it is attached is now known. It became clear, however—and Warburg was the first to point this out, against much opposition—that cytochrome c was not as readily autoxidizable as was Warburg's respiratory enzyme and therefore not identical with it; the respiratory enzyme was later shown to be a related compound termed cytochrome a. Subsequently several other cytochromes were identified. The probable structure of the haem moiety of cytochromes a, b, or c and the peptide chain immediately attached to the latter is shown in Fig. 1.

Despite the mass of irrefutable evidence which Warburg produced, his ideas on the central function of iron in cellular

respiration were vehemently attacked by nearly all the biochemical authorities on cellular respiration of the time, with a violence, prejudice, and irrational dogmatism which is difficult to understand to-day, even in the light of the then existing evidence. The main argument of Warburg's adversaries was the demonstration of the existence in all cells of iron-free enzymes capable of activating hydrogen in the substrates of oxidation, which led them to the conclusion that in cellular oxidation activation of hydrogen was more important than activation of molecular oxygen.

It fell to Warburg to demonstrate that both hydrogen activation and oxygen activation were equally important in cellular respiration and were, in fact, inseparably linked with each other as members of one chain of reactions, the respiratory chain. One of the crucial links between the two reactions was, as Warburg discovered, the fermentation cofactor of Harden and Young and of von Euler.

The laborious purification studies of this factor, as I have mentioned above, continued over two decades. Structural investigations on the most highly purified preparations obtained had shown that it was a nucleotide containing adenylic acid as one of its constituents, but the structure of the rest of the molecule remained obscure. Warburg, with his collaborator Christian, succeeded, in one operation of typical Warburgian brilliancy, not only in elucidating the complete structure of the coenzyme but at the same time its biological functions. He was able to isolate from a highly purified preparation which he obtained from yeast by a new method of his own a few milligrams of nicotinamide. This showed that the coenzyme was a dinucleotide in which one of the mononucleotides was adenylic acid and the other contained nicotinamide as base; both were linked in pyrophosphate linkage through their terminal phosphate groups. It is now termed nicotinamide adenine dinucleotide (NAD) (Fig. 2). Warburg recognized immediately

FIG. 2.—Nicotinamide adenine dinucleotide.

that this substance was a redox system and its capability to act as a cofactor for dehydrogenases resided in the nicotinamide group, which acted as electron and proton acceptor from substrate hydrogen, after activation by the respective substrate dehydrogenases. It became subsequently clear that the hydrogen in the thus reduced form of NAD did not react directly with the cytochrome and molecular oxygen, but through the intermediation of another redox system of diribonucleotide nature after activation through a specific dehydrogenase, termed NAD dehydrogenase. This diribonucleotide contained the alloxazine riboside riboflavin instead of the nicotinamide riboside moiety (Fig. 3). Riboflavin phosphate had previously been shown by Theorell to form part of the prosthetic group of another dehydrogenase, oxidizing glucose-6-phosphate to the lactone of the corresponding gluconic acid, an important enzyme discovered by Warburg and Christian and later shown by Dickens, in this country, to catalyse the basic reaction of an important metabolic pathway for the direct oxidation of glucose.

FIG. 1.—Cytochromes

Electrons from reduced FAD were shown to react with the cytochrome system, again after activation of its hydrogens through specific enzymes, termed cytochrome reductases, and the last member of the cytochrome system, cytochrome a or cytochrome oxidase, to pass on the electrons to molecular oxygen, reducing it to hydrogen peroxide.

FIG. 3.—Riboflavin adenine dinucleotide.

Biological oxidation was thus shown to be a flow of electrons in steps across a series of redox systems of decreasing electro-negativity. This series of redox systems is known as the respiratory chain, and this concept denotes one of the most important landmarks of biochemical research with wide ramifications. The exact sequence of the redox system in the respiratory chain in various cell types is still a subject of intensive investigations, and new members, such as coenzyme Q, non-haem iron, and copper-containing redox systems, are being discovered. Fig. 4 gives a picture of the respiratory chain in animal tissues, as it is proposed by some investigators in the light of the latest evidence. In anaerobic micro-organisms the final electron acceptor is not molecular oxygen but organic substances, or, in the case of some of the autotrophic bacteria, inorganic salts, such as nitrate and sulphate, which are reduced to ammonia, or sulphur or sulphide respectively. The respiratory chain explained the toxic action of substances such as cyanide, azide, sulphide, hydroxylamine, etc., in consequence of their formation of a firm complex with the iron of the final link of the respiratory chain, which is thus prevented from effecting the electron

transfer from substrate to oxygen so that the whole respiratory chain is put out of action.

