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CELLULAR RESPIRATION

NORMAN U. MELDRUM

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General Editor: G. R. de Beer, M.A., D.Sc.

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CELLULAR RESPIRATION

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WITH 17 DIAGRAMS

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PREFACE

THERE are few branches of biochemistry which appear to be more obscure or less well understood than that of Cell Respiration. It has accordingly been my aim rather to write in a didactic manner than to provide a detailed account of the subject. It may be that I have over-simplified matters; if so, the fault can be remedied by the reader turning to the original literature. This book is then to be regarded as an exposition: in a certain sense this is a confession, not so much of weakness, as of incompleteness. It has been necessary to omit much that is of interest, perhaps in the future to be of vital interest. This is in itself unfortunate, but I do not think it would have been expedient at the present time, nor suitable to this series, had I written a comprehensive and detailed treatise. I can only describe the outlines.

Most obvious is the omission of all reference to oxidation-reduction potentials. A considerable amount of work has been published thereon, but to describe it would be to enter upon a field where no two workers agree, either as to the accuracy of the experimental observations, or as to the interpretation to be placed upon them.

My thanks are due to a number of my friends who have assisted me in various ways, above all to Professor D. Keilin and to Dr. J. Needham.

N. U. M.

Sir William Dunn Institute of Biochemistry,
CAMBRIDGE

February 1933

NOTE

WHILE the plan and subject-matter of this book are the work of the late Dr. Meldrum, the MS. was not quite ready for press at the time of his death. The General Editor wishes to express his gratitude to Mr. David E. Green for editing the MS., completing the bibliography and making such alterations as seemed to be desirable, and for correcting the proofs. He is also indebted to Mr. R. B. Fisher for suggestions concerning the expression of certain formulae.

CONTENTS

NORMAN URQUHART MELDRUM

ABOUT the year 1929 the workers in the Cambridge biochemical laboratory were made aware of a person of small stature but enquiring and thoughtful countenance who would come into their rooms and ask them what they thought of such and such a recent piece of research, or of such and such a generally accepted biological theory. Answer being given, the questioner would thank them and depart before they could ascertain the reason for these questions or the general line of thought that prompted them. As is so often the case with people whose closer acquaintance is most worth while, this aloofness unduly lengthened the time taken for Norman Urquhart Meldrum to be fully absorbed into the lives of his fellow-researchers.

Born in 1907, schooled at the Edinburgh Academy, and having taken his degree at the University there, he came to Cambridge as a candidate for the research doctorate and was quickly caught up into one of the main streams of Cambridge biochemical research—the study of the oxidation-reduction reactions of the cell. Working in collaboration with Dr. Malcolm Dixon, he investigated the then newly purified specimens of the tripeptide, glutathione, and made great progress in our knowledge of the properties and reactions of this important substance. Throughout his research career he occupied himself with one or another aspect of the problem of glutathione, and published work on its reduction by a blood system, and on its biological significance. He also concerned

himself with the highly obscure and difficult protein-denaturation problem. Finally, in later times, he discovered, with Dr. Roughton, the enzyme present in blood which catalyses the liberation of carbon dioxide from bicarbonates, carbonic anhydrase, a system of great importance throughout the animal kingdom.

If Meldrum's polite withdrawing tendency was a barrier to some, those who penetrated it soon found that he possessed an intellectual vigour and assurance quite out of the ordinary, and a remarkably fine historical sense. The former spurred him to many pieces of research which he never found time to prepare for publication, and seemed to carry with it a wonderful power over the initial inertia so commonly felt by scientific workers. He never permitted himself to fall into the rut of routine manipulation. His interest ranged from the abstract realms of thermo-dynamics on the one hand to the chemistry and the natural history of the invertebrates on the other. And a great part of his charm lay, perhaps, in the fact that though fully conscious of his intellectual powers, he turned interior arrogance wholly towards external nature, and in converse with fellow-workers, or it might be, with the weaker brethren, offered them a generous and unceasing flow of admirable and searchingly critical ideas.

Penetrating further, we found something that might have been unexpected, namely, an entire devotion to an ideal of spaciousness not often met with today. Narrow methods and mean or bourgeois concepts were to him abhorrent ; to please him, everything had to have something of the grand manner about it ; but his attachment to the values of the individual prevented him from translating this into terms of modern social movements, and so again had the effect of insulating him from his immediate *milieu*. In details these tendencies led him to a passionate interest in the Napoleonic legend and in German poetry of

the romantic period, and to a special study of Carlyle and Carlylean theories, on which he became something of an authority. Theologically, they led him, too, to an affinity with Voltaire and the deist-atheist philosophy of the eighteenth century, especially in France, and he would have enthusiastically appreciated Jacques Loeb's dedication of *The Organism as a Whole* to Denis Diderot.

When a group of biochemists began regularly to go riding, it was inevitable that Meldrum should join them. In horsemanship he found, as it were, a new world of life, refreshing him for his work, and setting him in tune with the whole intellectual and historical background which he loved. But this happiness was not to last overlong. Early in 1933 he suffered a fall from his horse while jumping ; the cause of a severe concussion. Recovery proved to be slow, and feeling himself, perhaps, hampered with an intolerable drag on the progress of his thought and work, or fearing, it may be, an indefinitely prolonged illness, he put an end to his life in the early summer of that year. Against him the enemy might allege too great ambition, too little moderation in desire for fame ; but one ambition, and that not the least, was abundantly fulfilled, namely the love of his friends and fellow-workers, than which nothing is more to be prized, and nothing more difficult to attain by purchase.

J. NEEDHAM
D. E. GREEN

CAMBRIDGE
October 1933

CHAPTER I

INTRODUCTION

THE subject of this book is the description of the respiration of the cell. In the present state of biochemical knowledge little is known of the differences in respiration between various types of cells, and much less of the respiration of one part as contrasted with another part of the same cell. I shall then have little cause to speak of the size or shape or histology of the systems studied. In fact I shall have to discuss organisms as though they were chemical reagents or material from which such can be prepared. Such a state of affairs is unfortunate, but it is, of course, a stage preliminary to describing the cell as a whole, a position to which biochemistry has not yet attained.

When an organism moves, reproduces, digests, keeps itself warm, in short "lives", the energy necessary for this activity is obtained by the oxidation of substances present in the cells. An animal obtains foodstuffs which it digests and assimilates; part of the hydrolysed foodstuffs it uses to replace the worn-out tissues of the body, part it excretes, and part it uses as fuel from which to obtain energy. Of the energy of oxidation, the animal may use one fraction as mechanical work with which to perform movements, another it may use as heat with which to regulate its temperature. Two factors at least are needed for the cell to perform work: firstly substances which can be oxidised and from whose oxida-

tion energy can be obtained, and secondly oxygen with which to attack the molecules concerned. It is, of course, ultimately upon the absorption of solar radiation by plants that the dynamics of life are based, but this is not our problem : much more are we concerned with what happens to the organic molecules once they have been assimilated by the animal rather than with how they were originally formed in photosynthetic processes by plants or in what way they were derived from such synthetic materials. It must be realised clearly that the statement concerning the need of oxygen is true only in the broadest sense. All or most organisms, even aerobic cells, can perform work in the absence of oxygen, but they can perform only a small amount : ultimately (for example, in a runner) lactic acid accumulates, fatigue sets in, and finally death results if the anaerobiosis is too prolonged.

When an organism performs work, as for example by walking, or swimming, or flying, or produces heat to keep itself warm, it absorbs a certain amount of oxygen, burns a corresponding amount of metabolites (such as glucose), and excretes the oxygen in carbon dioxide and water. The end products of this system are precisely the same as when the metabolite is burned directly in a calorimeter, and the energy exchanges are the same in the two cases.[1] How this energy is used (as heat or as mechanical work) is, of course, a different matter. Our problem is to describe the mechanisms by which the molecules oxidised are burned in the cell : that is the object of this book. Before we pass on to a description of these mechanisms it may be well to work out the limits of our field.

It must be clearly realised that we are not con-

[1] This is true only in a limited sense, if the oxidation occurs reversibly. If the concentrations are different in the two cases the energy relationships will necessarily be different, but not greatly.

cerned with the problems of oxygen transport. Certain systems are known—in vertebrates haemoglobin, in some invertebrates haemocyanin—which have the power of absorbing oxygen at high pressures and of evolving it at lower. We know that haemoglobin forms a molecular complex with oxygen and that the oxygen can be split off by exposing the system to a vacuum, that in fact we have an equilibrium

$$Hb + O_2 \rightleftharpoons HbO_2$$

We know that in the lungs the oxygenation (to be distinguished from the oxidation) of haemoglobin takes place, that the oxygenated blood is carried through the arteries to the arterioles and capillaries and that there the oxyhaemoglobin dissociates to reduced haemoglobin and free oxygen which diffuses across the capillary walls to the tissues. All this does not concern us. Nor are we concerned with the fact that haemoglobin is found in some cells (e.g. in muscle). Further, our problem is not that of carbon dioxide excretion. We know that carbon dioxide diffuses from the tissues to the blood, that it is there carried (mainly as bicarbonate) to the lungs, and that when reduced haemoglobin becomes oxygenated the increase of acidity consequent thereon produces free carbonic acid according to the equation

$$HCO_3^- + H^+ \rightleftharpoons H_2CO_3$$

which then dissociates to water and carbon dioxide

$$H_2CO_3 \rightleftharpoons H_2O + CO_2$$

(the slow dissociation being accelerated by the enzyme carbonic anhydrase), and the carbon dioxide escapes from the blood into the alveolar spaces.

What then is our problem? Before we discuss this problem we must be sure that a problem does really exist at all. Given a living cell, respiring freely in excess supplies of metabolites and of oxygen—how is this cell able to keep warm and perform

mechanical work ? It is known that substances are oxidised, CO_2 and H_2O formed, work done and heat evolved. The system may be studied in two ways. We may ask

(*a*) How are the organic metabolites oxidised ?
and

(*b*) How is energy of oxidation converted into mechanical work ?

The first question alone concerns us.

Now the cell absorbs certain substances such as glucose, succinic acid and fatty acids, and then oxidises them to CO_2 and H_2O. If these substances are heated in a bomb calorimeter with oxygen they are converted, as in the cell, to carbon dioxide and water, and a definite fixed amount of heat is produced, characteristic of the system studied and of the conditions under which the reaction takes place. If these substances be used as fuel in an engine, less heat is evolved and the difference between the heat evolution in the calorimeter and in the engine is turned into mechanical work done by the latter.[1] Such molecules can also be oxidised by reagents like chromic acid, nitric acid, and other substances. Here too the end products are CO_2 and H_2O. Is our problem in any way different from that of the calorimeter or of the engine ? The answer is—profoundly so. Why, we shall see later.

Consider more closely what happens in the calorimeter. Suppose fat is being burned. Ultimately carbon dioxide and water are formed. The fat has been oxidised, the oxygen reduced. Now when we heat the fat in oxygen we cause its oxidation, but we are not initiating a new reaction ; we are merely accelerating an old one which has been going on all the time, though immeasurably slowly. The oxidation of

[1] This is true only in so far as friction is negligible. Even in a mechanical engine the efficiency cannot be greater than that of a perfect reversible engine working between the same temperature limits.

the fat has been proceeding continuously even when it was not in the calorimeter, and what we have done is to make this oxidation go much faster. It is an observed empirical rule that the rate of many chemical reactions is doubled by each 10° C. rise in temperature.

At ordinary temperatures the rate of oxidation of glucose, succinic acid and similar molecules is immeasurably slow, and it is quite clear that the carbon dioxide evolved by the cell cannot be due to the spontaneous oxidation of these molecules running its normal course unaffected by the rest of the cell. Such an oxidation would produce the most minute amount of CO_2. It has been made clear that our problem is not concerned with the absorption and transport of oxygen, nor with the carriage and elimination of carbon dioxide : we see now that it is not that of the spontaneous oxidation of organic molecules. For the cell oxidises molecules of great stability, it oxidises them at low temperatures, and it attacks them with great ease without the presence of violent reagents.

The end products of cell respiration are carbon dioxide and water : the intermediate stages are much harder to define. Thus it happens that when the cell oxidises, say, stearic acid, the intermediate stages between $C_{17}H_{35}\cdot COOH$ and CO_2 and H_2O are largely unknown. Acetic acid can be oxidised by many cells—how is largely a matter for conjecture. One of the methods by which the problem of intermediate metabolism is attacked is to feed to the normal animal some substance and study the excretions such as urine. In this way was established the theory of β-oxidation for the lower fatty acids [Knoop, 1904 ; Embden, 1905]. According to this view the phenyl derivatives of the lower fatty acids, such as hexoic acid, $CH_3\cdot CH_2\cdot CH_2\cdot CH_2\cdot CH_2\cdot COOH$, are oxidised first to the corresponding β-keto acids and ultimately (if the acid bore an even number of

carbon atoms) to γ-phenyl-β-ketobutyric acid. In the case of hexoic acid the process would be

$$C_6H_5\cdot CH_2\cdot CH_2\cdot CH_2\cdot CH_2\cdot CH_2\cdot COOH \longrightarrow C_6H_5\cdot CH_2\cdot CH_2\cdot CH_2\cdot CO\cdot CH_2\cdot COOH \longrightarrow C_6H_5\cdot CH_2\cdot CH_2\cdot CH_2\cdot COOH \longrightarrow C_6H_5\cdot CH_2\cdot CO\cdot CH_2\cdot COOH$$

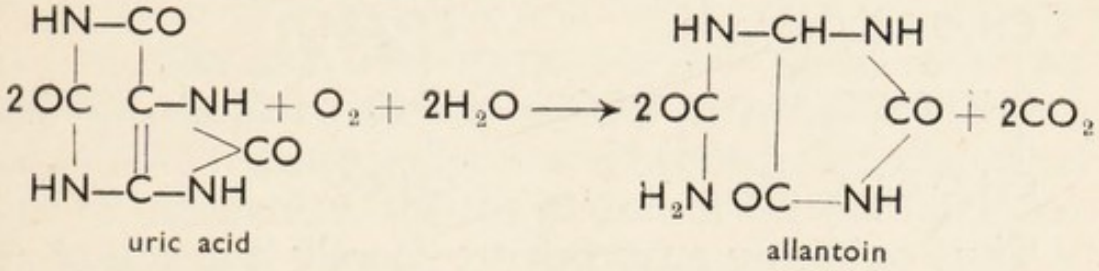
The objection to this method of study is that only those substances are excreted which are slowly oxidised, and that hence the more rapidly a substance is oxidised the less likely is it thus to be isolated from the urine. The same criticism applies to all attempts to isolate substances from respiring cells—the more easily a substance is attacked the less likely is it to be isolated and the harder is it to establish its rôle in respiration. Suppose a substance A is broken down in the cell to yield eventually a substance Z, then at a steady state the reactions are all going normally—there is no accumulation of any product at any one stage, nor any shortage at another. Under such conditions we can apply the equations

$$k_1[A] = k_2[B] = k_3[C] = k_n[Z]$$

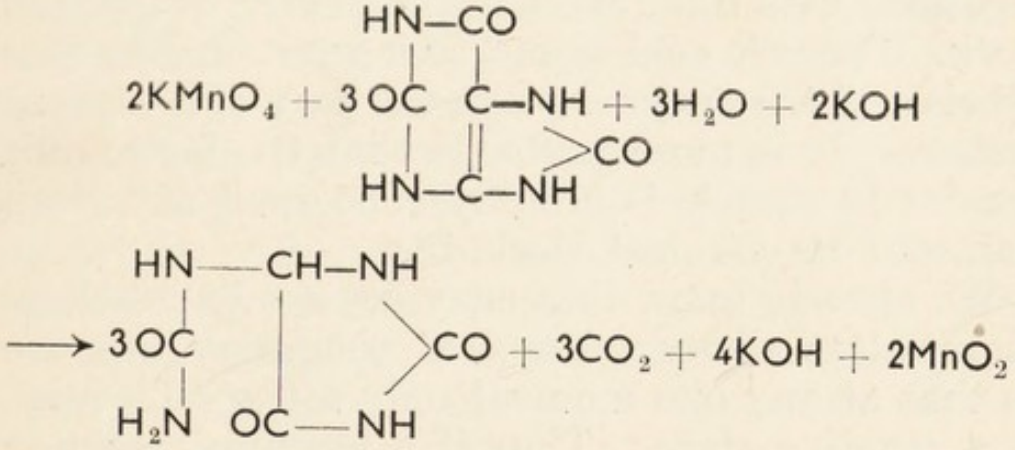
where k is a velocity constant and expresses the amount of material transformed in any one reaction per unit time when the concentration of reactant is unity. Thus the more rapidly any substance S is transformed the bigger k_S and the less [S], the concentration of S. It is for this reason that comparatively little work has been done on the intermediate substances which are the primary oxidation products of complex molecules. It is as yet impossible to say how a molecule breaks down to carbon dioxide and water.

What we have to study is the means by which the cell achieves these oxidations much rather than the path the oxidations follow. The effective agents are the catalysts known as " enzymes ". At the present moment it is impossible to define an enzyme except by the way in which it acts. We can say the oxidation of uric acid to allantoin is catalysed by (the enzyme) uricase but we are as yet unable to say what

the chemical nature of uricase is, and we cannot detect its presence by any chemical test other than the formation of allantoin from uric acid according to the equation below. Alkaline permanganate oxidises uric acid to allantoin, but alkaline permanganate is not uricase. There are several points of distinction between the two types of oxidation. In the first place, uricase catalyses the oxidation according to the equation



Here it is molecular oxygen which performs the oxidation, under the influence of the enzyme which is unaffected by the reaction, and which hence can transform an indefinite amount of uric acid in presence of excess oxygen. In the second place, boiling the enzyme solution stops its action. When alkaline permanganate performs the oxidation, the equation followed, viz.



shows that the amount of uric acid oxidised is strictly proportional to the amount of alkaline permanganate used. We must remember then that the enzyme is

(*a*) a catalyst and attacks an indefinite amount of uric acid ;

(*b*) destroyed by heat.

There is a third attribute of enzymes which must be emphasised, namely their inability to dialyse through parchment and other membranes. In other words they are colloids. Thus we come to the definition of an enzyme as a colloidal catalyst which is destroyed by heat and produced by living cells.

Besides catalysing oxidations, enzymes accelerate other changes such as hydrolysis of an ester, say tristearin to glycerol and stearic acid.

$$\begin{array}{l} CH_2O\cdot OC\cdot C_{17}H_{35} \\ | \\ CHO\cdot OC\cdot C_{17}H_{35} \\ | \\ CH_2O\cdot OC\cdot C_{17}H_{35} \end{array} + 3H_2O \longrightarrow \begin{array}{l} CH_2OH \\ | \\ CHOH \\ | \\ CH_2OH \end{array} + 3C_{17}H_{35}\cdot COOH$$

Thus oxidising enzymes are merely a group of cell catalysts, and we shall take it as demonstrated that cell-free extracts contain systems catalysing the oxidation of many substances normally found in the cell. That these systems are directly concerned in respiration and the production of mechanical work is less clearly established. Thus the enzyme uricase catalyses (as we have already seen) the oxidation of uric acid to allantoin, but it is unlikely that the organism uses this reaction as a source of mechanical work. The uric acid seems, as it were, to be chemical débris and the enzyme plays the part of a scavenging system. It is more probable that the lactic oxidase present in muscle is directly concerned with respiration and mechanical work.

We already know that enzymes act by accelerating chemical processes. Physical chemistry has taught us that at any one moment only a few molecules are in a reactive state. Thus if a chemical reaction be proceeding in a gas mixture only a certain (very small) number of molecules can react at any time, and of the potentially reactive ones, only a few actually do so react. When a catalyst is introduced into a system (as, for example, platinum black into a mixture of oxygen and hydrogen) the velocity of

combination is enormously increased. Exactly how catalysts act is still a matter of debate. Michaelis and Menten [1913] have suggested that when yeast saccharase acts upon sucrose and yields glucose and fructose

$$\underset{\text{sucrose}}{C_{12}H_{22}O_{11}} + H_2O \longrightarrow \underset{\text{glucose}}{C_6H_{12}O_6} + \underset{\text{fructose}}{C_6H_{12}O_6}$$

the enzyme forms a labile compound with sucrose which then adds on water and breaks down to glucose and fructose and free enzyme, which again combines with sucrose, and so on, till the reaction is finished. Haber and Willstätter have recently suggested another type of mechanism for the action of certain enzymes. The reader will do well to read the articles by Haldane and by Richter, for further information.