I myself was able to show that the powerful neurotoxic effect of certain snake venoms was due to their action on the respiratory chain. The link affected in this case was the nicotinamide adenine dinucleotide coenzyme, which, I showed, was rapidly and completely broken down into two mononucleotides by an enzyme of nucleotidase nature contained in the venom.

The techniques for studying the phenomena connected with cellular respiration were nearly all introduced by Warburg. Not only did he develop the manometric measurements to the fine art they have become—Warburg's manometric techniques have become an indispensable tool of every biochemical laboratory—but he was also responsible for the introduction of the spectrophotometric absorption techniques for studying the kinetics of the oxidation-reduction reactions of the respiratory coenzymes.

These spectrophotometric methods, which include measurements in the visible and the ultra-violet range as well as fluorescent spectrophotometry, have become an indispensable tool in the biochemical laboratory. They have been used extensively not only for kinetic measurements of isolated oxidative enzyme systems—and in this field the work of Hugo Theorell on substrate-enzyme relations in catalase, peroxidase, and alcohol dehydrogenase is an important landmark—but also for following the kinetics of single components of the respiratory chain in intact cells and sub-cellular fractions, and this became possible through the application of modern electronic methods by Britton Chance which rendered the spectrophotometric and fluorometric methods very much more sensitive.

I should like at this point to recall and pay tribute to the fundamental and pioneering investigations of Leonor Michaelis and Mansfield Clark on the thermodynamics of redox systems which have been of the greatest importance for the evolution of our basic concepts on biological oxidation.

5. Discovery of Identity of Cofactors and Vitamins

We have seen that the study of the yeast cofactor of Harden and Young and related substances led to the elucidation of the mechanism of biological oxidation.

Interest in the study of enzymic cofactors was very much stimulated and their position appeared in quite a different light when it was recognized that apart from their theoretical importance for the understanding of basic metabolic reaction mechanisms they have very great practical importance for therapeutic medicine. This knowledge was gained from biochemical studies of biological nature in the field of nutrition, which at first seemed totally unconnected with yeast fermentation or cellular respiration. In the 1890's the Dutch physician Eijkman made the important discovery that a severe and widespread disease, manifesting itself with symptoms of nervous dysfunction, called beriberi, by which people in the Dutch East Indies were affected, could be reproduced in fowls by feeding them a diet of polished rice from which the hulls had been removed and that this disease could be cured by feeding the birds with unpolished rice or an extract of the hulls. Here we have an excellent example of the validity of one of the remarks which I made at the beginning of my lecture, when I referred to the vital importance for biochemical research of choosing the right biological systems. The particular

FIG. 4.—The respiratory chain.

nutritional deficiency with characteristic neuropathological symptoms, such as head retractions, is readily demonstrated only in birds, best of all in pigeons (Fig. 5).

FIG. 5.—Thiamine-deficient pigeon showing typical head-drop symptom.

In 1912 Frederick Gowland Hopkins, in whose laboratory I had the good fortune to spend two very happy and fruitful years, reported his now famous experiments (Fig. 6) that rats supplied with a calorically adequate diet of carbohydrate, protein, and fat, would not grow unless 2 ml. of milk per day was added to their diet. The observation made it clear that there existed in the diet some hitherto unknown factor or factors, present in trace amounts, in the absence of which some important metabolic systems in the intact animal did not properly function, and that this caused characteristic pathological deficiency syndromes which could be cured by the addition of the lacking factor to the diet. The similarity between the deficiency diseases and the lack of activity of the fermentation-enzyme system in the absence of cozymase was evident.

FIG. 6.—Graph to illustrate the effect of milk on growth and life. Lower curve, six rats on artificial diet alone. Upper curve, six similar animals receiving in addition 2 ml. of milk each per diem.

The identity of vitamins and enzyme cofactors was proved when it was found that one of the vitamins, an animal-growth factor discovered in yeast by P. Gyorgi and termed vitamin B₂, was riboflavin. As has already been mentioned, riboflavin phosphate linked to adenylic acid in pyrophosphate linkage (Fig. 3) is the coenzyme of the dehydrogenase termed NAD dehydrogenase, which catalyses the transfer of electrons from reduced cozymase to the cytochrome system and therefore has a vital role in the respiratory chain. Later the flavine-adenine nucleotides were found to be cofactors of several other important oxidation enzymes.

The recognition of identity of vitamins and coenzymes was one of the most important achievements of biochemical research and opened up a field with vast perspectives which continues to be actively explored.

6. Isolation of New Cofactors

In these studies the ingenuity of the biochemist had to be primarily directed towards finding an appropriate biological test system and the right nutritional conditions in which nutritional deficiencies would show up. Once these conditions were found, the isolation of the factor responsible for the deficiency symptoms was a matter of routine, though sometimes laborious and time-consuming.

Once the close similarity of the basic metabolic reactions in cells of higher animal tissues and microbial cells had been demonstrated beyond doubt the possibility suggested itself of using nutritional deficiencies of microbial growth, which is more rapid than the growth of animals and can be followed under more readily controllable and reproducible conditions, for revealing the existence of new growth factors. Fildes was one of the pioneers of the development of this method, which was successfully used for the discovery of several important vitamins. This work has been recently recognized by the award of the Copley medal of the Royal Society.

Furthermore, the old method of Harden and Young of using cell-free enzyme systems for the recognition of new cofactors, either by dialysis or by other detection techniques, has continued to give rewarding results also in more recent times: the discovery of coenzyme A by Lipman, which had such far-reaching repercussions for our understanding of the biosynthetic pathways of fatty acids, steroids, rubber, and many other natural products, is a particularly brilliant example. He found that liver extracts were capable of acetylating sulphanilamide, lost this property on dialysis, and regained it when boiled liver extract or a concentrate of dialysate was added. Fig. 7 lists some of the important cofactors discovered by nutritional or biochemical techniques.

Discovery of New Cofactors

A Through Nutritional and Clinical Studies		B Through Studies of Isolated Enzyme Systems	
On Animals	On Micro-organisms	Cofactors	Enzyme Systems through which Discovered
Vitamin A Thiamine (B ₁) Riboflavin (B ₂) Nicotinic acid Pyridoxine Pantothenic acid Folic acid Vitamin B ₁₂ Vitamin C Vitamin K Tocopherol	Biotin p-Aminobenzoic acid Lipoic acid Folic acid Vitamin B ₁₂ Streptogenin Mevalonic acid	Mono- and dinucleotides NAD NADP FAD ATP UTP GTP Polynucleotides Transfer RNA Messenger RNA Coenzyme A Coenzyme Q	Alcoholic fermentation Glucose-6-phosphate dehydrogenase Glucose-6-phosphate dehydrogenase Glycolysis Galactose-isomerase Protein synthesis Protein synthesis Protein synthesis Acetylation of sulphanilamide Respiratory chain

FIG. 7

As can be clearly seen, animal biochemistry and chemical microbiology are inextricably mixed up in this field, for the revelation as well as the preparation of cofactors, and in many cases the isolation of the cofactors, involved large-scale production of micro-organisms. Each of these substances has opened up a new important chapter of biochemistry. The study of their function forms an essential part of the field of enzymology to which a large proportion of the biochemical research effort has been and still is devoted. Most of these cofactors have been shown to form part of important enzyme systems, and as such to possess a highly important metabolic function.

7. Elucidation of Biosynthetic Pathways

The elucidation of the pathways of metabolism is one of the most important tasks of functional biochemistry. Very great progress has been made in this field, and we are now familiar with the essential steps of most of the important metabolic pathways.

This achievement is a major landmark in biochemistry and has been brought about by enzymological studies in cell-free systems, in conjunction with studies designed to follow the fate of metabolites in the intact cell, organ, or organism.

Three phases in this research development can be distinguished: (1) the pre-isotope era, (2) the isotope era, and (3) the era of biochemical genetics.