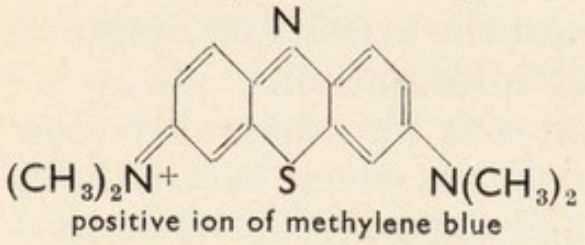
The oxidation of succinates is immeasurably slow at ordinary temperatures. Yet when succinate is added to a chopped, washed preparation of striped muscle, which respires slowly, the rate of oxygen uptake is greatly accelerated: the tissue has catalysed the oxidation of the succinate. If a great excess of succinate be added, then the rate of oxygen uptake is proportional to the amount of tissue, i.e. to the amount of catalyst, added. Thus if there be a large amount of metabolite present, the rate of oxygen uptake is governed by the amount of catalyst, which is said to be the "limiting factor". Anything which reduces the amount of this limiting factor will reduce the rate of oxygen uptake. Thus (to anticipate results later described) urethanes and cyanide inhibit cell respiration by knocking out the respiratory catalysts.

Every oxidation of any sort is accompanied by a corresponding amount of reduction, and conversely so. It is impossible to oxidise any substance without reducing an equivalent amount of another. Similarly if any substance be reduced some other molecule has to be oxidised. Thus when butyric acid is burned

to CO_2 and H_2O the acid is oxidised and the oxygen required is reduced,

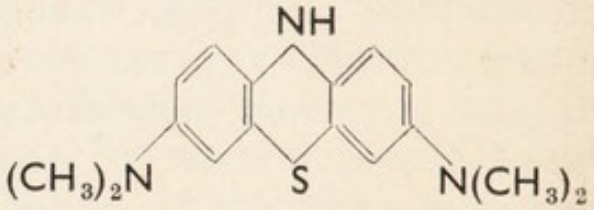
$$CH_3{\cdot}CH_2{\cdot}CH_2{\cdot}COOH + 5O_2 \longrightarrow 4CO_2 + 4H_2O$$

Every cell can perform oxidations. This also implies that it can carry out reductions too. In the case of living organisms the substance reduced is usually molecular oxygen. In exceptional circumstances other substances are reduced. Thus certain bacteria grow only in the presence of H_2S and of CO_2. Illumination of the bacteria results in reduction of the CO_2 and oxidation of the H_2S [Van Niel]. When methylene blue is added to a suspension of, say, B. *coli*, and the air removed from the suspension by evacuating, the methylene blue is rapidly reduced,[1]

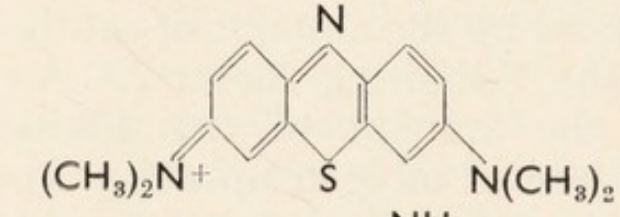


positive ion of methylene blue

$+ 2H \longrightarrow$

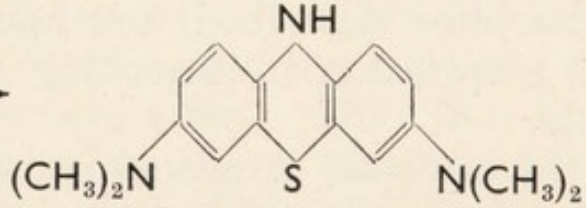


[1] Methylene blue chloride in neutral solutions dissociates into a positive cation and a chloride ion. The positive cation is the molecular species with which we are concerned. The following process takes place on reduction :—



$+ Cl^- + H + \ominus + H^+$

$\longrightarrow$

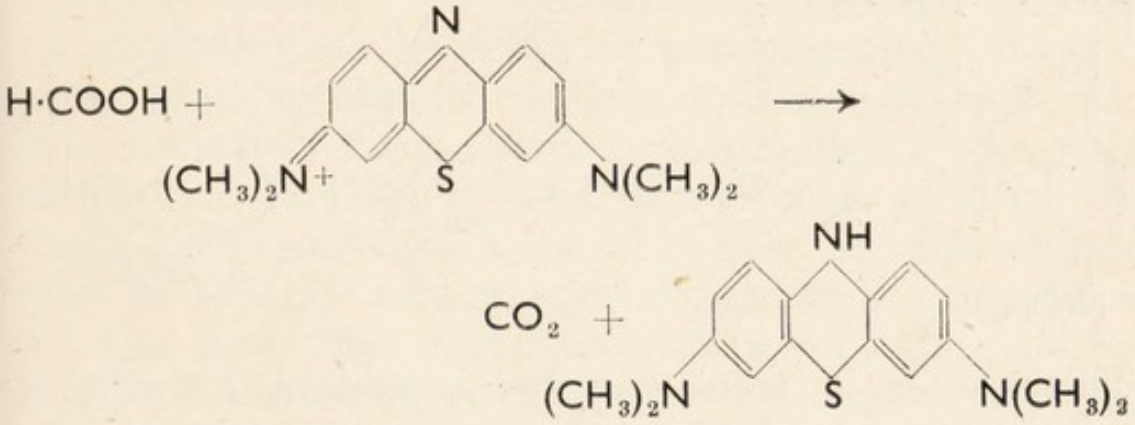


$+ HCl$

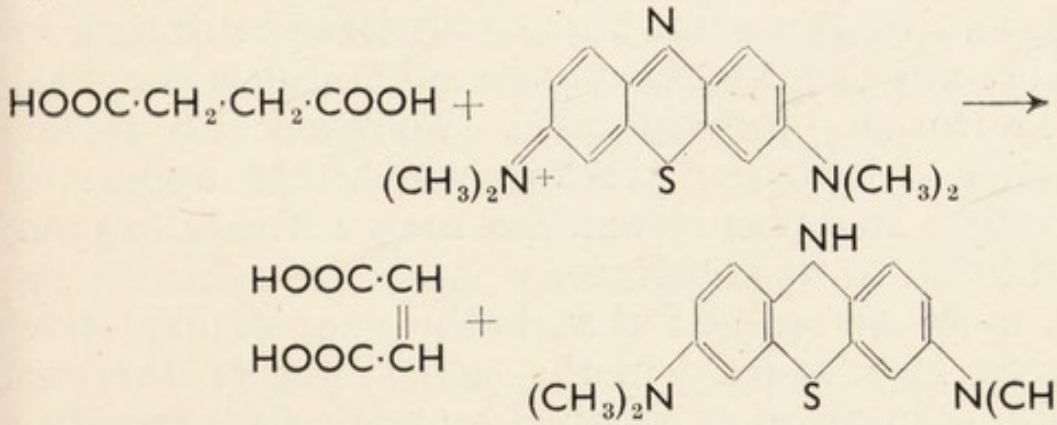
As the equation indicates, the reduction involves the transfer of one H atom and one electron. The remaining H^+ ion simply neutralises the Cl^- which is liberated by the conversion of the pentavalent nitrogen to the trivalent form. Thus only one H atom enters the methylene blue molecule on reduction although two equivalents of hydrogen are required.

leucomethylene blue being formed. The cell has reduced methylene blue instead of oxygen. Other substances can be used besides methylene blue and very many other cells may be used besides B. *coli*. One can use suspensions of chopped, unwashed muscle. Spermatozoa reduce methylene blue as they do nitrobenzene [Lipschitz]. Cell extracts likewise reduce dyes, and so do many secretions. Milk, for example, reduces methylene blue in the presence of aldehyde, but not in its absence.

Now when methylene blue is reduced to leucomethylene blue the net result of the reaction is as though two hydrogen atoms had been transferred to the dye from the substance oxidised. This is true no matter what substance is oxidised or what is the catalytic tissue. It is true when B. *coli* catalyses the reduction of methylene blue by formate,



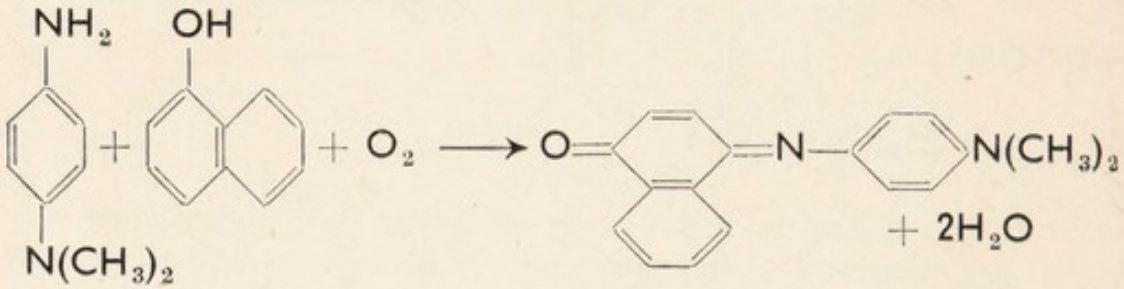
or when muscle tissue catalyses the reduction of the dye by succinate,



Because of this *apparent* transfer of two atoms of

hydrogen from the molecule oxidised to that reduced, the catalysts concerned are known as " dehydrogenases ". The system acting on succinate is called succinic dehydrogenase, that on lactate lactic dehydrogenase and so on. The substance on which each enzyme acts specifically is known as its " substrate ". Thus succinate is the substrate for succinic dehydrogenase, formate for formic dehydrogenase. These dehydrogenases appear to catalyse the transfer of hydrogen from one molecule to another.

Not only can cells reduce added dyestuffs: they can also perform oxidations on added substances. For example yeast, after it has been warmed in aqueous suspension to 52° for 1½ hours, can cause the formation in air of indophenol blue from dimethyl-*p*-phenylenediamine and α-naphthol. Many other cells act likewise, muscle for example, and brain. The action is



This action is catalysed by a system known as " indophenol oxidase ". Similarly other systems can be obtained from plants, and also from milk and various tissues, which have the power of oxidising guaiacum to guaiacum blue or nitrites to nitrates, in the presence of hydrogen peroxide. Such enzymes are known as " peroxidases ". They act only in the presence of hydrogen peroxide which they appear to activate. Indophenol oxidase may activate oxygen in addition to its substrate.

It must be realised that both types of oxidative catalyst may co-exist in the cell. Thus on the one hand a suspension of chopped muscle reduces methylene blue anaerobically (in the absence of oxygen),

while on the other hand the same suspension washed free of metabolites gives a strong reaction for indophenol oxidase when α-naphthol and dimethyl-*p*-phenylenediamine are added in alkaline solution. Brain behaves similarly. The reduction of methylene blue by tissues, and the oxidation of α-naphthol and dimethyl-*p*-phenylenediamine in alkaline solution (the combination is known as the " Nadi " reagent) do not, of course, represent oxidations which take place in the normal cell ; the two reagents, methylene blue and Nadi, are used merely as tests for catalysts, the dye for dehydrogenases and the Nadi for the oxidase. There is then a certain element of artificiality in much of the work which has been carried out on biological oxidation systems. The ideas of Wieland in particular have been reached by the medium of observations carried out upon systems of an entirely artificial nature. His experiments involved the use of reagents such as colloidal platinum and colloidal palladium, substances which could never enter into cellular respiration. Analogy has here played a part much greater than is usual in scientific theory. In itself there is nothing harmful in this, but the acceptance of analogy as a weapon of research (and above all the fact that the analogies seem often to be justified) has led to the uncritical acceptance of observations based on systems derived from living matter as applicable to the respiratory mechanisms of the normal cell. The mere fact that from the cell a system can be derived which gives an oxygen uptake when certain metabolites are added does not in itself justify the acceptance of this system as a respiratory catalyst. Thus haemoglobin, besides transporting oxygen, has the power of acting as a peroxidase and decomposing hydrogen peroxide with the production of oxygen in an active state. Yet in the blood it is unlikely that it ever exerts this enzymic activity, which it possesses in virtue of the haematin group. It must thus be borne in mind that many of the

catalytic activities possessed by cellular extracts are the expression of chance properties residing in molecules of quite other biological significance. It is for this reason that the observations of Keilin and of Warburg, consisting as they do for the most part of studies on whole yeast respiring more or less normally, acquire a significance greater than that of other work, carried out it may be on artificial extracts of various organs and tissues which have been irreversibly damaged by the processes inflicted upon them. Respiration is the organised oxidation of organic metabolites, whereby work may be derived and turned to the use of the cell. That so little is known of the processes in the normal cell, and that so much has to be inferred by analogy, is in itself unfortunate. Equally unfortunate is it that the differences between the respirations of different tissues are so little understood. It is, for example, impossible to describe the respiration of nervous tissue : the fact is that almost literally nothing is directly known on the subject. Whether the respiratory mechanisms of, say, liver, resemble those in brain is a matter for the future. Yet the problem is perhaps less complex than is here implied. There are certain mechanisms which almost certainly enter into the respiration of muscle tissue. These mechanisms are also present in brain. Though we have no definite knowledge of how they act there, it is being too critical to doubt their activity in brain, and introducing unnecessary complexity to regard them as possessing quite different functions in the two cases. We may study such systems in muscle, or in yeast, and apply the results to nerve and brain, without, however, suggesting that the two systems act identically in the two cases.

Let us recount what these systems are, common to both brain and muscle, or yeast. There are

(1) dehydrogenase systems ;
(2) the " Atmungsferment " of Warburg ;
(3) oxidases and peroxidases ;

(4) Keilin's cytochrome ;
(5) glutathione.

We shall discuss these systems in detail in the succeeding parts of this book.

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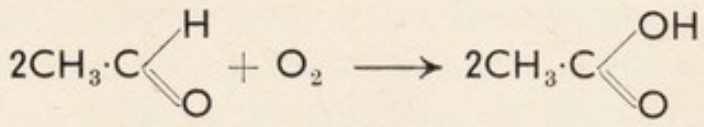
CHAPTER II

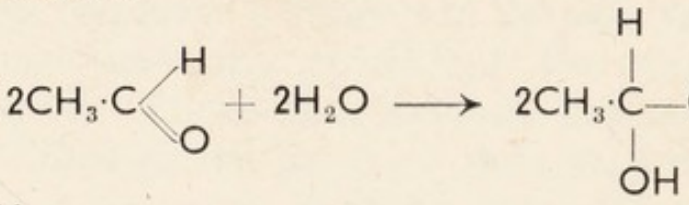
DEHYDROGENASE SYSTEMS

WE have seen in the introduction that every oxidation is accompanied by an equivalent reduction, and every reduction by the corresponding amount of oxidation. Thus when acetaldehyde oxidises in aqueous solution in the presence of oxygen we may write the action

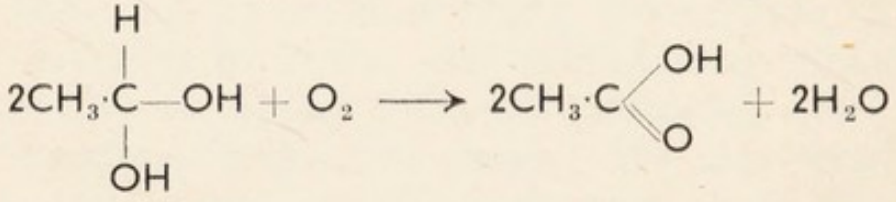


. . (1)

Here acetic acid is formed as end product, acetaldehyde is oxidised and oxygen reduced. We may also write the action

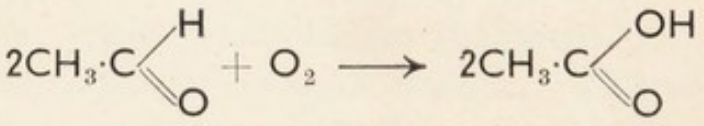


. (2*a*)



(2*b*)

and by adding the equations together we get the net result



. . (2)

which is identical with equation (1). Now in the first equation (1) we regard the oxidation taking

place by the *addition of oxygen*. According to equations (2*a*) and (2*b*) we regard the reaction as taking place first by the formation of an aldehyde hydrate and then (2*b*) by the *removal of hydrogen*. The net equation (2) formed by adding (2*a*) and (2*b*) is, however, identical with (1). This example teaches us three things :

(1) that the same end product (acetic acid) can be formed in equivalent quantity by different paths ;

(2) that the net equation tells us nothing of the actual molecular mechanism and the intermediate products ;

(3) that the oxidation of aldehyde by molecular oxygen can be pictured equally well as taking place by the *addition of oxygen* or the *removal of hydrogen* (from the aldehyde hydrate).

These statements apply equally to molecules other than aldehydes. Thus we can make the general statement that the mechanism of oxidation of organic molecules by molecular oxygen can be regarded as occurring by the addition of oxygen to the molecule concerned or by the removal of hydrogen from that molecule or from its hydrate.

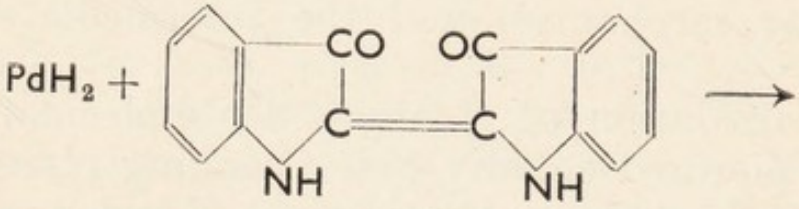
The view that the essential mechanism is the activation and removal of hydrogen has been advanced and maintained by Wieland [1912 and onwards]. Though Wieland's ideas have been based on a series of model experiments, with artificial reagents never, or rarely, found in the cell, yet the work of Thunberg [1920] with muscle has shown how closely analogous systems may be found in biology, and may actually play their part in cellular respiration. The views of Wieland must then be regarded as much more than mere speculations based on analogy. Let us briefly review what these ideas are, and how they were produced.

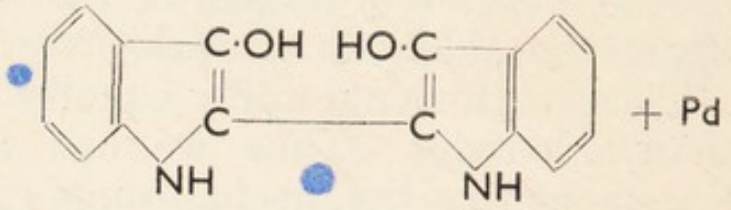
Wieland's *basic* experiment appears to be this. When an aqueous solution of aldehyde is shaken anaerobically with a suspension of palladium (palla-

dium black) the palladium becomes charged with hydrogen and the aldehyde is oxidised. The net equation is

$$CH_3{\cdot}CHO + H_2O + Pd \longrightarrow CH_3{\cdot}COOH + PdH_2$$

the reaction coming to an end when the metal has become saturated with hydrogen. If, however, another reducible molecule is present, the palladium loses its hydrogen and the second molecule becomes reduced, e.g.,





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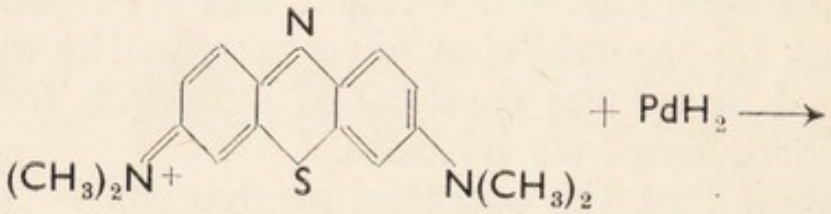

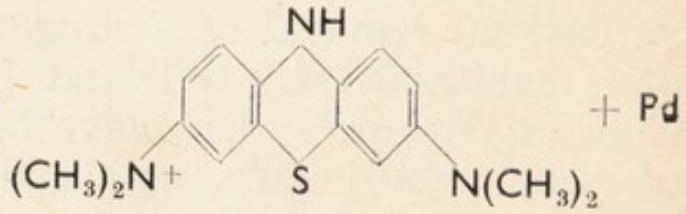

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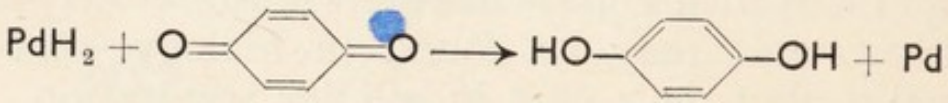
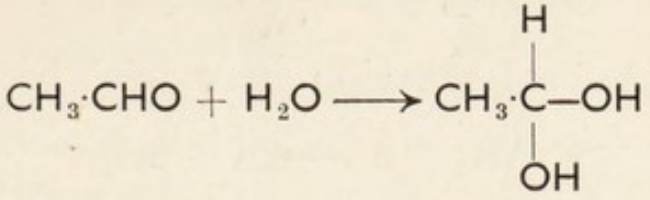


indigo white, leucomethylene blue, and *p*-hydroquinone being formed in the three respective cases from the three respective precursors, indigo blue, methylene blue, and *p*-quinone.