Pre-isotope Era

In the pre-isotope era the most important information was obtained from enzymological studies of alcoholic fermentation and glycolysis in cell-free extracts of the Buchner type, which led to the complete elucidation of the pathway involved (Fig. 8). Closely connected with and actually emanating from

FIG. 8.—Glycolytic Meyerhof-Embden system.

these studies was the discovery by the Coris of the enzyme phosphorylase, which brings about the phosphorylase of glycogen and starch to glucose-1-phosphate and, by reversal of this reaction, the synthesis of starch and glycogen through condensation of glucose-1-phosphate units in the presence of a small amount of polysaccharide primer with the elimination of phosphate (Fig. 8); glucose-1-phosphate was first found by the Coris as a breakdown product of glycogen in muscle extracts. Phosphorylase, as well as most of the enzymes involved in alcoholic fermentation and glycolysis, has been obtained in the crystalline state. These investigations led to the elucidation of function of several important new cofactors, with repercussions far beyond the limited field under study. The discovery of the function of vitamin B₁ as the coenzyme of carboxylase which catalyses the decarboxylation of pyruvate to acetaldehyde and CO₂, by Lohmann was a further important milestone; its importance for pyruvate oxidation in animal tissues had previously been recognized by Peters. The mechanism by which,

in the light of the latest experimental evidence, vitamin B brings about anaerobic and aerobic decarboxylation of pyruvate is shown in Fig. 9.

FIG. 9.—Non-oxidative and oxidative pyruvate decarboxylation.

The discovery of another cofactor of alcoholic fermentation and glycolysis with tremendous repercussions was that of adenosine triphosphate (ATP) (Fig. 10), which was shown to be the main chemical mediator for the storage and transfer of oxidative energy. The names of Meyerhof, A. V. Hill, Lohmann, Karl Meyer, Lipman, and many others are associated with the discovery of the functions of ATP.

FIG. 10.—Adenosine triphosphate (ATP).

Its importance became clear only slowly, over the course of many years, and to a large part in the second era of the study of intermediate metabolism, when isotopes became available. It was found capable of undergoing several types of reactions, of which the most important ones are (Fig. 11):

1. The transfer of the terminal phosphate to a wide variety of substances, sugars, phosphorylated sugars, nucleotides, nucleosides, pyruvate, creatine, arginine, cofactors such as pyridoxine and mevalonic acid and mevalonic 5-phosphate. In all cases the substances became more susceptible to enzymic attack and therefore metabolically more reactive through phosphorylation; in some cases the energy of the terminal ATP phosphate link is retained in the phosphorylated products and enables them to undergo energy requiring condensation reactions. Thus, for instance, Popjack has shown that molecules of mevalonic acid 5-pyrophosphate undergo condensation with each other to give squalene and higher terpenoids.

2. It can undergo enzymic pyrophosphorylytic cleavage to give AMP and a pyrophosphate ester, for instance in the reaction with thiamine, which leads to the formation of thiamine pyrophosphate, the biologically active form of thiamine or with UMP to give UTP. The latter, after condensation with glucose-1-phosphate, through the action of another pyrophosphorylase, to give UDPG, was found by Leloir to have an important metabolic function in carbohydrate metabolism as the coenzyme of galactose-4-isomerase, converting galactose phosphate into glucose phosphate, and as coenzyme of glycogen synthesis.

3. It can undergo pyrophosphorylytic cleavage to give mixed anhydrides, for instance, with carboxylic acids and amino-acids, which are thus activated for synthesis of long-chain fatty acids and of proteins respectively, the latter via transfer R.N.A. and "messenger" R.N.A. (see below).

4. It can undergo condensation with other nucleotides through enzymic pyrophosphorylysis to give the dinucleotide coenzymes of the respiratory chain NAD and FAD and mononucleotide triphosphates.

5. ATP can undergo enzymic condensation with other ribonucleotide triphosphates containing purine and pyrimidine bases to

give high molecular RNA under the action of a polymerase and the elimination of pyrophosphate. This reaction, like the above-mentioned polymerization of glucose-1-phosphate to starch or glycogen, needs a high polymer primer, which in this case is high molecular RNA or DNA. The primer also acts as template. If, for instance, DNA of different origin is used as primer, the resulting RNA corresponds in its composition and nucleotide sequence to the particular DNA added. This observation is obviously of great importance for the transmission of genetic information.