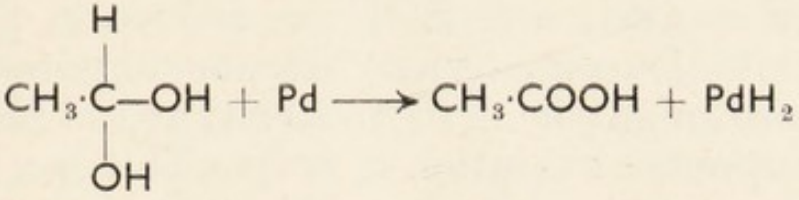
Let us consider this reaction more closely. A number of points must be emphasised.

(*a*) Wieland believes that a hydrated form of

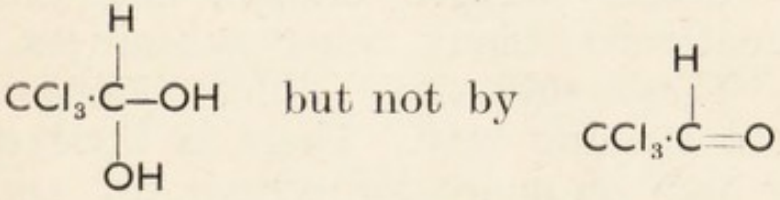
aldehyde reacts, and that the essential mechanism is the formation of an aldehyde hydrate, thus—



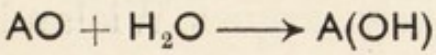
and the dehydrogenation of this hydrate by the palladium, thus—



In support of this view he was able to show that silver oxide was reduced in benzene solution to silver by chloral hydrate but not by chloral, i.e. by



(*b*) Though the oxidation of aldehyde results in the addition of oxygen to the –CHO group, the fundamental process consists in the activation of the hydrogen in the acetaldehyde hydrate previously formed. The affinity of the molecule for hydrogen is thus reduced and hydrogen passes to palladium. The formation of a hydrate is an assumption to satisfy the needs of this particular case, and with many, possibly the majority, of molecules Wieland regards the activation and transfer of hydrogen as taking place without any previous addition of water to give a hydrate. Thus, though in the particular case of aldehydes two reactions are involved, firstly the formation of a hydrate



followed by the dehydrogenation of the hydrate formed

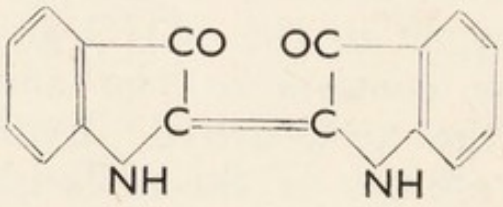
$$A(OH)_2 + B \longrightarrow AO_2 + BH_2$$

the more general reaction may best be written,

$$AH_2 + B \longrightarrow A + BH_2$$

In these equations AO stands for aldehyde, $A(OH)_2$ for aldehyde hydrate, and B for oxygen, indigo blue, methylene blue, or quinone. A represents acetic acid, and BH_2 the reduction product, indigo white, leucomethylene blue, or *p*-hydroquinone as the case might be. It is clear that the reduction of B involves the oxidation of AH_2. This is a general law of chemistry.

(*c*) Only some organic molecules can be oxidised in this way (with palladium black in anaerobic conditions). Yet a large number of substances can be reduced. Besides oxygen, indigo, and the others before mentioned, many other substances can be reduced. These substances differ amongst themselves in a striking way. There is little chemical similarity between molecular oxygen, O_2, and indigo blue,



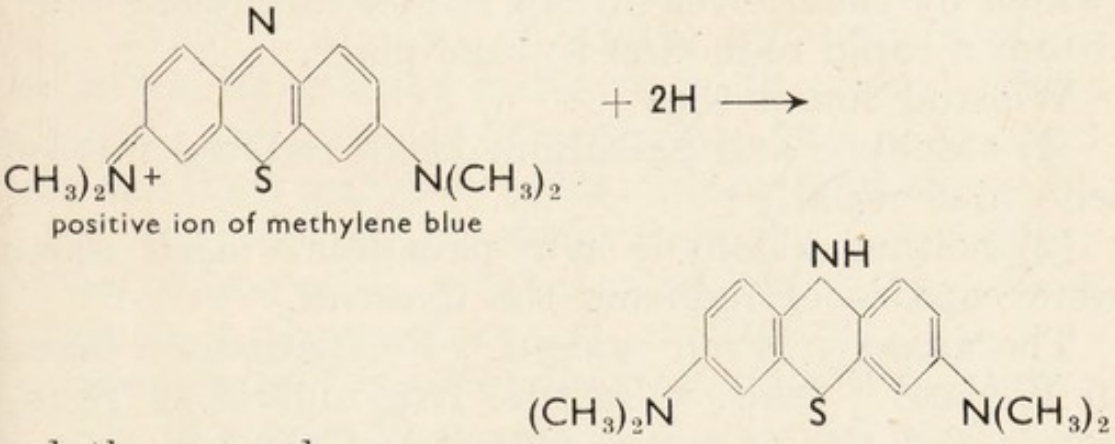
All they possess in common is their power of being reduced.

(*d*) The metal acts as catalyst and will cause the oxidation of an infinite amount of aldehyde, and the simultaneous reduction of an equal amount of oxygen, or quinone, or other substance. It thus has the characteristics of an inorganic ferment, and may be recovered at the end of the reaction in the same state as it was at the beginning.

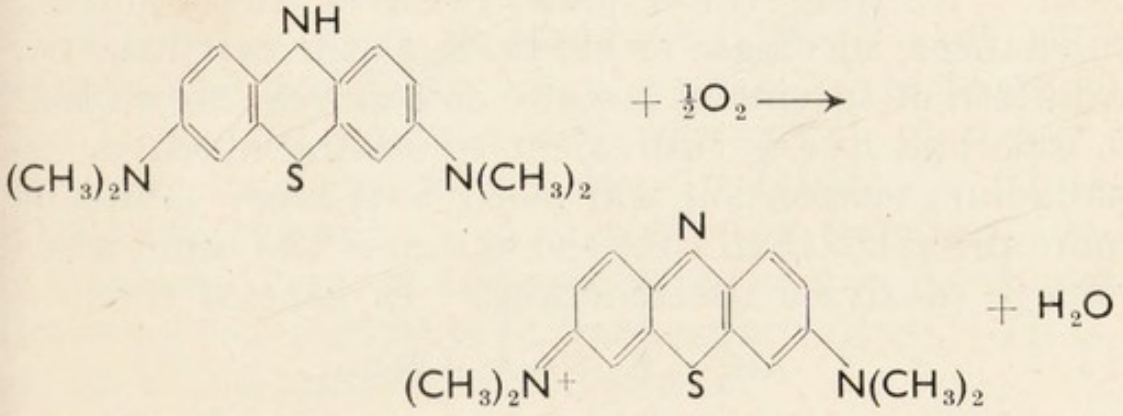
In some such way, argues Wieland, by the "acti-

vation" and removal of hydrogen from organic molecules, biological oxidations are carried out.

The work of Thunberg [1920] has shown that this idea may actually apply to cellular respiration mechanisms, and may represent what actually takes place. Thunberg studied the reduction of methylene blue by frog muscle. In order to do this he devised a special technique which has been applied widely within recent years. The method is simple, and the theory also. When methylene blue (which yields an intensely blue solution at dilutions of say 1/5000) is added to a suspension of chopped, but otherwise untreated, frog muscle and the whole system evacuated, a rapid reduction to colourless leucomethylene blue results. On shaking the colourless system with air, the blue tint is rapidly developed again as the leucomethylene blue reoxidises back to the dye. The first reaction is :



and the second



(The technique employed is described in the appendix.)

What Thunberg found was this. Unwashed chopped frog muscle reduces methylene blue rapidly under anaerobic conditions; washed frog muscle reduces methylene blue with extreme slowness if at all. Evidently merely washing the system destroys the power to reduce the dye. But (and this is the crux of the matter) the addition of substances such as sodium succinate, restores the reducing capacity enormously. What is the analogy between this system and that of Wieland? The answer is not far to seek. It may be best to recapitulate the facts.

Thunberg observed

(1) that chopped frog muscle reduces methylene blue rapidly;

(2) that washed frog muscle is unable to reduce the dyestuff; and

(3) that the addition to the washed chopped muscle of sodium succinate and various other substances (which by themselves do not reduce methylene blue) causes a rapid reduction to take place.

Wieland found that

(1) aldehyde and palladium black reduced methylene blue rapidly;

(2) neither aldehyde nor palladium black alone were capable of reducing the dyestuff.

The analogy is self-evident. For palladium black in Wieland's work, substitute frog muscle in Thunberg's and the apparent identity of the schemes is clear. Washing the muscle removes the reducing substances, succinate or aldehyde as the case may be. Addition of succinate, lactate, or aldehyde to washed muscle and dye is equivalent to adding aldehyde to palladium suspension and methylene blue. What is more probable than that the action is the same when muscle catalyses the reaction? In general then

$$AH_2 + B \longrightarrow A + BH_2$$

If AH_2 stands for succinic acid then fumaric acid might be expected to be formed. That this is so

was shown by Einbeck [1919]. The action is then

$$HOOC{\cdot}CH_2{\cdot}CH_2{\cdot}COOH + B \longrightarrow HOOC{\cdot}CH{:}CH{\cdot}COOH + BH_2$$

In such reductions by tissues and saturated substances like succinic acid other molecules besides methylene blue may be reduced; Abelous and Gerard [1899] used nitrates; Lipschitz [1921] used nitrobenzene; Mansfield Clark, quinones and oxidation-reduction indicators. Keilin [1929] has found a naturally-occurring substance, universally distributed amongst the tissues of higher organisms and present in almost all aerobic cells, which is rapidly reduced by washed muscle in the presence of succinate, but by neither muscle nor metabolite singly. This substance he calls "cytochrome", and it is apparently identical with the myohaematin, or histohaematin, of MacMunn. We shall see the significance of this material later.

Besides succinic and lactic acids and aldehydes, very many substances of widely different chemical nature can be oxidised in this way by washed tissues or cells in the presence of methylene blue. Amongst these are the lower fatty acids, dibasic aliphatic acids, sugars and their simple derivatives, e.g. hexosephosphates, amino acids, some purines and so on. Even molecular hydrogen can be oxidised in this way [Stephenson and Stickland, 1932]. Thus the action of dehydrogenase mechanisms is exerted upon very many chemical types, and many workers have each contributed a share in extending the list of substances oxidised. We must regard "dehydrogenation", i.e. the enzymatic decolorisation of methylene blue, as a general function of living cells which extends to substances of almost every chemical sort. In each case two, or some multiple of two, hydrogen atoms are removed from the molecule oxidised. Thus, we have the quite general reaction

$$RH_{2n} + n\mathrm{MB} \longrightarrow R + n\mathrm{MBH}_2$$

where RH_{2n} is the material oxidised, MB stands for

methylene blue and MBH_2 for leucomethylene blue. n is a whole number and in general $n = 1$.

Not only are all sorts of molecules oxidised by methylene blue in the presence of dehydrogenases, but very many types of organism possess such enzymes. Thunberg [1923] has studied the distribution of dehydrogenases in mammalian tissues. He finds them present in muscle, smooth and striped, in heart, in liver, in kidney, in nerve tissue, even in eye lens ; in short, their distribution is universal in mammalian tissues. They are found generally in vertebrates ; they are found generally in invertebrates ; and they are possessed by unicellular organisms. Thus many dehydrogenase systems have been extracted from yeast by Bernheim, Harrison and others, while Stephenson and her collaborators have studied several in B. *coli*. There is no reason to doubt their universal occurrence in bacteria. Holmes and Pirie [1932] have found that the ultrafilterable virus of bovine pleurine pneumonia is able rapidly to decolorise methylene blue in the presence of lactate. In short, dehydrogenases act on very many substances and they are universally distributed.

Besides methylene blue many other substances can be reduced by dehydrogenase systems : Lipschitz [1921] used *m*-dinitrobenzene ; Bertho [1929] used quinone. It is clear, however, that whether a substance is reduced or not depends essentially on the nature of the molecule oxidised rather than on the catalytic mechanism involved. If the oxidation of A to B has insufficient energy to reduce C to D, then the action *will not* occur, whereas if the energy exchanges are sufficient to reduce E to F, the action *will* occur, perhaps immeasurably slowly, perhaps rapidly if the appropriate catalyst be present and if the mechanism of oxidation permits of it.

The question next arises as to the multiplicity of dehydrogenases. We may ask, to put the question in its most extreme form, does one dehydrogenase

catalyse all dehydrogenations? Does one and the same enzyme catalyse the oxidation of succinic acid and of, say, glucose by methylene blue? The answer to so extreme a question is not far to seek. Bernheim [1928], for example, succeeded in separating these dehydrogenases. He extracted washed muscle, yeast and various plants, and obtained four different fractions, which we will call *A*, *B*, *C*, *D*. As a result of his observations the following table can be drawn up, where + means a rapid reduction on adding the metabolite and 0 no reduction, i.e. the presence or absence of the appropriate catalyst.

Extract	Succinate	Lactate	Citrate	Aldehyde
A	+	0	0	0
B	0	+	0	0
C	0	0	+	0
D	0	0	0	+

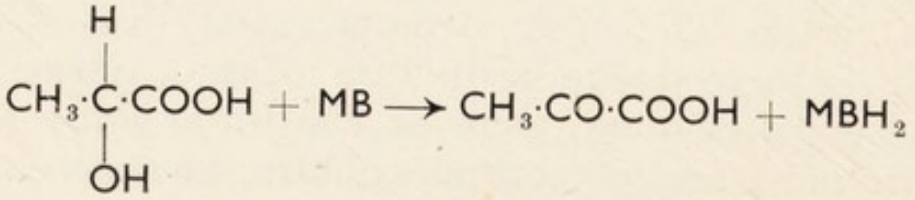
Thus when succinate was added to fraction *A*, a rapid reduction of dyestuffs occurred, but not when succinate was added to the other extracts. This was because fraction *A*, and fraction *A* alone, contained the dehydrogenase which activates succinic acid. Similarly citric acid is activated by fraction *C* alone. Hence it appears that each type of organic molecule oxidised has its own *catalyst*, but it is a matter of indifference which *substance* is used to oxidise it. Only fraction *B* *catalyses* the oxidation of lactate, but methylene blue, nitrobenzene or cytochrome can *oxidise it stoichiometrically*, becoming themselves quantitatively reduced. These substances are often known as hydrogen acceptors, the molecules oxidised, i.e. the lactate or succinate, as hydrogen donators. Again, when Thunberg demonstrated that washed muscle catalysed the reduction of methylene blue by many substances, such as the lower fatty acids, glutaric acid, and glycerophosphates, he succeeded in

destroying the different catalytic centres by cooling the muscle and then thawing it. He found that according to the temperature reached one or more of the substrates lost its power of reducing methylene blue, i.e. that one or more of the centres of activation had been destroyed.

It is hence quite clear that many different dehydrogenases exist, and thus having obtained an answer to the question " Is there only one dehydrogenase ? " we now ask, " Is there to each different substrate activated an appropriate specific catalyst ? " This is a much harder question and to answer it we must consider the work of Quastel. In actuality the cell has compromised. There is more than one dehydrogenase, and fewer dehydrogenases than substrates activated. Let us consider Quastel's work and see how he has demonstrated this.

When B. *coli* is grown on certain media, and washed free from reducing material, a suspension can be made in saline which reduces methylene blue very slowly, and reduces it rapidly when certain substrates are added to it. What Quastel did was this : to a washed suspension of B. *coli* he added methylene blue and a solution of a substrate, and incubated the whole in the absence of air, as a result of which the methylene blue became rapidly reduced (or not, as the case might be). Now of about 100 substrates tested in this way, almost 60 were found to reduce the methylene blue. Let us restate our question : " Is there to each of the 60 substrates active in reducing methylene blue a specific catalyst accelerating its oxidation ? "

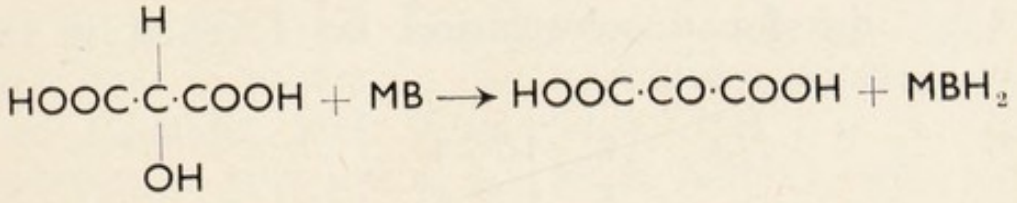
Suppose we add lactate to the suspension of bacteria and find the dye to be reduced in time t_1, the action is



and for the velocity of reduction we have the equation

$$v_1 = \frac{1}{t_1}$$

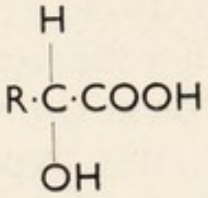
Now suppose we add, to another sample of the same bacterial suspension, a solution of hydroxymalonate and find the dye reduced in time t_2, then we have the equations



and

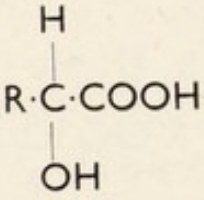
$$v_2 = \frac{1}{t_2}$$

There is a certain similarity in structure between lactic acid and hydroxymalonic acid ; both can be written



In the case of lactic acid R is CH_3–, for hydroxymalonic it is HOOC–.

We can now state our problem again in terms of structural organic chemistry : Is there one enzyme which catalyses the oxidation of substances of the general type



or is there an enzyme specific for each modification of R, i.e. one specific to lactate (when R is $-CH_3$) and one for hydroxymalonate (where R is –COOH) ? Suppose there is an enzyme for each molecule. Then

if solutions of lactate and of hydroxymalonate be simultaneously added to a washed suspension of B. *coli* and incubated in the presence of methylene blue, the rate of reduction of the dye should be the sum of the rates of reduction with hydroxymalonate and lactate individually, i.e. if V be the velocity of reduction

$$V = v_1 + v_2$$

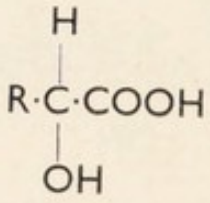
If the time for decolorisation be T, then if this hypothesis be correct,

$$\frac{1}{T} = \frac{1}{t_1} + \frac{1}{t_2}$$

or

$$T = \frac{t_1 t_2}{t_1 + t_2}$$

Now when Quastel carried out such experiments he found no such relationship. He was unable to find evidence that two catalysts existed, specific the one for lactate and the other for hydroxymalonate. How can this be explained from the view that one catalyst only exists, affecting the type R·CH(OH)·COOH? If only one such centre exists, it will combine both with lactate and hydroxymalonate, and the partitioning of the enzyme between those two species will depend on the relative affinities for each. Hence the rate of reduction of methylene blue will be intermediate between t_1 and t_2, as was indeed observed. It appears then that one centre catalyses the oxidation of



Suppose now we add a molecule of a different type, say succinic acid, to the suspension of B. *coli*. The reduction time is t_3. Now in the combined presence

of succinic acid, $HOOC{\cdot}CH_2{\cdot}CH_2{\cdot}COOH$ and of lactic acid, $CH_3{\cdot}CH(OH){\cdot}COOH$, the reduction time is actually

$$\frac{1}{T} = \frac{1}{t_3} + \frac{1}{t_1}$$

i.e. two centres are concerned. Thus it appears probable that the catalysts of B. *coli* are type specific rather than molecule specific.

So much for the identity of catalysts. Their multiplicity is easier to demonstrate. Suppose we expose [Quastel and Wooldridge, 1927] a B. *coli* suspension to the action of dilute $NaNO_2$ solution, and that we then centrifuge down the bacteria, wash and resuspend them in ordinary saline, and measure their catalytic powers thereafter. We obtain the following data :

Concentration of $NaNO_2$ to which the organism was exposed g. %	pH of exposure	Time of exposure (hours)	t glucose (mins.)	t succinic acid (mins.)	t lactic acid (mins.)	t formic acid (mins.)
1	6·8	22	60	∞	12·5	3·8
1	6·8	46	∞	∞	18	26
5	7·0	20	∞	∞	60	8
2	7·3	24	—	∞	33	54
1	6·8	30	—	∞	19	58

Here the nitrite has inactivated the succinoxidase after 22 hours' and the glucose oxidase after 46 hours' action. The separate identity of these catalysts is thus demonstrated. By the use of other reagents such as phenols, alcohols, potassium permanganate and so forth, Quastel and Wooldridge were able to demonstrate the separate identity of other catalytic centres. It is not improbable then that the muscle systems studied by Thunberg and others have a narrow range of specificity and each catalyses the

activation of a particular *type* of molecule. Quastel attempted to explain the dehydrogenation of organic molecules in terms of molecular polarity, and considered that the enzyme induced an electric strain in the molecule "activated" which led to drift of two protons. It seems, however, somewhat premature to speculate on matters of such complexity. Stephenson and Stickland [1932] have, however, obtained data of great interest with regard to the oxidation of formates by B. *coli*. They have shown that three enzymes can (at least potentially) be concerned in the dehydrogenation, and that the same end-products, namely CO_2 and leucomethylene blue, are formed from formates and methylene blue by entirely different paths. While they do not discuss the problem of intra-molecular activation, Stephenson and Stickland's observations have very real relevance with regard to the molecular path followed when formate is oxidised. They were able to demonstrate two separate enzymes from B. *coli* grown under certain definite conditions. These are

(*a*) hydrogenase,

(*b*) hydrogenlyase.

Hydrogenase acts on molecular hydrogen which then reduces methylene blue

$$H_2 + MB \longrightarrow MBH_2 \quad . \quad . \quad . \quad . \quad . \quad (1)$$

Hydrogenlyase liberates free molecular hydrogen from formate,

$$H{\cdot}COOH \longrightarrow H_2 + CO_2 \quad . \quad . \quad . \quad . \quad (2)$$

On adding equations (1) and (2), equation (3) is obtained. Is it not possible that the action of so-called formic dehydrogenase is due to the combined effect of formic hydrogenlyase liberating free hydrogen from formate and of hydrogenase then activating the molecular hydrogen with reduction of methylene blue ?

It is, however, possible to separate a formic dehy-

drogenase which catalyses the reduction of methylene blue in the presence of formate,

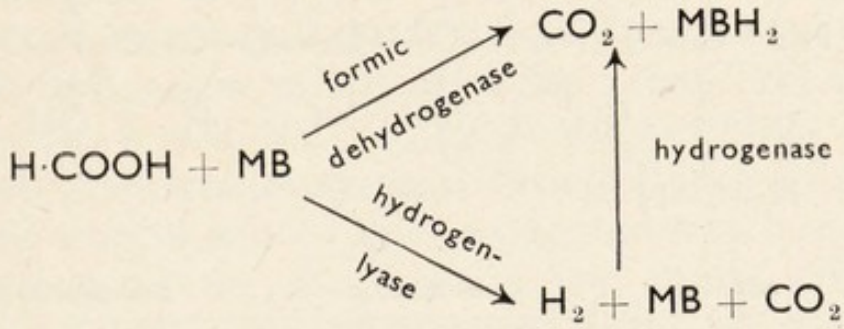
$$H{\cdot}COOH + MB \longrightarrow CO_2 + MBH_2 \quad . \quad . \quad . \quad (3)$$

but which is unable

(*a*) to reduce methylene blue in the presence of hydrogen,

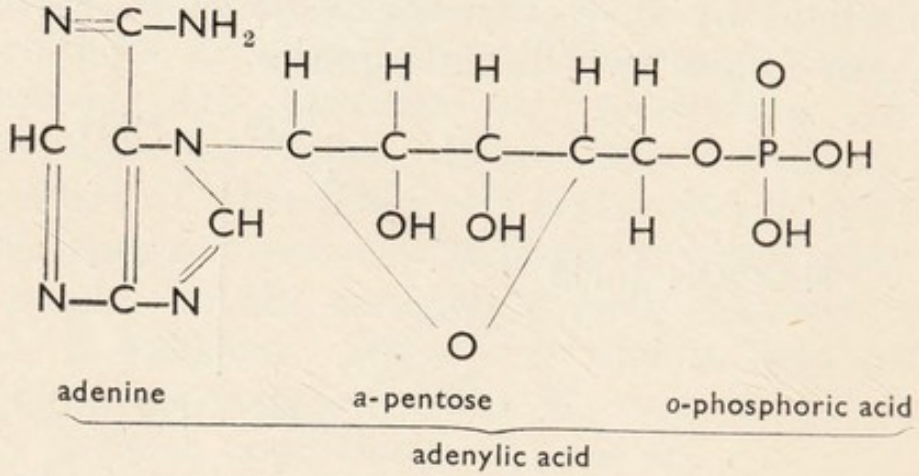
(*b*) to liberate free hydrogen from formate.

Thus the separate identity of formic dehydrogenase is demonstrated and we must accept the possibility that the oxidation even of so simple a molecule as formic acid by B. *coli* in the presence of methylene blue can follow two distinct paths



The idea of dehydrogenase does not, however, imply merely the notion of a heat-labile, colloidal catalyst, possessing catalytic properties of a certain type. The work of Szent-Györgyi has thrown light on the properties of lactic dehydrogenase. When muscle is washed well after thorough mincing, the addition of lactate fails to cause the rapid reduction of methylene blue. Meyerhof made acid extracts of boiled muscle, the so-called " Kochsaft ", and showed that it contained substances which increased the respiration of chopped muscle. This he attributed to the presence of oxidisable material therein, which he called the " Atmungskörper ". Szent-Györgyi has analysed Kochsaft more closely and has shown that besides the Atmungskörper it also contains a thermostable dialysable substance which he has succeeded in isolating. This substance has the property of accelerating the reduction of methylene blue by

lactate in the presence of washed muscle. It is thus a co-enzyme for lactic dehydrogenase. It is hard to say at present to what extent other dehydrogenases contain co-enzymes, and we must be content to remember that the term "dehydrogenase system" implies the complete system of colloidal enzyme, of its appropriate substrate, and of any dialysable co-enzymes which may be concerned. Szent-Györgyi's co-enzyme seems to be a mononucleotide, but its precise nature is at present undecided. The general formula of such molecules is shown below



The co-zymase of alcoholic fermentation (and perhaps vitamin B_1) appear to be of a similar chemical nature, and we thus appear to have some chemical connexion between the action of B_1 and of the co-enzyme of lactic dehydrogenase.

So far we have been content merely to accept empirical observations and limit ourselves to the statement that dehydrogenase systems (i.e. of enzyme, substrate, perhaps co-enzyme) reduce methylene blue anaerobically. We must now penetrate further and discuss their more intimate nature.

Before proceeding further, we must define in chemical terms if possible what we mean by the word "dehydrogenase". Now when Quastel studied dehydrogenations by "resting" bacteria, he was studying a surface phenomenon. The catalysts existed on the surface of the cells, and could not be

separated from them readily. Stickland showed that the tryptic digestion of B. *coli* led to a great increase in activity of the formic dehydrogenase. In other words, some of the enzyme was incorporated into the cell wall, some existed in the interior. The enzyme may be free or it may be built into internal structures. Penrose and Quastel studied dehydrogenations effected by M. *lysodeikticus*, a bacterium particularly sensitive to lysis. They found that intact washed cells exerted powerful oxidising activity towards the usual added metabolites, but that lysis of the cells destroyed the catalytic action. In other words, the activity of the enzymes depended on the integrity of the cell walls. Thus it seems impossible to define accurately what is meant by the term dehydrogenase when used for a catalyst incorporated into a cell wall. Nor is the problem much simplified when the enzyme is brought into solution. Thunberg and his school have extracted succinoxidase from muscle tissue, Stephenson has made cell-free solutions of lactic dehydrogenase from B. *coli* and so on. But in each case the enzyme is accompanied by impurities: when the impurities are precipitated, frequently the enzyme precipitates as well. It is true that Northrop has claimed to have produced crystalline pepsin and trypsin, and Sumner crystalline urease. In each case the crystals are of protein nature, but there are criticisms . . . and in any case, what is a protein? We are thus up against the problem, firstly, of obtaining a crystalline dehydrogenase, which has never been done, and, secondly, of defining its precise chemical nature, which is impossible if it is really protein. So when we speak of a solution of a dehydrogenase we really mean a system which exerts a more or less specific catalytic action and decolorises methylene blue in the presence of its substrate. It may well be that, for example, lactic dehydrogenase from B. *coli* is quite another enzyme to that from yeast. An enzyme cannot then

be defined by its chemical nature, but only by the action it exerts. It is evident then that the properties of a dehydrogenase solution will be considerably affected by the nature of the accompanying impurities, and will not be so reproducible as the properties of, say, normal hydrochloric acid in water.

Let us now see how a solution of a dehydrogenase can be prepared and handled. Suppose we remove the skeletal muscle from, say, a rabbit immediately after it has been killed (not by an anaesthetic), cool to 0° C., chop finely and wash with a large volume of water, repeating the process of washing several times, then we finally obtain a muscle preparation which contains little water-soluble material and which cannot reduce methylene blue. Suppose we suspend this mass of washed muscle in five times its weight of $M/15$ K_2HPO_4 and shake very gently at 10° for about an hour, then we obtain (after centrifuging down the muscle) a rather opaque solution of succinic dehydrogenase. On adding succinate the solution rapidly reduces methylene blue. Thunberg has obtained solutions of dehydrogenases from seeds in a similar way — by extracting the ground material with $M/15$ K_2HPO_4. What are the general properties of such solutions ? (It must clearly be understood that the following description is not intended to apply to any one specific enzyme.) In the first place the enzyme is non-diffusible, i.e. it does not pass through collodion or parchment membranes, and in the second place the catalytic activity is destroyed by heating to, say, 85° for 10 minutes. The enzyme has been made inactive by the high temperature. Now this destruction has been going on all the time, even at 0°, and the increase in temperature to 85° has merely increased the rate of inactivation. Thus solutions of dehydrogenases gradually lose their activity at rates depending on the temperature, on the particular enzyme, and on the individual specimen of that enzyme. The increase in velocity of inactivation by

a rise of 10° may be very great, perhaps 50 times, so that the effect is a large one. Now in the introduction we said that an increase in temperature increased the velocity of reaction in a system. Suppose now we add substrate to an enzyme, e.g. succinate to succinic dehydrogenase, and study the decolorisation of methylene blue at several temperatures. At each higher temperature there will be

(*a*) an increased rate of enzyme inactivation ;

(*b*) an increased velocity of reaction catalysed.

Thus there will be an " optimal temperature " where the effects counterbalance. This optimum temperature will be dependent on salt content and on the pH of the solution. In general, however, for pH 6–8 it is about 37°. Some catalytic mechanisms are highly resistant to heat. Tarr [1933] has recently studied the dehydrogenase mechanisms in the spores of B. *subtilis*, and finds them resistant to 30 minutes at 90°. This is probably true for the corresponding catalysts of thermophilic bacteria.

If the solution of enzyme be made very acid or very alkaline and allowed to stand at that reaction for some time and then brought back almost to neutrality, considerable destruction will be found to have taken place. Dehydrogenases are thus sensitive to excess acidity or alkalinity in a degree dependent on the particular catalyst and on the individual sample of that enzyme. Change in reaction of the solution also affects the rate of the catalysed reaction. Thus alteration in pH (pH is the negative logarithm of the H^+ concentration) affects an enzyme in two ways :

(*a*) it may alter the rate of the catalysed reaction ;

(*b*) it may destroy the enzyme.

It is thus clear that dehydrogenases are far from stable systems and cannot be left almost indefinitely as can museum specimens.

It is a little difficult to generalise here, and it must be emphasised that the precise conditions under which

an enzyme works in the cell are completely obscure. For one thing the pH of a cell is not necessarily that of the surrounding medium and for another it is unlikely that the pH is constant throughout. There may be interfaces where the acidity is high, or very low, where active catalyses occur.

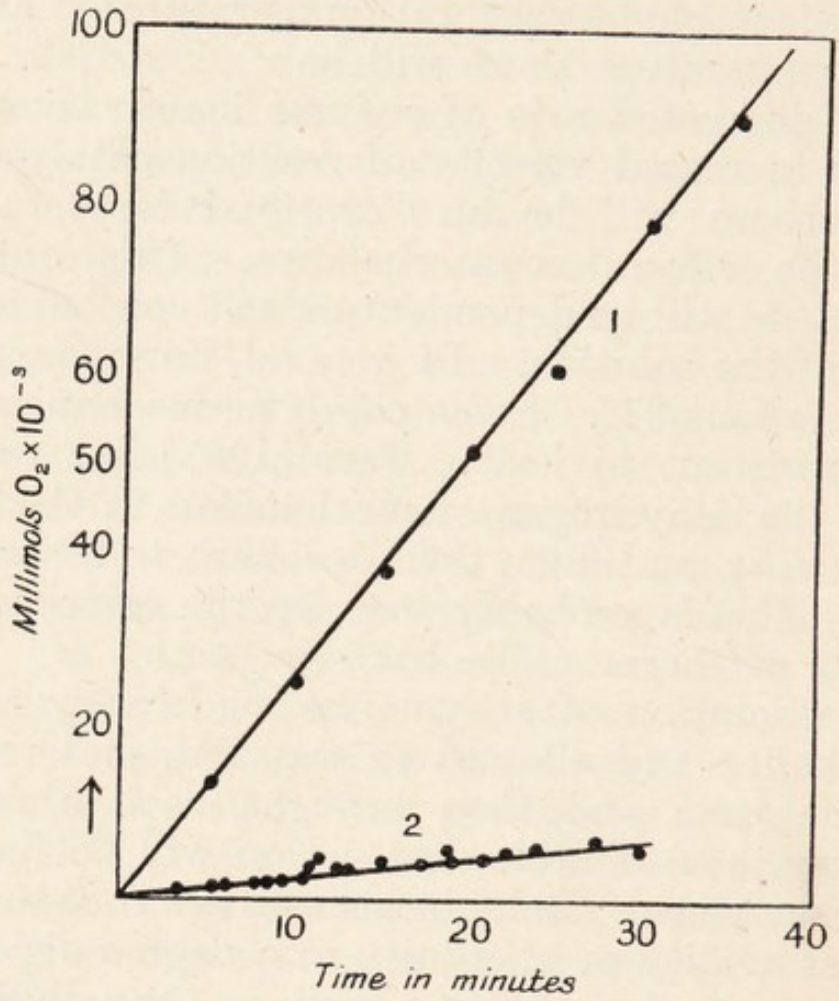


FIG. 1.—Comparison of oxygen uptake and methylene blue reduction by 100 mg. dry yeast in presence of $M/20$ ethyl alcohol. 2·5 cc. $M/200$ methylene blue added in second case. Curve 1 shows reduction of oxygen, curve 2 of methylene blue

(WIELAND and CLAREN [1932]. *Ann.* **492,** 183)

All these facts emphasise the difficulty of handling dehydrogenase solutions and of reconstructing their behaviour *in vivo* from that observed *in vitro*. The same type of behaviour is found with oxidases, with peroxidases and catalases, and these remarks must be taken to apply to these enzyme classes in general.

It must, however, be emphasised clearly that each different dehydrogenase must be studied individually and that there appears as yet to be no means of foretelling how any particular system will behave. We have previously spoken of dehydrogenase systems as reducing methylene blue, or indigo, or quinone, to leucomethylene blue, indigo white, or hydroquinone, and we have spoken of all three reactions being equivalent, and taking place with equal readiness. Now there are systems which can reduce methylene blue but not quinone : thus the succinic dehydrogenase of muscle rapidly reduces methylene blue, but not quinone ; the xanthine oxidase of milk reduces both dye and quinone, but with different rates. Nor is it merely a question of *chemical potential*, but much rather of *mechanism*. The glucose oxidase from Aspergillus which was isolated and studied by Müller [1931] oxidises glucose to gluconic acid

in the presence of oxygen, but not with methylene blue ; yet the glucose dehydrogenase of liver [Harrison, 1931] uses methylene blue with ease in order to form gluconic acid from glucose. Even when an enzyme system reduces several different substrates it does not follow that it reduces all at the same velocity, and the rate at which any one substance is reduced depends on a number of as yet indeterminate factors.

We have found that dehydrogenases, like most enzymes, are destroyed by excessive acidity or alkalinity and by heat : this is merely a result of their colloidal nature. There are however a number of reactions which appear to be limited (or almost limited) to these enzymes, and we shall now turn to those all the more as they may explain the behaviour

of the cell under certain exceptional circumstances. Most dehydrogenases appear to be inhibited

(*a*) by narcotics,

(*b*) by certain dyes,

(*c*) by traces of some heavy metals.

The action of narcotics is perhaps most specific, and dehydrogenases can sharply be distinguished from oxidases by their greater sensitivity to narcotics. The other reagents—dyes and heavy metals—are less specific in action.

Sen [1931] studied the action of narcotics on the reduction of oxygen and of methylene blue by the succinic dehydrogenase system. He found narcotics strongly inhibited the enzyme activity and compared his results with those of Warburg on the respiration of nucleated erythrocytes. The following table shows his data :

Concentration of Narcotic which Inhibits about 50 % (*M*)

Narcotic	Oxygen uptake in presence of succinic system	Respiration of nucleated erythrocytes	Reduction of methylene blue
Vanillin	0·011	0·02	0·022
Ethylurethane . .	0·65	0·33	0·6
Phenylurethane . .	0·003	0·003	0·002
Diethylurea . . .	0·35	0·52	0·2
Phenylurea . . .	0·028	0·018	0·028
Propionitrile . . .	0·48	0·36	—
Valeronitrile . . .	0·08	0·06	—

It must, however, be remarked that the xanthine oxidase of Dixon (which reduces methylene blue in presence of xanthine or hypoxanthine) was unaffected by these narcotics. Besides narcotics such as urethanes, inhibiting dehydrogenases, it seems probable that other substances act likewise. Sherif [1930] studied the action of caffeine and other substances upon the respiration of nervous tissue. He found the

oxygen uptake and the methylene blue reduction to be inhibited to the same extent. Quastel and Wheatley [1932] studied the action of hypnotics of the barbituric acid series and observed a somewhat similar effect. It hence appears likely that the action of narcotics and hypnotics may in part be exerted by their depressive action upon the dehydrogenase mechanisms of the nervous system. Similarly (as Quastel and his collaborators showed) antiseptics such as toluene or chloroform inhibit the surface catalysts of B. *coli* and may exert their " antiseptic " power in this way.

The effect of dyes upon bacterial dehydrogenases has been studied by Quastel and Wheatley [1931]. They measured the inhibition produced by about thirty dyes on the oxidation of glucose, succinate, lactate and formate by B. *coli*. They found that basic dyes alone were effective inhibitors and that there was a manifest specificity of behaviour in their effect on the enzymes. The behaviour of the dyes is illustrated in the following table :

PERCENTAGE INHIBITION BY DYESTUFFS OF OXYGEN UPTAKES BY B. *coli* IN PRESENCE OF VARIOUS SUBSTRATES
pH 7·4. Temp. 37°

Dyestuff (1/5000)	Glucose	Lactate	Succinate	Formate
Malachite green . .	100	92	72	71
Brilliant green . .	68	66	67	46
Methylene blue . .	57	33	75	28
Acid fuchsine . .	0	0	2	0
Congo red . . .	0	15	10	0

It may well be that the action of chemotheropeutic reagents is exerted (in some instances) upon dehydrogenase systems.[1]

[1] It is interesting to note that Voegtlin claims the action of arsenoxides is exerted by combination with suphydryl glutathione.

The "oligo-dynamic" action of some metals is well known. Thus traces of copper in solution inhibit the growth of bacteria, or of barnacles upon ships. Nothing is known as to the mechanism of this action. It is, however, plausible to suggest that the inhibition of growth is exerted by inactivation of catalytic mechanisms. Besides invertase [Myrbäck, 1926], carbonic anhydrase (unpublished observations of Meldrum and Roughton) and other enzymes, some dehydrogenases are sensitive to the action of metallic inhibitors. Wieland and Mitchell [1932] have shown that xanthine oxidase is particularly sensitive to Cu^{+}, Hg^{++}, Ag^{+} and Au^{+++}, and Quastel has demonstrated a like effect when Cu^{++} acts on B. *coli*.

To sum up—we must regard dehydrogenases as catalysts essential to the respiration of every aerobic cell, and which act upon the substrates oxidised rather than upon oxygen or upon the substance reduced.

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CHAPTER III

THE WORK OF WARBURG

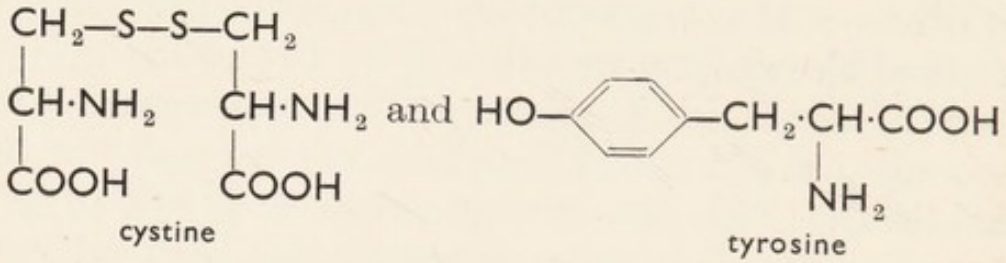
THE work of Warburg has been concerned mainly with the rôle of heavy metals in cell respiration. He has maintained and developed the thesis that iron in the form of a certain haematin derivative is intimately connected with the catalysis of cellular respiration. Recently, however, the views expounded by Warburg have tended to coalesce with those of Keilin, who, as we shall see later, has succeeded in incorporating the "iron mechanism" of Warburg and the "dehydrogenase system" of Thunberg and Wieland into one comprehensive scheme.