REACTIONS OF ATP

1. Kinases

Reactant	Product
Glucose	Glucose-6-phosphate + ADP
Glucose-6-phosphate	Glucose-1,6-diphosphate ..
Pyruvate	Phosphoenol pyruvate ..
Creatine	Creatine phosphate ..
Pyridoxine	Pyridoxine phosphate ..
Mevalonate	5-Phosphomevalonate ..
5-Phosphomevalonate	5-Pyrophosphomevalonate ..
Ribonucleosides	Monoribonucleotides ..
AMP	2 ADP

2. Pyrophosphokinases

Reactant	Product
Thiamine	Thiamine pyrophosphate + AMP
UMP (uridinemonomophosphate)	UTP (uridinetriphosphate) + AMP

3. Carboxyl activation through pyrophosphorylation

Reactant	Product
$R-CH_2-COOH$	$AMP-O-CO-CH_2R + PP$
$R-CH-(NH_2)COOH$	$AMP-O-CO-CH(NH_2)R + PP$

4. Dinucleotide formation through pyrophosphorylation

Reactant	Product
NMP (nicotinamide ribose monophosphate)	NAD + PP
FMP (flavine monophosphate)	FAD + PP

5. Polynucleotide formation

FIG. 11

Kornberg discovered that DNA itself can be synthesized enzymically from a mixture of deoxyribonucleotide triphosphates, also with the elimination of pyrophosphate. A primer is required, too, for this reaction.

Finally, mention must be made in this connexion of the phosphorylase discovered by Ochoa and Grunberg-Manago which condenses nucleotide diphosphates containing both purine and pyridine bases with the elimination of phosphate, and does not need a primer. This enzyme condenses single nucleotide diphosphates of one base composition to give polyadenylic, polycytidic, etc., acids, or of different base compositions, giving rise to high molecular ribonucleic acids with varying proportions and varying sequence of bases. As will be discussed later, the artificial synthetic nucleotides have proved very useful tools in studies of protein biosynthesis.

ATP is generated principally by oxidative energy—that is, the energy generated during electron transfer at some step of the respiratory chain; the mechanism of this process has been, and still is, the object of intense study by many investigators, but despite the immense amount of work expended is still very imperfectly understood.

We ourselves are actively engaged in a study of this problem, and shall continue for some time to come, in connexion with

our studies, which I shall briefly mention later, on the metabolism of nervous tissue, the mode of action of insulin, and other questions regarding intermediate metabolism.

The spectrophotometric studies of Britton Chance are of particular interest in this connexion; quantitative spectrophotometric scanning of some of the most important known redox components of the respiratory chain and simultaneous polarographic determination of oxygen consumption show that addition of ADP immediately leads to increased respiration and characteristic changes in the state of oxido-reduction of the redox components until it is fully phosphorylated (Fig. 12).

The respiratory chain and the machinery for oxidative phosphorylation are carried by a subcellular fraction, obtained by centrifugal fractionation of broken-up cells: the mitochondria. We owe very largely to the work of Green and his colleagues,

FIG. 12.—Influence of substrate and ADP on redox systems of respiratory chain. (Britton, Chance, and Williams, G. R., *J. Biol. Chem.*, 1955, **217**, 409.)

The investigations of Krebs are characterized by extensive use of manometric analytical techniques for the determination of metabolites, using the apparatus introduced by Warburg.

Not only were many of the concepts of the pre-isotope era confirmed—for instance, the existence of the urea and tricarboxylic acid cycles—but much new knowledge accumulated.

Fig. 15.—The tricarboxylic acid cycle.

Fig. 16.—Biosynthesis of the porphyrin ring via δ -aminolaevulinic acid.

Fig. 13.—The pentose phosphate cycle.

forming agent as DNA, already referred to ; (2) biochemical and genetic studies on viruses and bacteria ; (3) the enzymatic synthesis of polynucleotides ; (4) the discovery that protein synthesis can be brought about in cell-free enzyme systems, of mammalian and bacterial origin, when suitably supplemented. It has been recognized (Fig. 21) that this occurs in several stages, the first of which is the activation of amino-acids already referred to. This is followed by the attachment of the activated amino-acid to a ribonucleic acid of low molecular weight, termed transfer nucleic acid (each amino-acid having its own transfer ribonucleic acid), and finally the amino-acid is transferred from the transfer of nucleic acid to the ribosomes and incorporated into the protein. This is done under the directing influence of a ribonucleic acid, of molecular weight of about 150,000, which, as has already been mentioned, is formed under the directing influence of the chromosomal DNA and is its copy in miniature ; it has been termed "messenger nucleic acid" because it conveys the genetic message for specific protein synthesis for the chromosomes to the amino-acid-assembling machinery of the protein-synthesizing enzyme system. This itself is non-specific, like a printing machine which can reproduce any set of type. The artificial ribonucleotides have proved very useful tools in the elucidation of the role of messenger