Warburg's work all ultimately turns on analogy, but on an analogy based on the behaviour of living cells. Let us see what these early observations are. The respiration of sea-urchin eggs is inhibited by HCN at extremely low concentrations and by urethanes at high. This respiration is accelerated by the addition of small amounts of iron salts, to the extent of 50–110 % when 0·01 mg. Fe is added at 23° C. to a cell suspension containing 22 mg. N. Addition of further amounts of iron increases the respiration but not in proportion. Now this induced oxidation was inhibited by urethanes in precisely the way as was the natural oxidation. Other tissues are, however, not affected by added iron in this manner, and Warburg assumed that free metallic ions have little catalytic activity, but that in the case of sea-urchin eggs there exists a small amount of some

compound which combines with added free iron ions to form the normal respiratory catalyst. He thus pictures the respiration as being catalysed by iron in combination with some unknown substance, and assumes that only in the case of sea-urchin eggs does the second substance exist free, in excess of the iron already in the cell. He regards the absence of effect when iron is added to other tissues as due to the absence of the second substance to combine with the free iron and form a catalytically active complex. The theory further explains the action of HCN in poisoning cell respiration, for it may plausibly be assumed that the cyanide combines with the catalytic iron compound to form an inactive complex.

Then Warburg developed his charcoal model. The first observations were published in 1913–14. Warburg described the system mentioned by Freundlich [1909]. When oxalic acid is shaken with water and blood charcoal, a rapid oxygen uptake occurs and the acid is oxidised. This observation in itself is not of great interest. The charcoal acts catalytically, that is all. A further study, however, yields matter of importance. Not only does the charcoal act catalytically, but it behaves like erythrocytes, or like sea-urchin eggs : it is poisoned by cyanide, and inhibited by urethanes just as are living cells. The urethanes are adsorbed in proportion to the extent to which they lower surface tension, and they inhibit oxygen uptake in the same way. Cyanide is scarcely adsorbed at all, yet it inhibits powerfully. There thus appears a close analogy between the behaviour of sea-urchin cells and the charcoal-oxalic acid system. The question arises, is it merely an analogy, or is there some common factor involved ? Warburg inclined to the second view. Let us compare the systems. The problem is to find a common factor to sea-urchin eggs, avian erythrocytes, and blood charcoal. It will help us in our search to remember that all three systems are inhibited by cyanides, and that the addition of

Fe to sea-urchin eggs accelerated their respiration. The answer to the problem lies in these two observations. Iron certainly exists in sea-urchin eggs, and it certainly exists in blood charcoal : it further exists in blood charcoal in small amounts so that even though cyanides combine with it, yet only a small amount of poison would be absorbed or adsorbed. Warburg assumed that the similarity between sea-urchin eggs and the charcoal system was more than a mere analogy and expressed a real similarity between the two cases, this similarity residing in the presence in both systems of catalytically active iron, bound in some complex. Besides oxalic acid, several other substances were found to be oxidised by charcoal : cystine, cysteine, and tyrosine.



cystine tyrosine

If iron be indeed responsible for the catalytic activity of charcoal in oxidising these amino acids, then by controlling the iron content it should clearly be possible to reduce the oxygen uptake. The problem was attacked by manufacturing charcoals from various substances. When charcoal was formed by heating pure cane sugar with silica in clean vessels the resulting product was catalytically inactive. Active material could not even be obtained when iron salts were added to the mixture before heating, but only if some nitrogenous material was added as well. A similar result was obtained when haematin was added to the sugar and silica in place of iron salt + nitrogenous compound. Let us recapitulate the observations of Warburg :

(1) the respiration of sea-urchin eggs is accelerated by the addition of small amounts of inorganic iron salts ;

(2) the respiration of sea-urchin eggs is inhibited by urethanes at high concentration and by cyanides and sulphides at low ;

(3) when oxalic acid is oxidised by molecular oxygen in the presence of (catalytically) active charcoal, the system is affected by HCN, H_2S, and by urethanes precisely as is the respiration of sea-urchin eggs ;

(4) for the production of active charcoal from pure nitrogen-free substances, the presence of iron alone is insufficient—nitrogen must be there too ;

(5) the active iron common to both eggs and charcoal is thus bound in some complex, or complexes, containing nitrogen.

To some extent the chemical nature of the active centre in charcoal has been elucidated. It is an iron-nitrogen complex. Another reagent must be employed for further elucidation of the ferment in living cells. This reagent is carbon monoxide, CO, the well-known blood poison. Another type of cell, too, has to be used : this cell is yeast. With the aid of these two systems, carbon monoxide and yeast, the most striking advances have been made. For the present, however, we must anticipate results, and consider the action of carbon monoxide on oxyhaemoglobin. When carbon monoxide is shaken in a closed vessel with oxyhaemoglobin an equilibrium is reached : carbon monoxide, oxygen, carboxyhaemoglobin and oxyhaemoglobin coexist,

$$HbO_2 + CO \rightleftharpoons HbCO + O_2$$

where HbO_2 stands for oxyhaemoglobin and HbCO for carboxyhaemoglobin. If K be the equilibrium constant then

$$\frac{[HbO_2]}{[HbCO]} \cdot \frac{p_{CO}}{p_{O_2}} = K$$

This means that the *ratio* p_{CO}/p_{O_2} decides how much haemoglobin is to exist as oxy- and how much as carboxy-. Suppose that the pressure of oxygen be 10 times that of carbon monoxide, i.e. $p_{O_2} = 10p_{CO}$,

then the ratio $[HbCO]/[HbO_2]$ will be constant no matter whether the *actual* pressure of oxygen be 1 atmosphere or 100.

Suppose a suspension of yeast be made up in 1% glucose and its respiration studied, and suppose further that the action of CO be tested. At low CO pressures no action on the respiration can be observed : at higher pressures a considerable inhibition is found. Now this inhibition is a function of the *ratio* p_{CO}/p_{O_2} just as was the case with haemoglobin. There is, however, one distinction between the two cases, and while this difference is of minor importance from the purely scientific aspect, it is fundamental from the applied, biological, side of the matter. It is this—that the ratio p_{CO}/p_{O_2} must be much bigger for an appreciable inhibition of yeast respiration than for the formation of carboxyhaemoglobin. When a human being is poisoned by CO it is because the carriage of oxygen is stopped, *his cellular respiration not being affected by these low* CO *pressures.* Thus the affinity of haemoglobin for carbon monoxide is much higher than that of the ferment in yeast.

In these experiments Warburg [1927, 1928, 1929] used a dilute suspension of yeast respiring freely in a 1% solution of glucose. The rate of respiration then depended on the yeast itself and was not a function of extraneous factors, such as rate of diffusion of oxygen, or absence of oxidisable material. Thus the rate of respiration becomes limited by the amount of iron-containing catalyst. If this be written as Fe, then

$$-d(O_2) = k.Fe$$

i.e. a linear proportionality is assumed between the amount of active catalyst and the rate of respiration. In the presence of CO the respiration of yeast is lower, presumably because the iron-containing ferment is inactive when combined with that gas.

Hence the respiration decreases. If concentrations of reactants be expressed with the suffix 1 before CO inhibition, and with the suffix 2 after, we have

$$-d(O_2)_1 = k.Fe_1$$

and

$$-d(O_2)_2 = k.Fe_2$$

If now

$$Fe_1 = 2Fe_2$$
$$-d(O_2)_2 = \tfrac{1}{2}\{-d(O_2)_1\}$$

From the experimental data it was found that the ratio $[CO]/[O_2]$ determined the extent of inhibition, just as it does the ratio $[HbCO]/[HbO_2]$. It appears then that carbon monoxide and oxygen compete for the respiratory ferment in yeast just as they compete for haemoglobin.

Temp. ° C.	Gas mixture in vols. % CO	O_2	Inhibition of respiration %
20	80·1	19·9	35
	88·6	11·4	61
20	78·8	22	22
	90·6	9·4	55
37·5	80·7	19·3	38
	87·7	12·3	60

The inhibition is reversible. When yeast poisoned with CO is replaced in a CO-free atmosphere the rate of oxygen uptake goes back to its normal level.

Let us turn to the properties of carboxyhaemoglobin. When illuminated in aqueous solution the compound tends to split up, and the equilibrium

$$HbCO + O_2 \rightleftharpoons HbO_2 + CO$$

alters with the intensity of illumination [J. S. Haldane]. This sensitivity to light appears to be characteristic of the CO compounds of iron and iron alone. This can be demonstrated by the effect of

CO on various catalyses by iron derivatives. The amino acid cysteine readily oxidises to cystine (see p. 86) in the presence of Fe, Cu, haematin and various haemochromogens. CO inhibits this oxidation when haemochromogens are catalysts, the ratio p_{CO}/p_{O_2}

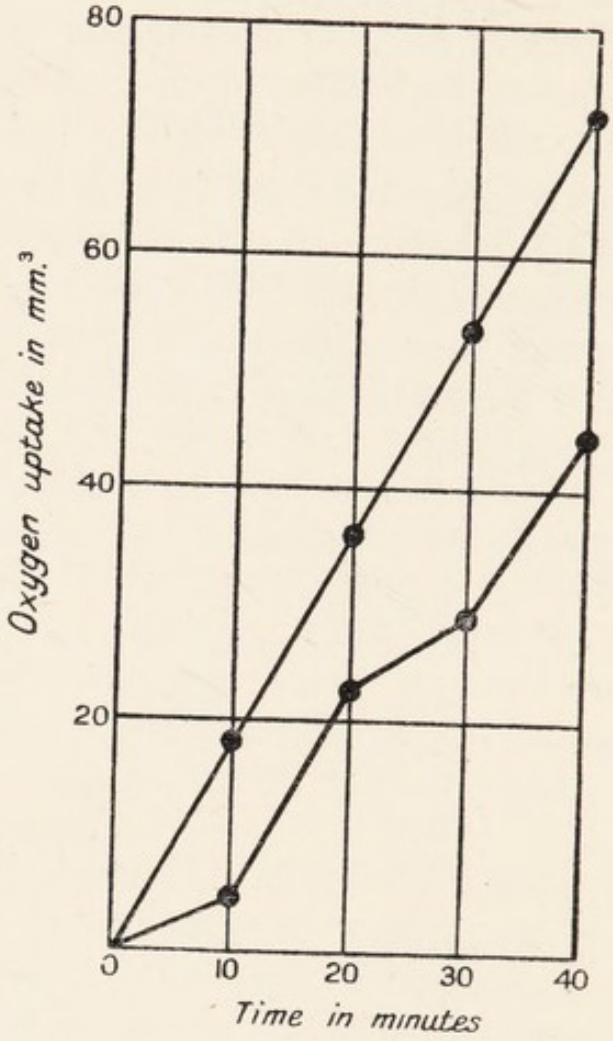


FIG. 2.—Effect of light on the CO inhibition of cysteine oxidation catalysed by pyridine-haemochromogen. Upper curve in air, lower in 21% oxygen + 79% carbon monoxide. Solutions contain $M/4$ borate buffer pH 10·3, 3 mols. pyridine, $0{\cdot}77 \times 10^{-6}$ mol. haemin, $3{\cdot}8 \times 10^{-2}$ cysteine, $1{\cdot}8 \times 10^{-6}$ $FeSO_4$.

(KREBS [1929]. *Biochem. Zeit.* **204,** 322)

decides the extent of the inhibition, and light dissociates the CO complex, thus annulling the inhibition. Fig. 2 illustrates this.

The respiration of yeast respiring in presence of CO is increased on illumination, in the same fashion

that carboxyhaemoglobin and CO-haemochromogen show increased dissociation. There is then a fairly close analogy between various reactions catalysed by haemochromogens and the respiration of yeast—at least as regards their common behaviour to CO and to light.

Since light dissociates the CO-ferment complex, a study of the relative efficiencies of different wave-lengths should throw light on the nature of the ferment. Warburg has analysed the phenomenon

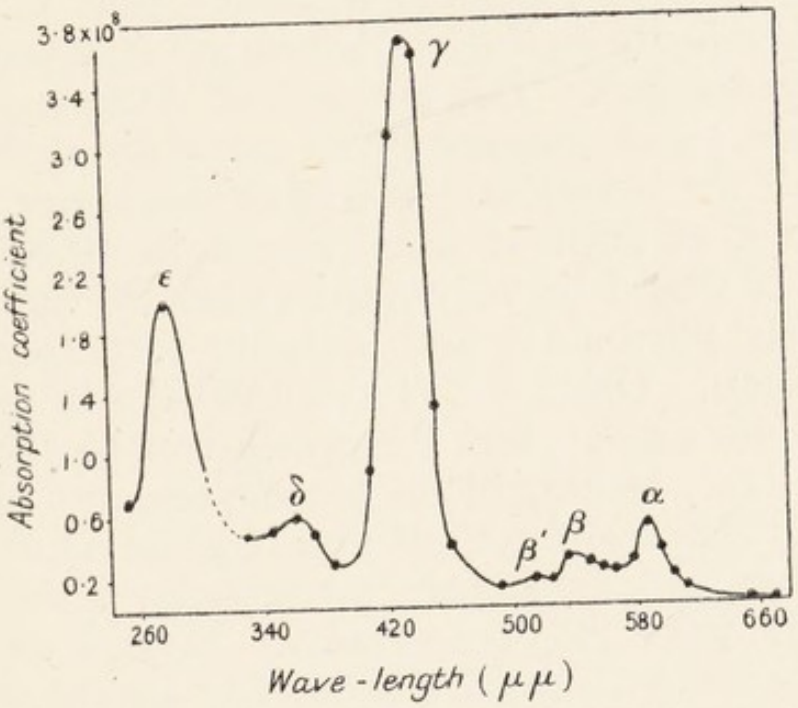


FIG. 3.—Absorption spectrum of the "Atmungsferment" (WARBURG [1932]. *Zeit. f. Angw. Chem.* **45,** 1)

in some detail, and has succeeded in calculating the absorption curve of the ferment. This curve resembles the absorption spectrum of CO-haematins and is quite distinct from that of other substances, as for example CO-ferrous-cysteine.

His conclusions may profitably be summarised:

(1) cellular respiration can be simulated with charcoal containing iron combined with nitrogen;

(2) the respiration of yeast is reversibly inhibited by CO, the inhibition depending on the ratio p_{CO}/p_{O_2} just as does the action between haemoglobin, oxygen

and carbon monoxide, and the catalysis of cysteine oxidation by haemochromogens;

(3) the CO-inhibition of yeast respiration is light-sensitive, the ferment having less affinity for CO when illuminated, just as in the cases of compounds between Fe and CO, carboxyhaemoglobin and CO-haemochromogen;

(4) the absorption spectrum of the respiratory ferment (i.e. of the Atmungsferment) resembles strongly that of the CO derivatives of haematin.

There is thus plausible evidence that a ferment allied chemically to the haematins governs respiration in the yeast cell. The amount of Atmungsferment present in yeast appears (from quantum data) to be too small for isolation. Warburg calculates there is about 1 g. of ferment iron present in 10,000,000 g. yeast. It would be very hard to isolate the enzyme in a fairly pure state and actually to determine its constitution. Other cells have not been investigated to the same extent, but this much can be said—that cells of liver, retina, blood leucocytes and platelets, chorion, tumours of rat, rat and chicken embryo, and other systems, all contain an enzyme which reacts reversibly with HCN and H_2S. Retina alone has been studied with regard to its CO-sensitivity. Its respiration appears to be reversibly inhibited by CO, the reaction being light-sensitive.

It may be asked to what extent all the respiration of the cell is governed by this ferment? To this no satisfactory answer can as yet be given. The problem to some extent devolves upon the meaning and size of HCN-stable respiration. (It will be remembered that HCN and CO are largely equivalent.) When cells are poisoned with HCN (which stops the Atmungsferment) a large part of their respiration is stopped. In the case of yeast the inhibition is almost complete. With most other tissues, however, a considerable (and rather variable) percentage remains. In such cases it is difficult to

accept the view that the respiratory ferment of Warburg accounts for more than about 50 % of the total respiration. It may be, however, that the whole respiration normally goes through the Atmungsferment and that the use of cyanide has called into action processes widely different from the normal. (See the chapter on Modern Developments.)

Different CO-haematins (or CO-haemochromogens) show similar but not identical spectra, and Warburg

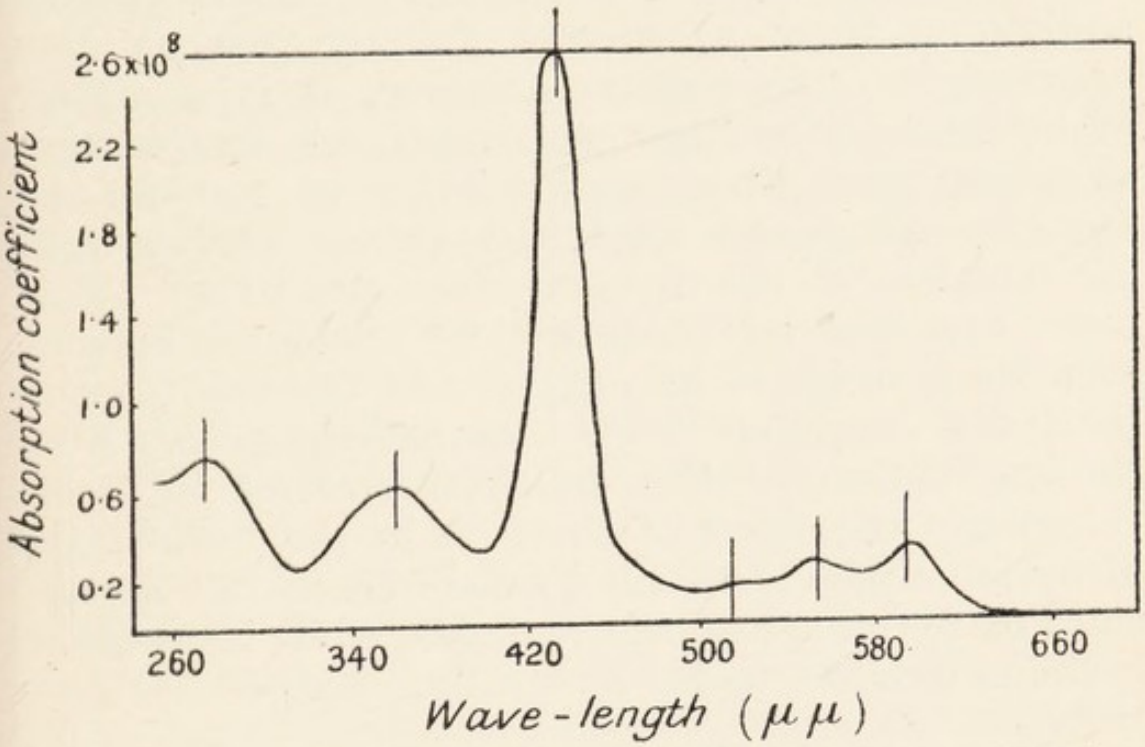


FIG. 4.—Absorption coefficient of CO derivative of Spirographishaemin

(WARBURG [1932]. *Zeit. f. Angw. Chem.* **45,** 1)

has compared that of the Atmungsferment with those of known substances. So far he has been unable to identify the absorption of the Atmungsferment with that of any known compound. It may, however, be that the haematin fraction is united with a unique base which is found nowhere else and which alters the spectrum of an otherwise readily recognisable haematin. Now the question arises as to what is implied by the expression "Atmungsferment". Its properties have been defined by its

reactivity to CO, to HCN and to H_2S. Does it imply one substance common to many sorts of cell, or several substances differing in their haematin fractions, or in their basic parts, or in both ? Warburg inclines to the view that—just as with haemoglobin—there is only one haematin concerned, but possibly many different protein components.