FIG. 21.—Protein synthesis.

ribonucleic acids for specific protein synthesis. With their help it could be shown that the sequence in which the amino-acids are incorporated into proteins is determined by the sequence of nucleotide units in the messenger ribonucleic acids, each unit probably consisting of three nucleotides (so-called nucleotide triplets). With the help of synthetic nucleotides of different nucleotide composition and sequence it could be established which specific sequence of ribonucleotides in the triplets was required for the incorporation into proteins of each individual amino-acid. As the messenger ribonucleic acids are formed under the influence of chromosomal DNA and are their exact copy, nucleotide triplet sequence determines the specific genetically determined amino-acid sequence in proteins. The sequence of ribonucleotides in the ribonucleotide triplets for incorporation of individual amino-acids is known as "genetic code" (Fig. 22), which appears to be universal.

Fractionation of the protein-synthesizing cell-extracts by centrifugation in a density gradient has shown that the amino-acid-assembling system is associated with a subcellular particulate fraction, the ribosomes, which needs a high gravity field for sedimentation.

High-speed ultracentrifugation in density gradients in which gravity fields of 100,000 *g* and more are reached and which has to be continued in some instances for periods of several days has become, despite the complexity and cost of the equipment, an indispensable and widely used technique, not only for the preparation of subcellular fractions, but also in the

nucleic-acid field, for analytical as well as preparative purposes aimed at the separation of the metabolically active nucleic acids of different molecular weight. The ultracentrifuge was developed many years ago by Svedberg for the determination of molecular weights of proteins and used only for this limited purpose by a small number of protein chemists specializing in this field. Now it has become a standard piece of biochemical equipment and represents a good example of the ever-increasing complexity and costliness of modern biochemical methodology.

Amino-acid	"Modified" Base Pairs	Suggested Functional Triplets		
		° = U	° = A	° = C
Lysine	... A°A	AUA	AAA	
Asparagine	... °AA,C°A	CUA,UAA	CAA	
Histidine	... A°C	AUC		ACC
Glutamine	... °AC,GG°	UAC	AAC,GGA	
Glutamic acid	... A°G	AUG	AAC	
Tyrosine	... A°U	AUU		ACU
Threonine	... °CA,°GC	UCA	ACA	CCA,CGC
Proline	... C°C	CUC	CAC	CCC
Alanine	... C°G	CUG	CAG	CCG
Serine	... C°U,°CC,°CG	CUU,UCC	ACG	
Aspartic acid	... G°A	GUA		GCA
Methionine	... °GA	UGA		
Arginine	... G°C,G°A	GUC	GAA	GCC
Glycine	... G°G	GUG	GAG	GCG
Tryptophan	... °GG	UGG		
Cysteine	... G°U	GUU		
Isoleucine	... °UA,°AU	UUA	AAU	CAU
Valine	... U°G	UUG		
Leucine	... U°C,°CU,°AU,°GU	UUC,UAU,UGU		CCU
Phenylalanine	... U°U	UUU		UCU

FIG. 22.—Suggested assignments of messenger RNA triplets to coding functions in the amino-acid code.

Era of Biochemical Genetics

The introduction of microbial genetic methods for the study of intermediate metabolism by Tatum and Beadle is another milestone in biochemical research. The concept that enzymic reactions are gene-controlled was arrived at on the basis of the study of certain metabolic abnormalities in man (inborn errors of metabolism) and the genetic transmission of anthocyanine pigments of known constitution in plants ; the names of Garrod, Rose Moncrieff, and Haldane are prominently associated with this idea. Beadle and Tatum, using the fungus *Neurospora crassa*, were able to demonstrate that each enzymic step in a chain of biosynthetic events is controlled by one gene. Here again much of their success depended on choosing this particular organism.

The general principle of the method which they used for studying the metabolic pathway of a given metabolite—for instance, an amino-acid, a purine base, etc.—was the following : a wild strain of *Neurospora crassa* which was capable of growing in a simple medium was treated with mutagenic agents (α rays, ultra-violet rays, chemicals). Mutants with blocks in the biosynthetic reaction sequence of the metabolite under study were thus obtained which were not able any more to grow, or grew only at a retarded rate, unless supplemented with the metabolite under study, or its precursors after the genetic block. In some cases precursors of the metabolite in the reaction chain before the metabolic block accumulated and could be isolated and their constitution elucidated. It was also found possible in some cases to demonstrate the absence of an enzyme bringing about the reaction interrupted at the genetic block of the biosynthetic reaction sequence. These metabolic genetic blocks were later shown to be due to changes brought about in one single Mendelian gene by the mutagenic agent.