How does the Atmungsferment act ? We have spoken throughout as though it combines directly with molecular oxygen and with carbon monoxide, adding on these substances directly just as does haemoglobin. In reality, however, it is a matter of indifference whether we regard the combination as taking place literally or whether we suppose the ferment undergoes a continuous valency change from the trivalent to the divalent state and so on. This point can best be illustrated by analogy. Ferrous cysteine is oxidised by oxygen and the oxidation is catalysed enormously by haemochromogens. Now the mechanism of this catalysis involves the stoichiometric reduction of the iron in the ferric state of pyridine parahaematin to the iron in the ferrous state of pyridine-haemochromogen, while the ferrous cysteine oxidises to ferric cysteine, i.e.—

$$(Fe^{+++})_H + (Fe^{++})_{Cys} \longrightarrow (Fe^{++})_H + (Fe^{+++})_{Cys}$$

and the ferric cysteine rearranges to ferrous-cystine, which dissociates. This is the *mechanism* of the catalysis : it is due to the *valency change* of the metal. So far all seems simple. Yet CO inhibits the reaction, it stops the oxidation and it slows the catalysis competitively with oxygen. CO inhibits as though it competed with oxygen for pyridine-haemochromogen just as it certainly competes with oxygen for haemoglobin. Thus the action might be *regarded* as

$$(Fe^{++})_HO_2 + CO \rightleftharpoons (Fe^{++})_HCO + O_2$$

But $(Fe^{++})_HO_2$ is quite unknown and has no observ-

able existence as has HbO_2 or HbCO. CO probably inhibits by forming the stable pyridine-CO-haemochromogen, which does not autoxidise to pyridine-parahaematin with ease.

So far we have talked of respiration as involving "oxygen activation." But methylene blue reduction undoubtedly occurs. How then can this be reconciled with the views outlined in this chapter? Warburg [1930] has attempted to answer this in his more recent researches. He regarded methylene blue reduction as a "Hämineisen" phenomenon, and to demonstrate this view has studied the interaction between haemoglobin and methylene blue. Let $(Fe^{++})_{Hb}$ stand for reduced haemoglobin and $(Fe^{+++})_{Hb}$ for methaemoglobin. Then

$$2(Fe^{++})_{Hb} + MB \longrightarrow 2(Fe^{+++})_{Hb} + MBH_2$$

i.e. methaemoglobin and leucomethylene blue are formed. If the leucomethylene blue is oxidised to methylene blue, e.g. by oxygen, the process becomes catalytic and a small amount of dye will oxidise an indefinitely large amount of haemoglobin, as for example when added to whole erythrocytes, whose haemoglobin ultimately changes entirely into methaemoglobin. Now, on adding glucose to such a suspension of erythrocytes the methaemoglobin is reduced back to haemoglobin and an oxidation of the glucose results. As, however, the reduced haemoglobin is constantly being oxidised back to methaemoglobin by the methylene blue and the methaemoglobin reduced by the glucose, a continuous oxygen uptake results. We have, then,

$$MB + 2Hb \longrightarrow MBH_2 + 2MetHb$$
$$MBH_2 + \tfrac{1}{2}O_2 \longrightarrow MB + H_2O$$
$$MetHb + \text{glucose} \longrightarrow Hb + \text{oxidation products of glucose}$$

So far we have followed the arguments of Warburg. The last equation is misleading. Methaemoglobin

and glucose do *not* react together *in vitro*, and the equation should be written

$$\text{MetHb} + \text{glucose} \xrightarrow{\text{enzyme}} \text{Hb} + \text{oxidation products of glucose}$$

Hence the methaemoglobin is playing the part

(*a*) not of an oxidation catalyst, but

(*b*) of a stoichiometric hydrogen acceptor.

Just as methylene blue or dinitrobenzene act as hydrogen acceptors and become reduced by dehydrogenase systems, so here the methaemoglobin—formed in this case fortuitously by methylene blue and oxygen—becomes reduced by the dehydrogenase systems working with glucose.

The implication of the work of Warburg on erythrocytes is rather obscure, but, once we realise the fact that in reality a dehydrogenase mechanism is involved, the observations become simpler. The work of Warburg is concerned with the " oxygen end " of respiration, and does not include the reducing mechanisms of Thunberg and of Wieland.

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CHAPTER IV

OXIDASES, PEROXIDASES AND CATALASE

AS their name implies oxidases are enzymes which catalyse oxidations (which means that they also catalyse reductions), and their peculiarity is that they catalyse the reduction of molecular oxygen only. The class of enzymes we are studying is to be distinguished from aerobic dehydrogenases, like xanthine oxidase, which reduce not only oxygen but also dyes such as methylene blue and which are unaffected by HCN, and from anaerobic dehydrogenases, such as succinic dehydrogenase, which reduce only dyes and not oxygen. Thus we define oxidases here as oxidative catalysts which reduce only molecular oxygen and whose action is independent of hydrogen peroxide. The latter condition differentiates oxidases from peroxidases, which need hydrogen peroxide for their action, and imposes the further condition that all reagents must be free from peroxide when testing for oxidases.

The " oxidase " system of Bach and Chodat [1904] was a more complex idea. According to their view, oxidations in many plants were catalysed by an involved system which acted by forming a peroxide which was in turn attacked by a peroxidase in presence of an oxidisable molecule. The system which formed the peroxide they called " oxygenase " : it consisted of an autoxidisable molecule which yielded in air a peroxide. When guaiacol (or guaiacum) was added the peroxidase in the system attacked

the peroxide, which then oxidised the guaiacol. Thus

$$\text{autoxidisable molecule} + O_2 \longrightarrow \text{peroxide}$$

$$\text{peroxide} + \text{guaiacol} \xrightarrow{\text{peroxidase}} \text{oxidised guaiacol} + \text{autoxidisable substance}$$

Onslow [1911] emphasised the fact that the oxygenase system was itself of an enzymic nature, and consisted of an oxidisable substance in association with an enzyme which oxidised it in presence of air. Szent-Györgyi [1925] showed that the Bach and Chodat system could oxidise guaiacol without the intervention of peroxidase. He added catechol to a suspension of potato oxidase and allowed the system to interact for a few minutes ; then he precipitated the enzymes and added the enzyme-free solution to a solution of guaiacol, which was coloured immediately.

The concept of " oxidase " outlined by Bach and Chodat has thus undergone much modification, and now has become limited to that catalyst which oxidises phenolic substances in presence of oxygen. The work of Bach and Chodat, Onslow, and Szent-Györgyi was limited to plants. Oxidases are known to exist in mammalian tissue, but of the mechanism of their action nothing is known. Thus, though the polyphenol oxidase of potato [Szent-Györgyi, 1925] oxidises *p*-phenylenediamine when traces of catechol are added, but not otherwise, there is no evidence that the indophenol oxidase of mammalian tissues [Vernon, 1911] which also oxidises *p*-phenylenediamine does so in virtue of the presence of catechol in association with it. There is no evidence that catechol does exist side by side with the enzyme in the mammalian cell.

The best-studied animal oxidase is the indophenol oxidase of Vernon [1911]. Keilin [1929] has demonstrated its function *in vivo*. Suppose we wish to test for indophenol oxidase in brain tissue—the process would be this : the tissue would be washed, perhaps

ground with sand, or else cut into very thin slices. Several reagents may be used for the detection of the enzyme. The " Nadi " reagent is perhaps most convenient. It consists of a mixture of $M/100$ *p*-phenylenediamine, $M/100$ α-naphthol (each in 50 % alcohol), and of 0·25 % sodium carbonate in water. Equal volumes of each solution are mixed immediately before use and the mixed solution is added to the tissue to be investigated ; a dark blue colour rapidly develops if the enzyme be present and if the system be saturated with oxygen. Even though the oxidase be absent the blue colour may develop if there be traces of metal present as impurity. Unless the action is rapid and the colour appears in a few minutes, the enzyme must be taken as absent. This is not merely a qualitative test for oxidases: the same reaction can be used to study the system from a quantitative aspect, and to measure the oxygen uptake when indophenol blue is formed from dimethyl-*p*-phenylenediamine and α-naphthol. The net reaction is shown by the equation on p. 12, indophenol blue being formed. When such a test is carried out on brain tissue it is found that grey matter has much more oxidase than white : the enzyme is entirely lacking from peripheral nerve.

Besides α-naphthol other phenols can be used for the study of oxidases [Lillie, 1902]. Similarly, other diamines can be used besides dimethyl-*p*-phenylenediamine. Thus it happens that oxidases have been studied by the use of artificial reagents which never play a part in the processes of cell respiration. These reagents are, of course, merely used as tests for the enzymes.

Only oxygen can be reduced by the oxidases, dyestuffs cannot. Now we saw that narcotics slowed down the velocity of dehydrogenase action, but that cyanides and sulphides were without action [Keilin, 1929]. Oxidases, on the other hand, are left comparatively unaffected by the presence of narcotics,

but are entirely inhibited by HCN and by H_2S. Thus cyanide, even in $M/1000$ concentration, stops the action of indophenol oxidase in all tissues studied ; sulphides act similarly. Just as cell respiration is affected by the action of cyanides and sulphides, so is the action of oxidases inhibited by these reagents. There is a close parallelism between the action of these reagents on the respiration of cells and on the activity of oxidases. Is this parallelism merely fortuitous, or is it the expression of some fundamental fact connecting the activity of oxidases with the general respiration ? To answer this question we must turn to the work of Keilin.

When the Nadi reagent is added to a suspension of washed heart muscle a blue colour rapidly develops. The action is stopped by the presence of HCN, H_2S, and by CO. When the rate of oxygen uptake is measured and the effect of CO studied, it is found that the CO competes with oxygen, and the ratio p_{CO}/p_{O_2} determines the amount of inhibition. The oxidase has the same affinity for CO as has the respiratory ferment of yeast. Now when such a preparation of heart muscle is used to catalyse the oxidation of cysteine to cystine in the presence of cytochrome, it is found that the rate of oxygen uptake is as great as in rapidly respiring muscle. The preparation contains all the factors for producing an oxygen uptake that is as great, quantitatively, as is found in normal conditions.

Normal yeast gives no Nadi reaction. Treating it in various ways causes the appearance of indophenol blue when Nadi reagent is added : warming to 52° for $1\frac{1}{2}$ hours has this effect, as has cooling to $-2°$; starvation till the rate of respiration falls to about 1,500 mm.^{3} O_2 per g. yeast per hour (in place of the usual 40,000 mm.^{3} in glucose) ; and treatment with urethanes. With yeast, as with muscle, the oxidation of the Nadi reagent to indophenol blue is inhibited by CO to an extent depending on the ratio

p_{CO}/p_{O_2}. The K values are the same as for muscle oxidising Nadi reagent and for yeast respiring in glucose. The CO inhibition of the indophenol oxidase of muscle and of yeast is light-sensitive as is the normal respiration of yeast. (The polyphenol oxidase of the potato is inhibited by CO, but the inhibition is not light-sensitive.)

To recapitulate — we see that the indophenol oxidase is an enzyme system which is inhibited by HCN, by H_2S, and by CO, precisely as is the system studied by Warburg, and as is the respiration of the yeast cell. What the oxidase attacks in the cell, and what relationship there is between it and the respiratory enzyme — those problems remain for later chapters.

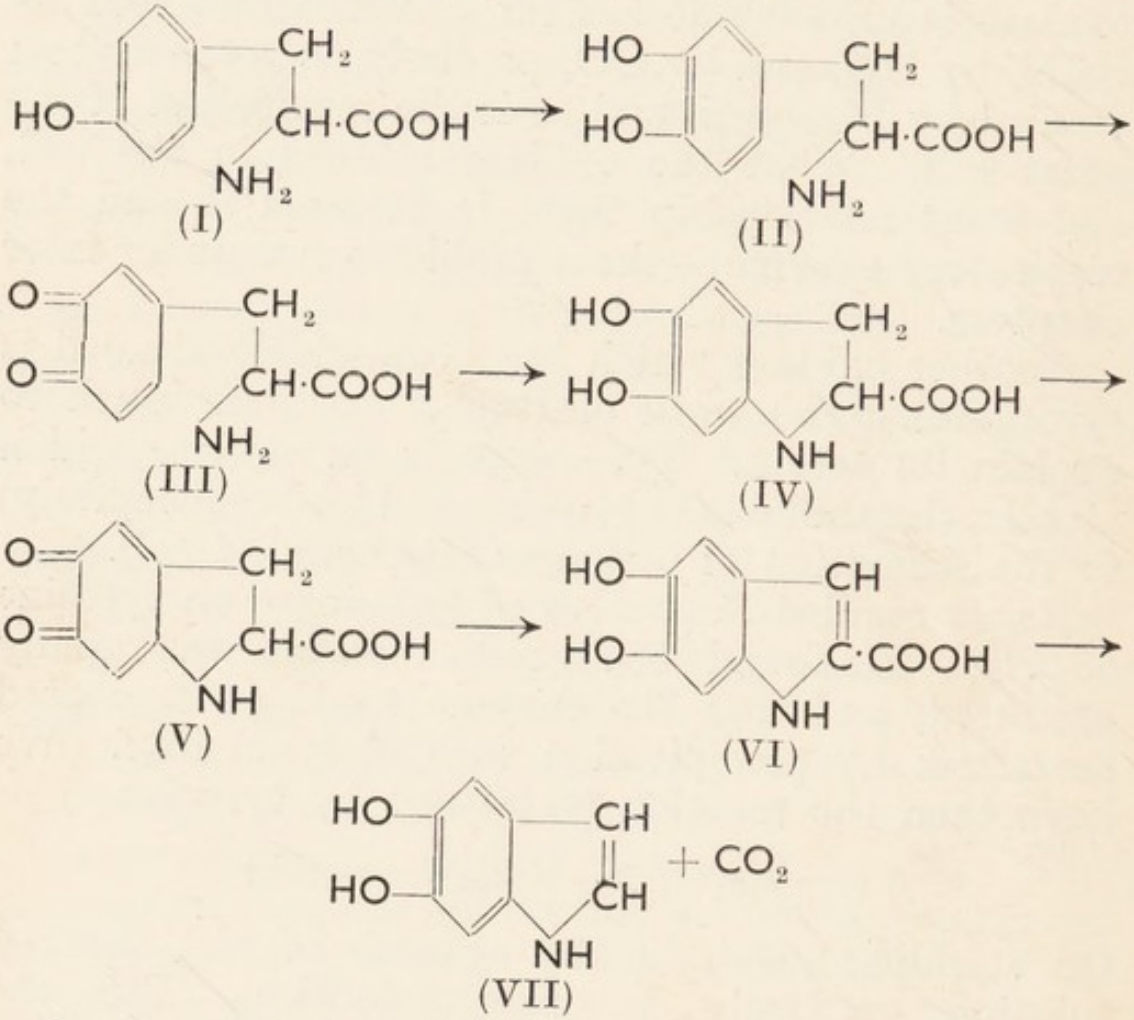
Another oxidase which has been widely studied is tyrosinase. The work of Raper has done much to explain its action. Tyrosinase is an enzyme which attacks the amino acid tyrosine and leads (ultimately) to the formation of melanins [Bertrand, 1896].

Raper studied the action of tyrosinase on tyrosine at pH 6. He found that a red substance was rapidly produced, and that the enzyme could be separated therefrom by precipitation with dialysed iron. We have then the reaction (catalysed by tyrosinase)

$$\text{tyrosine} + n\text{O}_2 \longrightarrow \text{red substance}$$

On standing (even in the absence of air) the red solution gradually becomes colourless. For the moment we shall leave the reaction at this stage and study the chemistry. We shall have, for the purposes of this book, merely to accept the conclusions which Raper reached and to leave the arguments by which he attained them. He supposes that tyrosine first forms 3:4-dihydroxyphenylalanine (II), which then forms an *o*-quinone (III). This *o*-quinone then rearranges to form 5:6-dihydroxydihydroindole-2-carboxylic acid (IV), which oxidises to 5:6-quinodihydroindole-2-carboxylic acid (V) which is supposed

to be the red material formed in solution when tyrosinase acts on tyrosine. This then rearranges with or without decarboxylation to 5:6-dihydroxyindole-2-carboxylic acid (VI) or 5:6-dihydroxyindole (VII), both of which are colourless. Now 5:6-dihydroxyindole is rather unstable in presence of oxygen and rapidly oxidises to form black melanin. This reaction occurs independently of tyrosinase.



Onslow [1915] studied the pigmentation of rabbits. He succeeded in showing that melanin deposition occurred as a result of the action of an enzyme on tyrosine, and extended his observations to explain dominant and recessive whiteness. He showed that

(*a*) in dominant whiteness there is an enzyme-inhibitor present which prevents the formation of melanin ;

(*b*) in recessive whiteness (albinism) there is a complete absence of catalyst.

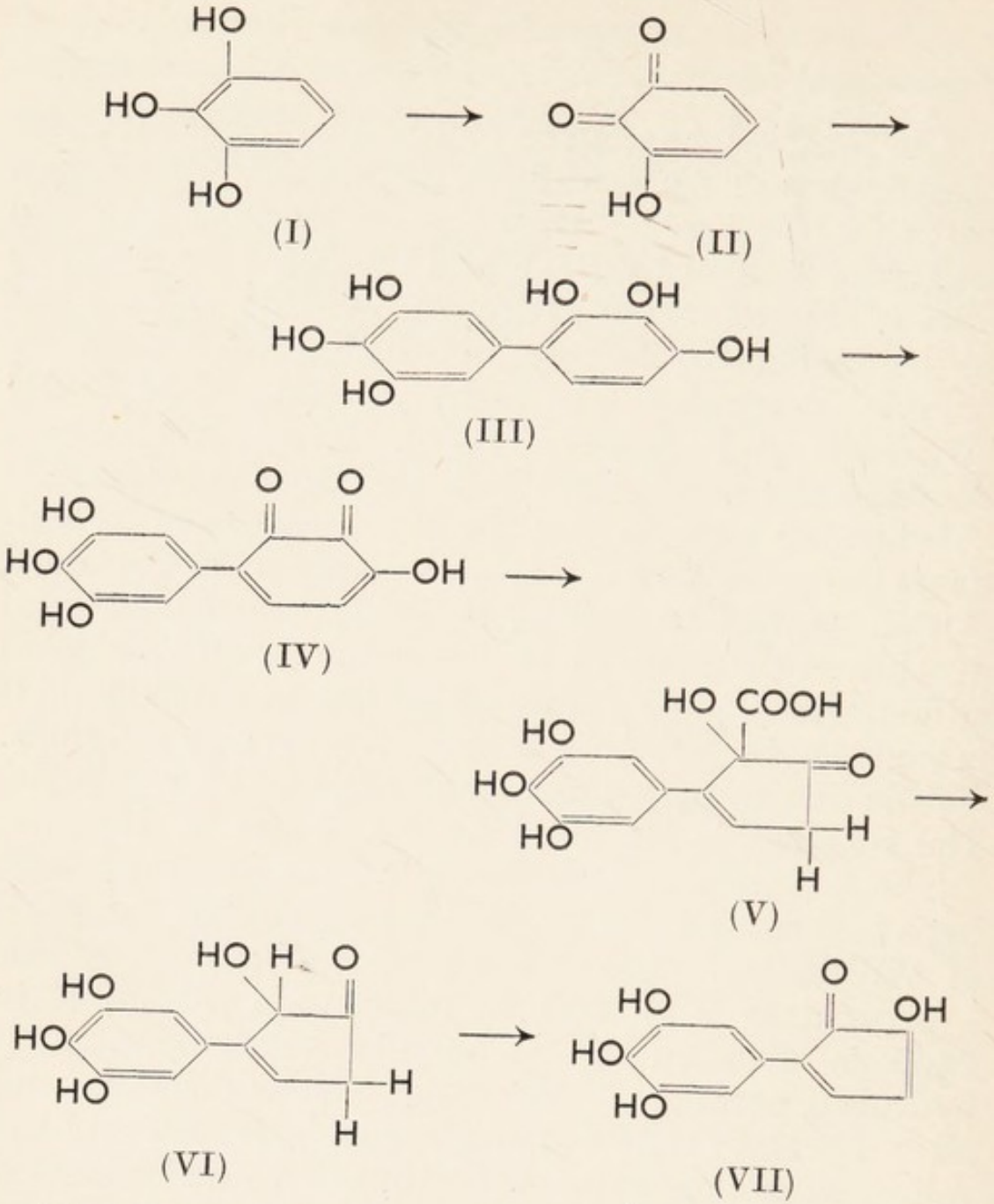
Peroxidases

The Nadi reagent can be oxidised by other catalysts than oxidases : such catalysts can act only if hydrogen peroxide be present ; they are known as peroxidases and were studied by Bach and Chodat. Peroxidases appear able to attack hydrogen peroxide rapidly but to be ineffective largely in causing oxidations by organic peroxides like ethylperoxide, $C_2H_5O{\cdot}OH$ [Wieland and Sutter, 1930]. They are, then, enzymes showing a very high degree of specificity.

When we wish to test for the presence of peroxidases the procedure is this—the tissue to be examined is well washed to remove reducing substances,[1] and a solution of guaiacol or benzidine in 50 % alcohol added with a few drops of hydrogen peroxide. If peroxidase be present a deep colour rapidly develops. This is due to the oxidation of guaiacol to a quinone. The reaction is inhibited by HCN and by H_2S.