With this method steps of the biosynthetic pathways of many important metabolites were established—amino-acids, vitamins, purines and pyrimidines, sulphate, nitrate, etc. A particularly important outcome of this work, emanating from its application to the biosynthesis of the amino-acids phenylalanine, tyrosine, and tryptophan, was the discovery of the pathway of biosynthesis of the aromatic ring system via shikimic acid (Fig. 23). The analytical techniques employed in the study of pathways of intermediate metabolism have also become very complex.

Chromatographic separation methods of all kinds—paper, column, and gas chromatography—have made a tremendous impact in this field, as they have in all other fields of analytical and preparative chemistry. Used in combination with various methods for determining the isotope content of tracers, they dominate the field. For determination of ^{14}C in the form of $^{14}\text{CO}_2$ the low background anticoincidence counter has found

without first having to dry aliquots or to combust aliquots to CO_2 .

The chromatographic as well as the isotope-determination methods have been automated to a large degree. For the study of protein biosynthesis and similar problems the automatic amino-acid analyser has become an indispensable tool, which allows not only the neat separation of complex mixtures of amino-acids and their individual quantitative determination but also simultaneously the determination of their specific activity. Heavy isotopes are also extensively used in studies of metabolic pathways and the mechanism of action of the enzymes involved, particularly ^{18}O and ^{15}N , but also ^2H . For the quantitative determination of the former mass spectrometric methods are used.

Looking back from our present position on the achievements attained in the field of metabolic pathways in the course of only thirty years, the progress is certainly impressive. However, despite all this progress, we are still as far away as ever from our final goal, to understand the biochemical functioning of the cell.

It is true we have discovered a great number of new biochemical reactions and reaction sequences, but it is surprising how little we know about their significance in the living cell, their relation to the specific functions in a particular cell kind, and the way in which the various reaction rates are regulated and interdependent. Yet these are problems of the very greatest importance, theoretical as well as practical, for all fields of biology, and not least for medicine—for metabolic disorders are caused by changes in metabolic reaction rates.

This brings me to the discussion of a field of research in which we are actively engaged at present and which will form one of the main topics of research in the newly constituted Metabolic Reactions Research Unit of the Medical Research Council, to be housed in our new Biochemistry Department: the mode of action of hormones.

[The conclusion of this Inaugural Lecture will be published in next week's issue.]

FIG. 23.—Biosynthesis of aromatic ring system.

wide application, but is rivalled by the considerably more costly scintillation counter which is suitable for β as well as for γ radiation, and therefore allows the determination of tritium, iodine, etc., in addition to ^{14}C . It has the great additional advantage that it allows the determination of radioactivity directly in fractions coming from column chromatography,

Primula Dermatitis

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Most doctors and many gardeners know that primulas may cause dermatitis, but general practitioners are often unfamiliar with the pattern of the rash produced. It was felt, therefore, that a clinical description would be helpful.

The Primulaceae

The primula family comprises some 22 genera and 600 species. The genera include *Primula*, *Androsace*, *Soldanella*, *Lysimachia*, *Glaux*, *Anagallis*, and *Cyclamen*.

The genus *Primula* contains over 200 species, including the common primrose and the cowslip. Those most commonly grown as house-plants are *P. obconica* (Fig. 1) and *P. malacoides*. *P. sinensis* is retailed in smaller quantities, while *P. kewensis*, *P. floribunda*, *P. denticulata*, and *P. mollis*

also appear in florists' shops from time to time. With very rare exceptions only *P. obconica* causes dermatitis.

Historical

P. obconica (Fig. 1) was introduced to Britain from China in 1880 and rapidly became established as a popular greenhouse plant throughout Europe and the United States. The first accounts incriminating this plant as a cause of dermatitis were published in American horticultural journals and were received sceptically in England. However, reports of similar experiences in this country soon followed; one indignant gardener vigorously condemned "this vegetable viper." A year or two later reports began to appear in the medical journals, first in America and soon after in Britain (Sym, 1890; Clarke, 1890; Ferguson, 1890). So numerous were the reports that it was predicted (Cooper, 1899) that such a poisonous plant would be grown less and less frequently; but this prophecy

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