The quantitative estimation of peroxidases is perhaps best carried out by the " purpurogallin " method of Willstätter. The essence of the method is this —peroxidase is allowed to react upon pyrogallol in aqueous solution in a definite concentration of hydrogen peroxide. An orange colour develops and after a certain time the enzyme is destroyed and the yellow substance extracted with ether and estimated colorimetrically. The chemical reactions are complex. Pyrogallol is oxidised to vicinal hydroxy-*o*-benzoquinone, which then reacts with a second molecule of pyrogallol to form a hexahydroxydiphenyl (III) which is in turn oxidised to an *o*-quinone (IV) and then to a *cyclo*pentane derivative (V) which loses CO_2 to yield substance (VI) and finally purpurogallin (VII).

[1] Elliott [1932] has noted that the –SH groups of denatured proteins reduce the blue quinone to colourless forms and hence obscure the reaction.



All known haematin compounds are able to act as peroxidases, i.e. to oxidise guaiacol in the presence of hydrogen peroxide, to a greater or lesser extent. Thermostable peroxidases such as haematins (e.g. cytochrome) are much less active than the true thermolabile enzymes.

Two questions confront us :

(1) To what extent do peroxidases catalyse oxidations in aerobic cells ?

(2) What oxidations do they catalyse ?

Now peroxidases appear able only to activate

hydrogen peroxide, and the extent to which they enter into cell respiration seems limited by the amount of hydrogen peroxide formed in cells. But how much hydrogen peroxide is formed? The question is hard to answer. Only in one case has hydrogen peroxide ever been detected as a product of a living cell, namely in suspensions of anaerobic bacteria brought into oxygen [Callow, 1923]. It is true that an aerobic dehydrogenase such as xanthine oxidase can and apparently does produce H_2O_2 when it oxidises hypoxanthine (or xanthine) in air, and it is also true that peroxidases will activate the peroxide so formed and oxidise added metabolites [Harrison and Thurlow, 1925]. We have no evidence that such actions ever occur *in vivo*.

The answer to the second question—what oxidations does peroxidase catalyse?—is almost as obscure. To say that peroxidase oxidises guaiacol in no way settles the problem: it is unlikely that peroxidase ever meets guaiacol in the cell, but it does perhaps hint at a solution. Elliott [1932, 2] endeavoured to settle the matter, but of a large number of substances investigated only tyrosine and histidine were oxidised. These yielded melanin-like substances. It may be that peroxidase oxidises these substances in the cell, but the question is still open.

Catalase

Most aerobic cells contain another enzyme besides peroxidase which acts upon hydrogen peroxide. The action of each enzyme is entirely different from that of the other. Peroxidase performs oxidations in the presence of peroxide,

$$A + H_2O_2 \xrightarrow{\text{peroxidase}} AO + H_2O$$

but catalase decomposes H_2O_2

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

Peroxidase affects H_2O_2 only in the presence of an oxidisable molecule, but catalase destroys it independently of another substrate, and is quite unable to catalyse oxidations. Thus when hydrogen peroxide is added to a solution of catalase and a few

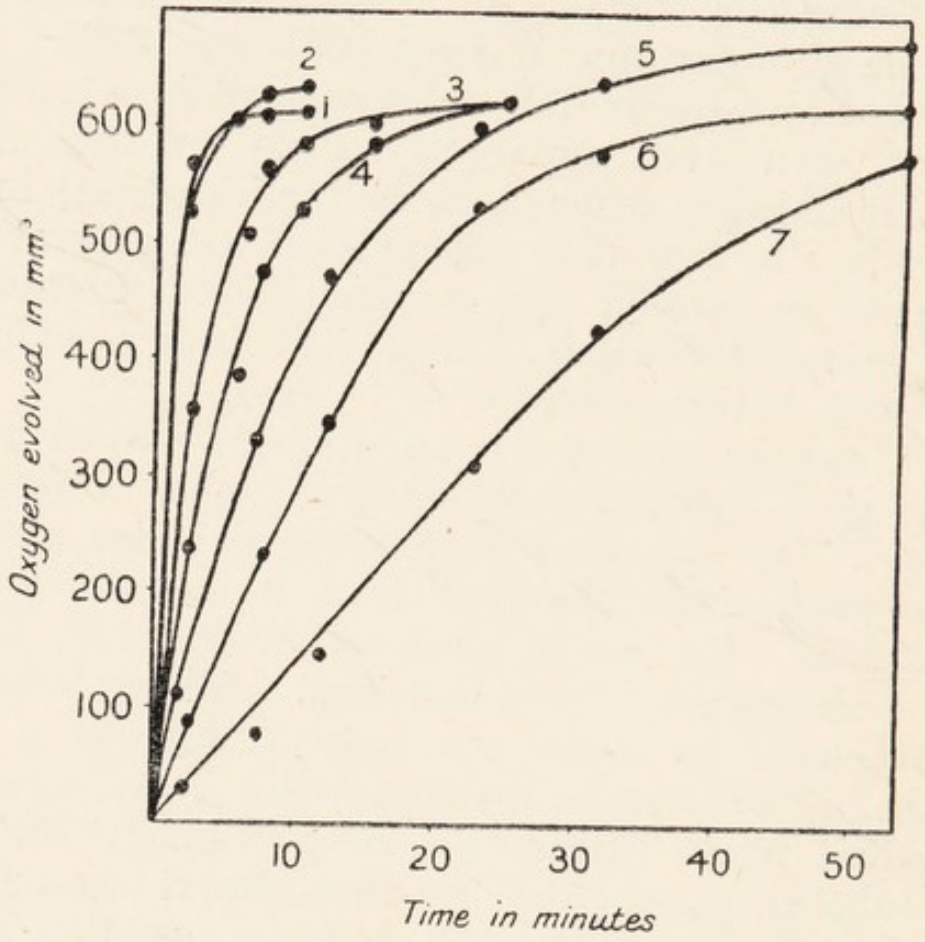


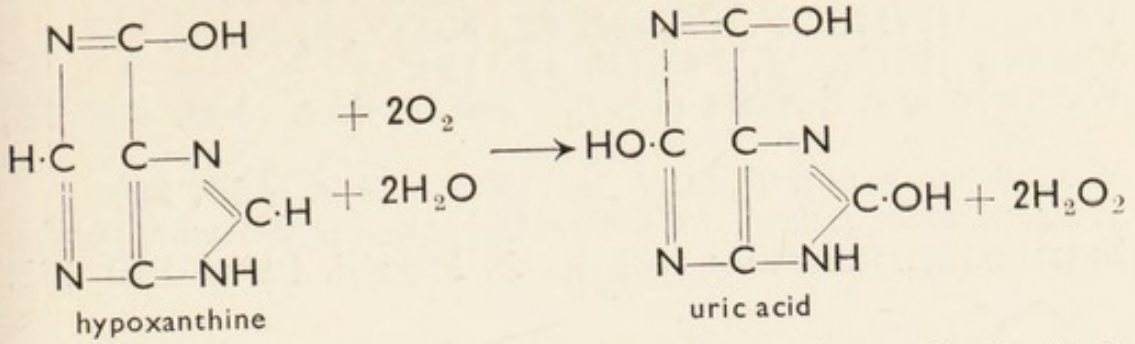
FIG. 5.—Influence of HCN on catalase action. 0·5 g. moist muscle tissue in 4 cc. phosphate pH 7·4, 2 cc. $M/30$ H_2O_2. Temp. 37° C.

Curve	1	2	3	4	5	6	7
Conc. HCN	0	$M/1000$	$M/500$	$M/200$	$M/100$	$M/75$	$M/50$

(WIELAND and LAWSON [1931]. *Ann.* **485,** 193)

drops of guaiacol as well, there is only decomposition of the peroxide to oxygen and water, and no colouring of the guaiacol. Catalase is inhibited (as is peroxidase) by HCN and H_2S. Dixon showed that it can behave as a protective agent and prevent xanthine oxidase from being destroyed by the H_2O_2

formed when it oxidises hypoxanthine aerobically,



Let us recapitulate the similarities and dissimilarities of peroxidase and catalase. Both enzymes act on hydrogen peroxide and both seem to be haematin compounds [Willstätter and Pollinger, 1923; Zeile and Hellström, 1930; Kuhn, Hand and Florkin, 1931]; the action of each is stopped by cyanide and sulphides. Peroxidase catalyses oxidations and attacks peroxide only when an oxidisable molecule (nitrite, tyrosine, etc.) is present, but catalase decomposes H_2O_2 independently of the presence or absence of such molecules and is unable to perform oxidations. It cannot attack peroxide at the low concentrations at which peroxidase acts.

The function of catalase in the cell is as obscure as that of peroxidase. Its importance seems to depend on the extent to which H_2O_2 is formed in the cell.

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CHAPTER V

THE CYTOCHROME SYSTEM

WE have seen how anaerobic dehydrogenase systems reduce methylene blue but not oxygen; we have seen how Keilin has studied a system which oxidises the Nadi reagent to indophenol blue, that this system is reversibly inhibited by CO (which competes for the enzyme with oxygen) by HCN and by H_2S; and we have discussed the work of Warburg (who emphasises the necessity for oxygen "activation" in respiration). Now we shall turn to a substance whose properties have shown that cellular respiration involves the combined and simultaneous action of dehydrogenase systems on their substrates and of oxidase on its substrate. This substance is cytochrome: its oxidation by molecular oxygen under the catalytic action of indophenol oxidase has been described by Keilin [1929].

In 1884–86 MacMunn described investigations into the absorption spectra of various tissues. (It has for long been known that the absorption spectrum of any one chemical substance is characteristic of that substance, under certain well-defined conditions, and it is a principle of physical chemistry that molecules of any one chemical class possess similar but not identical spectra.) With the aid of the microspectroscope he examined the tissues, and extracts therefrom, of animals killed by exhaustive bleeding and observed absorption bands which evidently belonged to substances of the haematin group. In

appearance the bands resembled those of haemochromogen derived from haemoglobin. But MacMunn's observations were criticised by several workers, notably Hoppe-Seyler. It was maintained that the myohaematin and histohaematin of MacMunn (for so he called the substances generating the bands) were merely decomposition products of haemoglobin. MacMunn argued that this could not be so since the haemochromogen derived from haemoglobin, though similar in spectrum, was not quite identical with myohaematin and histohaematin, and since the latter are present in many places, e.g. in insects, where haemoglobin is not found. Ultimately these observations fell into disrepute.

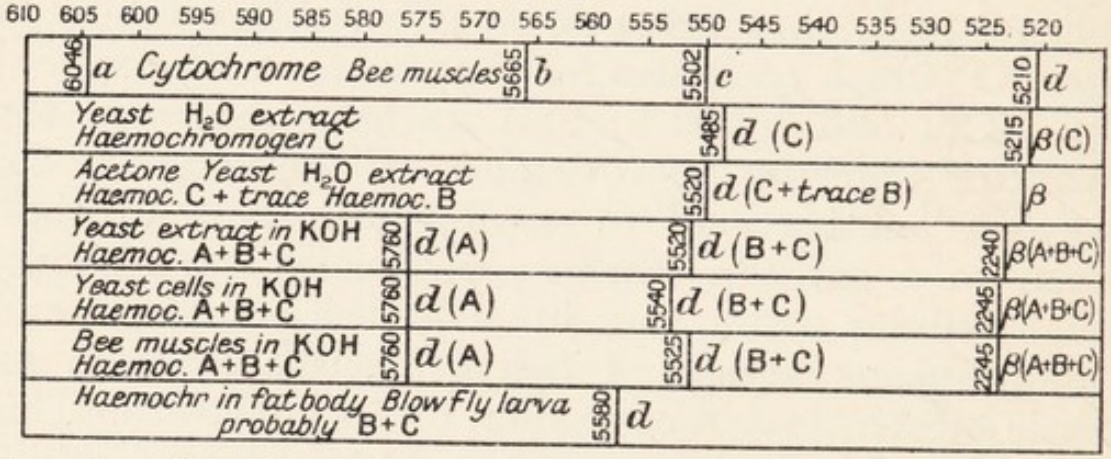


FIG. 6.—The absorption bands of reduced cytochrome compared with those of various haemochromogens

(KEILIN [1929]. *Proc. Roy. Soc.* B **104,** 206)

In 1925 Keilin described the presence of bands like those of MacMunn in many aerobic organisms. Fig. 6 shows the positions of these bands compared with those of various protohaemochromogens. When a suspension of baker's yeast is subjected to anaerobic conditions and examined with the microspectroscope four bands are clearly seen, *a*, *b*, *c* and *d*. Close examination of band *d* shows it to be composite in nature, and actually to consist of three almost coincident lines. These bands are those of cytochrome. They are shown in fig. 7. Keilin regards

cytochrome as consisting of three separate haemochromogens each contributing two bands to the spectrum : cytochrome *b* yields band *b* and one of *d* ; cytochrome *c* gives band *c* and another of *d*. These studies were prosecuted with the microspectroscope entirely, and the evidence that cytochrome was a haematin derivative based solely on the similarity of its spectrum to that of haemochromogens.

It will now be necessary to digress somewhat and consider cursorily the chemistry of the haematin derivatives. We have said that spectral evidence indicated cytochrome to be a " haemochromogen " ; we shall now examine more closely what that state-

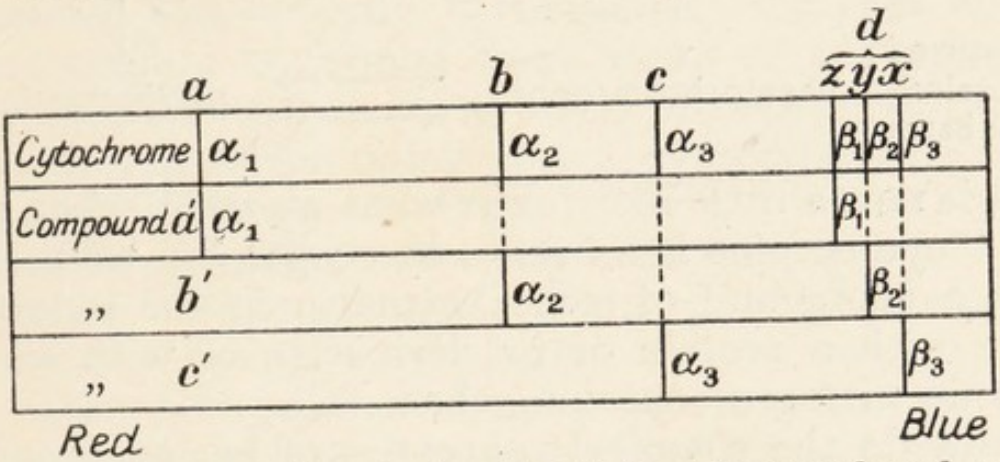
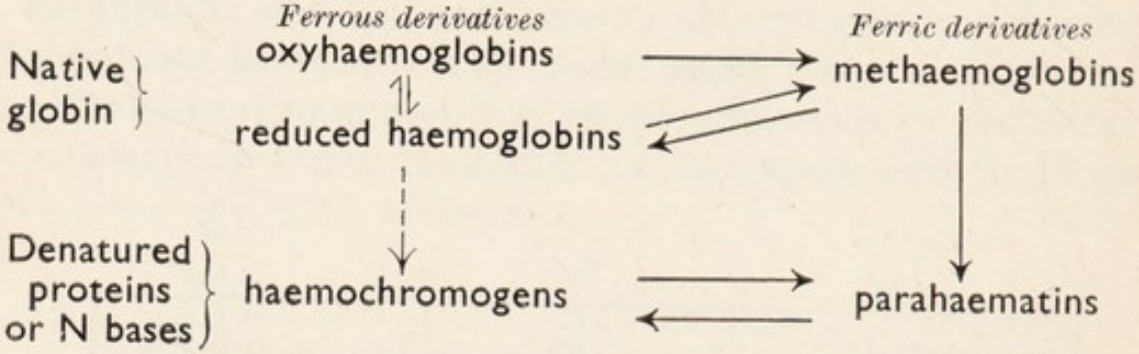


FIG. 7.—The constituent bands of the three reduced cytochromes.

ment means even though we shall finally have to admit that our ideas are not much clarified by such examination. Haemoglobin consists of protohaematin united to globin, four molecules of haematin (of molecular weight about 650) to one molecule of globin (of molecular weight about 64,000). Now haematins contain iron which can exist in the divalent state as in reduced or oxyhaemoglobin, or in the trivalent state. *Oxidation* (as opposed to *oxygenation*) of reduced haemoglobin gives methaemoglobins.

What precisely protohaematin is does not matter : suffice it to say that its constitution is known, as is that of other haematins, haematohaematin or mesohaematin, for example, and that all haematins when

united to native globin give haemoglobins or methaemoglobins according as their iron is reduced or oxidised. Besides combining with globin haematins can unite with other proteins or their derivatives and with such bases as pyridine or nicotine. In such cases haemochromogens or parahaematins (= kathaemoglobins) are produced according as the iron is reduced or oxidised. We thus have the scheme outlined below :



It is now a little more clear what we mean when we say " cytochrome is a haemochromogen " : we mean it is a compound of some haematin in the reduced state with a protein or its derivative or with some organic nitrogen-containing base.

How do the chemical properties of haemochromogens compare with those of haemoglobins ? In the first place they *cannot* reversibly absorb and *evolve* molecular oxygen. They can be *oxidised* to parahaematins, which can be reduced (e.g. by hydrosulphite) to haemochromogens, but they do not form loose molecular complexes with oxygen of the nature of oxyhaemoglobin. The relationship between haemochromogen and parahaematin is precisely akin to that between haemoglobin and methaemoglobin. In the majority of cases, however, the oxidation to parahaematin occurs more readily than that to methaemoglobin.

Hill and Keilin [1930] described a method for extracting from yeast that component of cytochrome which yields the *c* band. Such extracts contained more substances than cytochrome *c*, but the spectrum

showed no bands other than those characteristic thereof, i.e. no other haematin derivative was present. Let us now describe the physical and chemical properties of cytochrome. In appearance it is red in solution and will not pass through a collodion membrane. It is probably colloidal in nature, and the nitrogenous base united to the haematin is presumably a protein. The nature of the prosthetic group (i.e. of the haematin moiety) is worthy of closer attention. Hill and Keilin were able to show that the haematin contained is similar to, but not identical with, that of ordinary haemoglobin (i.e. of protohaemoglobin) and that by the action of acids protohaematin can actually be obtained. To sum up—cytochrome *c* is formed from union of a haematin allied to, but not identical with, protohaematin and some nitrogen-containing base ; cytochromes *a* and *b* are of similar general type, but whether they differ from *c* in haematin, or in base, or in both, is at present unknown. So much for the chemistry of cytochrome : let us now discuss its properties in solution. It can be isolated in reddish aqueous solution and can be reduced by substances such as hydrosulphite or cysteine, when the bands *c* and (one of) *d* become clearly visible. Aeration of the solution, however, does not yield oxidised cytochrome. In this respect it differs largely from ordinary haemochromogens, which almost all oxidise rapidly in air to the parahaematin.

Cytochrome is found in most aerobic organisms and tissues. It is found in most mammalian tissues, notable exceptions being peripheral nerve and possibly the white matter of brain ; it is found in plants, in insects, in yeasts and in most aerobic bacteria. It can almost be stated that cytochrome is a component of every aerobic cell, and conversely that it is absent — or almost absent — from cells which live anaerobically. " Cytochrome " is a generic name for a group of three haemochromogens, and

when it is said to be present almost universally in aerobic organisms it is not implied that all the three components are present to the same extent. This is particularly obvious in bacteria, where the absorption spectrum varies from species to species, according as cytochromes *a*, *b* or *c* are present in greater or lesser amounts. Thus, according to Yaoi and Tamiya [1928] B. *coli communis* shows bands *b* and *d* alone, whereas B. *subtilis* and B. *diphtheriae* show *a*, *b*, *c*, *d*. In the case of B. *subtilis* the *c* band is most intense; with B. *diphtheriae* the *b* band is most intense.

There is a rather rough parallelism between the amount of cytochrome present in a tissue and the rate of metabolism. There is, for example, much more pigment in the thoracic muscles of the male of the Winter Moth (C. *brumata*) than in those of the wingless female. There is much cytochrome in the grey matter of the brain, little in white and none visible in peripheral nerve: this is also the order of respiratory activity.

Let us now turn to the behaviour of cytochrome in the living cell. The Wax Moth (Galleria *mellonella*) contains cytochrome. It forms a peculiarly convenient subject for the study of cytochrome *in vivo*, and the data thus obtained are equally valid for other systems. The chitin of this species is readily transparent, and the insect can easily be examined with a microspectroscope. Keilin removed the scales from the thorax, fastened the ventral surface to a glass slide and examined it with a microspectroscope. By this means it is possible to study the behaviour of cytochrome in a living insect subjected to various influences. When such a system is left in nitrogen the bands of reduced cytochrome are clearly visible, and when the nitrogen is replaced by air or oxygen all the bands disappear. When the insect struggles, then the bands appear even when the insect is respiring in air, and disappear when it becomes quiescent:

thus work produces an effect similar to anaerobiosis. Compression of the thorax (which chokes the tracheae) has the same effect as anaerobiosis — the bands become visible and vanish when the exciting cause is removed.

Yeast is also an extremely convenient subject for study. A suspension of yeast made up in buffer solution shows the four-banded spectrum of cytochrome under anaerobic conditions : aerobiosis causes it to vanish as does the addition of oxidising agents, or the presence of traces of HCN or H_2S. Let us recapitulate these facts :

(*a*) when Galleria or yeast is subjected to anaerobiosis, the four-banded spectrum of cytochrome is especially visible ;

(*b*) the spectrum appears when Galleria does work by struggling ;

(*c*) the presence of the respiratory poisons HCN and H_2S induces the cytochrome spectrum ;

(*d*) the addition of reducing agents such as sodium hydrosulphite reduces oxidised cytochrome to reduced ;

(*e*) removal of the cause of anaerobiosis *in the living cell*, whether it has been caused by absence of oxygen in surrounding gas, by struggling of the insect, compression of the thorax, or the presence of HCN or H_2S, at once makes the bands to disappear.

Under anaerobic conditions then cytochrome is found in cells in the reduced form. Removal of anaerobic conditions causes a rapid oxidation of the cytochrome. We have, however, seen that the substance is not autoxidisable in aqueous solution. How then does it become oxidised ? Presumably by some catalyst, which we shall presently discuss. When HCN and H_2S inhibit the respiration of yeast or of Galleria *mellonella*, the bands of reduced cytochrome at once appear. Is it possible then that these reagents inhibit that catalyst which oxidises cytochrome ? and accordingly that cytochrome passes

over into the reduced form (the reduction being unaffected) ? The three enzymes known to be inhibited by HCN and H_2S are indophenol oxidase, catalase and peroxidase. CO inhibits the respiration of cells, as it does the activity of indophenol oxidase, but not the activity of peroxidase and catalase.[1] Keilin prepared suspensions of washed heart muscle. Such suspensions oxidise the Nadi reagent to indophenol blue, i.e. they contain indophenol oxidase (which we shall term "oxidase" in brief for the succeeding pages of this chapter) ; the oxidation of the Nadi reagent is inhibited by HCN, by H_2S and by high pressures of CO (of the order of $\frac{1}{2}$ atmosphere). But the oxidation of cytochrome in yeast or in muscle, or of cytochrome *c* added to washed muscle suspensions, is affected in precisely the same way by these three reagents. Not only this, but oxidase and cytochrome appear to co-exist in cells, and each to accompany the other. From such evidence it seems likely that indophenol oxidase is cytochrome oxidase. The evidence has been very greatly strengthened. Keilin investigated the suspension of heart muscle in greater detail. He measured the oxygen uptake in various conditions. Such suspensions of muscle respire very slowly : addition of cytochrome scarcely affects the rate, but addition of cytochrome + cysteine results in a very rapid uptake, as great as is found in rapidly-respiring muscle, i.e. in normal intact tissue. Fig. 8 shows this. We have then the four systems—

(1) oxidase + cysteine
(2) oxidase + cytochrome
(3) cytochrome + cysteine
(4) oxidase + cytochrome + cysteine.

Of the four systems the fourth and last shows a very rapid oxygen uptake, great enough to account for the normal respiration of the intact cell. Now

[1] Recent statements that CO inhibits peroxidases and catalases are erroneous.

this system is affected by HCN, by H_2S and by CO precisely as is the respiration of yeast: the ratio of p_{CO}/p_{O_2} for half inhibition is the same as for yeast. If the oxidation of the Nadi reagent be studied, the results are similar to those above. There is every probability then that cytochrome is oxidised by indophenol oxidase. Now in normally respiring cells cytochrome is found mainly oxidised, and this must mean that it is oxidised much faster than it is reduced.

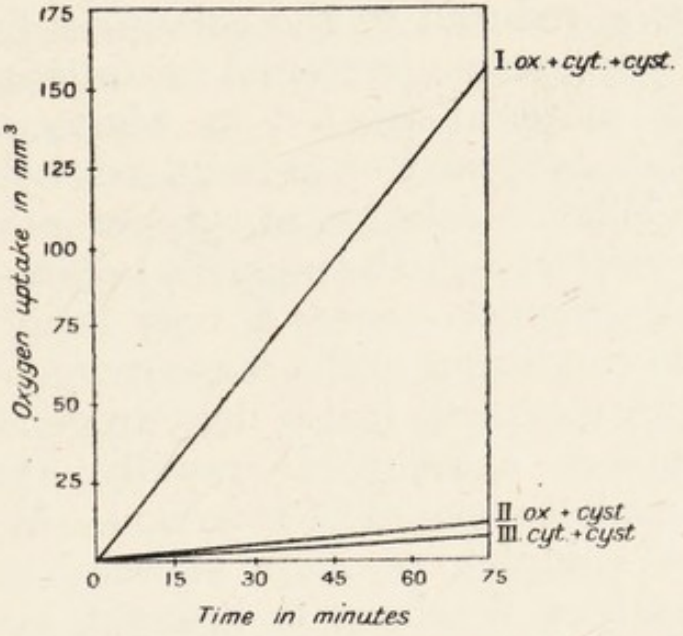


FIG. 8.—The action of oxidase-cytochrome upon the oxidation of cysteine
Curve I: oxidase + cytochrome + cysteine;
Curve II: oxidase + cysteine;
Curve III: cytochrome + cysteine
(KEILIN [1929]. *Proc. Roy. Soc.* B **104,** 206)

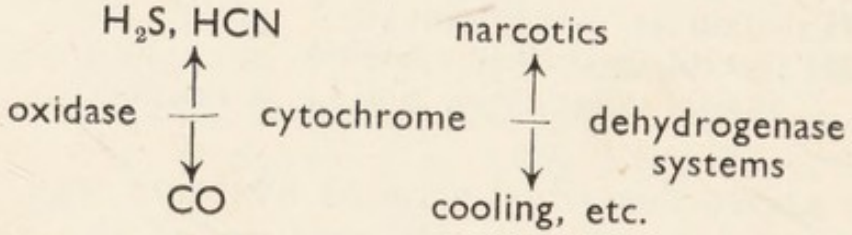
In the above-described scheme cysteine was used as reductant, but what is the nature of the reducing mechanisms in the cell?

To answer that question we must turn to yeast. We shall study the production of indophenol blue by yeast oxidase. When the Nadi reagent is added to a yeast suspension, no blue colour appears even in aerobic conditions. Yet, as we know, yeast contains oxidase. If, however, the yeast be treated beforehand by warming to 52° for 1½ hours, a blue colour

rapidly develops on adding Nadi reagent and aerating ; cooling has the same effect as has the addition of urethanes ; starvation of the yeast also stimulates the formation of indophenol blue. We see then that

(*a*) warming at 52° for $1\frac{1}{2}$ hours,
(*b*) cooling to 0°,
(*c*) action of urethanes,

cause the formation of indophenol blue. Now all these factors inhibit dehydrogenase systems much more than they inhibit oxidase systems. The indophenol blue is reduced to the colourless form by the active dehydrogenase systems in untreated yeast, but not in material treated as above. Is it not possible that cytochrome is reduced by the same systems ? When a solution of cytochrome *c* is added to washed heart muscle the pigment becomes oxidised. Addition of succinate causes a very rapid reduction in anaerobic conditions and a large oxygen uptake in aerobic ; the complete lactic dehydrogenase system likewise reduces cytochrome rapidly as does the hexosediphosphate system of Harrison. We can now construct a complete system whereby the oxidase of Keilin and of Warburg is brought into contact with dehydrogenase mechanisms by the action of cytochrome.



We now see why HCN, H_2S and CO inhibit cell respiration. They do so by stopping oxidase and the oxidation of cytochrome ; urethanes and warming, or cooling, stop dehydrogenases and reduction of cytochrome. We thus picture the latter as undergoing continuous oxidation and reduction.

It may be well to enlarge upon the concept. The so-called succinic dehydrase of Thunberg and of

Wieland is able rapidly to reduce oxygen, and its action is stopped by cyanide and by sulphide. Is this consistent with the Keilin scheme ? The answer is that such enzyme preparations probably contain all the factors—oxidase, cytochrome and dehydrogenase—necessary for an oxygen uptake. It is well to remember that only in some few cases has the dehydrogenase been truly separated from the oxidase-cytochrome mechanism.

The case of aerobic dehydrogenases is somewhat different. The xanthine oxidase (Schardinger enzyme?) is unaffected by HCN or H_2S and contains neither cytochrome nor oxidase. It is thus of a different type from succinic dehydrogenase, and is able to reduce cytochrome rapidly. It seems likely that such dehydrogenases work directly with oxygen and do not need the mediation of cytochrome and oxidase for the reduction of oxygen.

When methylene blue is added to a dehydrogenase solution and the oxygen uptake measured, the presence or absence of cyanide or sulphide is found to be without effect. Let us examine this more closely. The reducing action of dehydrogenases is unaffected by cyanide and the oxidation of leucomethylene blue, unlike that of cytochrome, is likewise unaffected. Hence there is a large oxygen uptake. In this sense methylene blue is equivalent to cytochrome and oxidase.

Cytochrome is oxidised by cytochrome oxidase, an enzyme which is inhibited by HCN, H_2S and by CO, the degree of inhibition by this last depending on the *ratio* p_{CO}/p_{O_2}. Now, as we have seen, the "Atmungsferment" of Warburg possesses precisely those qualities, and the question arises, is the indophenol oxidase ($\equiv$ cytochrome oxidase) identical with the Atmungsferment ? Both systems possess the same properties. More than that it is impossible to say, but their similarity is so close that it is hard to believe in their separate identity. On this point

Keilin and Warburg are agreed. The next point is harder to solve—it is this : " What is the chemical nature of the Atmungsferment ? " Warburg believes it to be a haematin derivative, which is disputed by Keilin. The evidence of the former worker is this :

(*a*) the Atmungsferment is inhibited by HCN and by H_2S ;

(*b*) the Atmungsferment is inhibited by CO, the degree of inhibition depends on the ratio p_{CO}/p_{O_2} and the CO-compound is dissociated by light, *the absorption spectrum resembling that of a haematin.*

These properties are characteristic of haematin derivatives. Now oxidase preparations are unable to oxidise cysteine in the absence of cytochrome : but this catalytic property is possessed by every known haematin, and hence, Keilin argues, the oxidase is probably not a haematin. It must be remembered that cytochrome does not combine with HCN or H_2S or CO, but Keilin believes that it does so when in association with oxidase. He thinks that the properties of the Atmungsferment are those of the oxidase-cytochrome complex, although the oxidase is not a haematin, and cytochrome alone is unable to combine with HCN, H_2S or CO. The main prop to Warburg's contention that oxidase contains haematin is the fact that the CO-oxidase spectrum resembles that of a haematin. Such observations as he has made have however been carried out on whole yeast cells, which contain much haematin, which may act as a photochemical sensitiser. To sum up—it seems clear that the Keilin-Warburg system accounts for most of the cyanide-sensitive respiration of cells, and the system is dominated by cytochrome, which is continually oxidised by oxidase and reduced by dehydrogenases ; but as to the nature of oxidase we are as yet uncertain.

In recent years pigments from divers sources have been isolated and shown to play a rôle in respiratory processes. Though differing widely in chemical

constitution, they all seem to function according to the following scheme :

$$\text{substrate} + \begin{matrix}\text{oxidised}\\ \text{pigment}\end{matrix} \xrightarrow{\text{dehydrogenase}} \text{leuco-pigment} + \begin{matrix}\text{oxidised}\\ \text{substrate}\end{matrix}$$

$$\text{leuco-pigment} + O_2 \longrightarrow \text{oxidised pigment}$$

These pigments differ from cytochrome in that their oxidation does not require the intermediation of an enzyme. Thus they offer an alternative path of respiration to the general path involving cytochrome. It must be emphasised, however, that these pigments are not universally distributed, and are restricted to special cells and tissues.

Warburg and Christian [1932] isolated a yellow pigment from bottom yeast with the following characteristics :

(1) the prosthetic pigment moiety is combined with a protein-like substance ;

(2) heating to 60° destroys the catalytic activity of the pigment ;

(3) the reduction can be brought about by a dehydrogenase system involving three components : (*a*) enzyme, (*b*) co-enzyme, and (*c*) hexosemonophosphate as substrate. This dehydrogenase system may be obtained from yeast, blood corpuscles, or liver ;

(4) neither CO nor HCN affect the reduction of the yellow pigment by the dehydrogenase system ;

(5) the leuco form is autoxidisable.

The addition of the yellow pigment to a solution containing the dehydrogenase system results in a rapid oxygen uptake. The yellow pigment is reduced at the expense of the oxidation of the hexosemonophosphate ; and the leuco-pigment reacts directly with molecular oxygen. Thus the pigment is constantly regenerated and oxygen used up.

There is no question but that the residual respiration observed in cells poisoned with HCN is due in the main to accessory catalysts of the type of the yellow

pigment. Since HCN inhibits only oxidase activity and these pigments are autoxidisable, respiration is not eliminated completely in those cells containing accessory catalysts. Warburg and Christian [1932] maintain that in the living cell the yellow pigment does not oxidise spontaneously but that the Atmungsferment brings about the oxidation. This assumption is gratuitous for the reoxidation of the yellow pigment by oxygen is as rapid as the reoxidation of methylene blue. There is no necessity for postulating oxidase activity.

Ellinger and Koschara [1933] have claimed that the yellow pigment isolated from bottom yeast is only one of a class of "lyochrome" pigments found in milk, liver, kidney, urine, muscle and the yolk of egg. Besides cytochrome, the yellow pigment is the most widely spread respiratory catalyst.

Michaelis and Friedheim [1931] extracted the blue pigment of B. *pyocyaneus* and showed that the pigment behaved as a reversible oxidation-reduction indicator. That is to say, the potentials at inert electrodes recorded by solutions containing the pigment agreed with the equations for reversible oxidation-reduction systems. Friedheim [1931] demonstrated the rôle of this pigment in the respiration of B. *pyocyaneus*. The bluish suspension of the bacteria in anaerobic conditions became colourless; readmission of air restored immediately the original blue colour. Thus it is clear that the pigment is reduced by some system in the bacterial cell, and the leuco form is reoxidised by molecular oxygen. As yet there is no knowledge of the exact mechanism in B. *pyocyaneus* which brings about the reduction of pyocyanine.

The addition of pyocyanine to red blood corpuscles or non-pigmented strains of B. *pyocyaneus* results in an enormous increase in the oxygen consumption. These results can be explained by assuming that the limiting factor in the respiration

of these two systems is the rate of oxidation of the intermediary carrier (presumably cytochrome). That is to say, dehydrogenase activity is more intense than oxidase activity. By adding pyocyanine, yellow pigment, or any other suitable oxidation-reduction indicator, we are presenting to the cells respiratory catalysts which depend only on dehydrogenase action. The autoxidation is spontaneous, and the result is an increase in oxygen consumption. It is noteworthy that there is apparently no specificity of respiratory catalyst. The pigment from B. *pyocyaneus* increases the respiration of red blood corpuscles as well as that of bacteria. Likewise the yellow pigment from bottom yeast is not type-specific for yeast, and exercises an effect on a variety of cells.

There still remains unanswered the fundamental question of what properties determine whether a pigment can function in a respiratory capacity. The work of Barron [1929] indicates that the oxidation-reduction potential is an important factor in determining whether the pigment can play a rôle in respiration. It is not within the province of this book to discuss either the theory or practice of oxidation-reduction potentials. Suffice it to say that the potential assigned to an indicator is a measure of the reducing tendencies. That is to say a group of indicators can be arranged in a scale of potentials according as the indicator is more or less reducing. Barron has established the empirical rule that artificial indicators can act as respiratory catalysts when their potential lies within a restricted range. Indicators above or below that range are not efficient as respiratory catalysts. Whether this empirical rule applies to natural pigment still awaits further studies.

In addition to the yellow pigment and pyocyanine, many more have been described though not studied in as great detail; echinochrome from sea-urchin

eggs [Cannan, 1927]; hermidin from the plant Mercurialis *perennis* [Cannan, 1928]; the pigment of B. *violaceus* [Friedheim, 1932]; and the pigment of the nudibranch mollusc Chromodoris *zebra* [Preisler, 1930].

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CHAPTER VI

THE GLUTATHIONE SYSTEM

IN this chapter we have to study a substance which occupies a unique place in biochemistry. It is found in most aerobic cells, from which it can be isolated in a crystalline state and its constitution determined, it can be reversibly oxidised and reduced, it possesses catalytic properties which we shall study in detail, it combines with certain heavy metals, and it acts as a co-enzyme to various thermolabile catalysts. This substance is glutathione. It is a peptide isolated in 1921 by Hopkins. He obtained it from liver and from yeast by a complex method of isolation. Since then it has been obtained from other sources such as blood [Holden, 1925], and a similar substance appears to exist in many organisms. The distribution of glutathione is almost universal in aerobic tissues, as judged by the nitroprusside reaction for the sulphydryl group. (This test was devised by Mörner in 1901. It is best carried out as follows. To the solution to be tested an excess of a mixture of ammonium sulphate freshly ground with a little sodium nitroprusside is added. A drop of strong ammonia is added and the whole mixed. If glutathione or a cysteine peptide be present, a purplish red colour appears and quickly fades.)

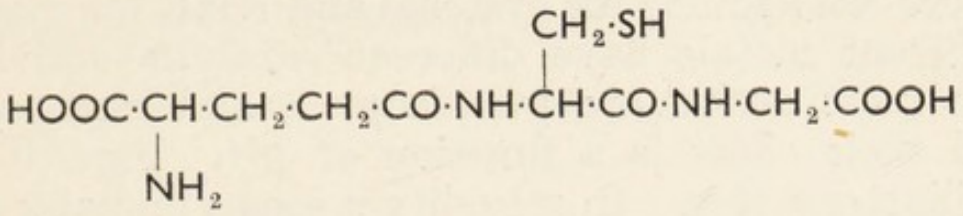
The product obtained in 1921 by Hopkins was amorphous and deliquescent, but that isolated in 1929 was crystalline and quite without any tendency to deliquesce. The method of 1929 is of the

most elegant simplicity. Yeast is warmed with 0·1 % acetic acid, filtered through kieselguhr, and the filtrate precipitated with the Hopkins-Cole reagent (10 % $HgSO_4$ in 7 % H_2SO_4). After decomposition of the mercury precipitate with H_2S, Cu_2O is carefully added to the sulphide-free filtrate which has been made 0·5 *N* with H_2SO_4 and warmed to 60°. A cuprous-glutathione complex falls out of solution. After having been washed free from sulphate, the cuprous salt is gassed with H_2S, the filtrate evaporated *in vacuo*, and finally over sulphuric acid. The glutathione crystallises out. The method has been still further simplified by Pirie [1930]. Kendall, MacKenzie and Mason [1929] have also succeeded in isolating crystalline glutathione, their method however being much less simple than that of Hopkins.

At first it was thought that glutathione was a dipeptide of cysteine and glutamic acid. A study of the crystalline material showed it to be a tripeptide containing cysteine, glycine and glutamic acid. When we talk of " crystalline glutathione " we mean those preparations which have been obtained crystalline, and respond to the usual chemical tests for purity, and which differ in certain respects from the non-crystalline material. When we talk of " amorphous glutathione " we mean those preparations which were made by the earlier methods, or which cannot be crystallised, and which appear from the property of deliquescence to be impure. Besides being impure these amorphous forms of glutathione possess properties which the crystalline material exhibits to a lesser degree. The terms " amorphous " and " crystalline " hence imply far more than mere difference in physical state—much rather do they refer to the possession of catalytic properties to a greater or lesser extent. It must, of course, clearly be realised that these terms apply to the behaviour of glutathione *in vitro* (after it has been separated, in

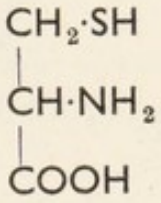
the crystalline or amorphous state, from the cell) much rather than to its behaviour in the cell, which is an entirely different problem.

Crystalline glutathione is glutamylcysteinylglycine,

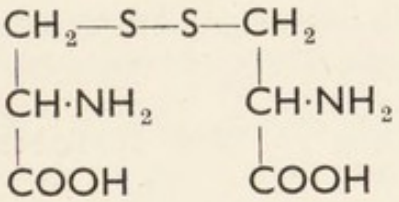


[Pirie and Pinhey, 1929; Kendall, MacKenzie and Mason, 1929; Kendall, Mason and MacKenzie, 1930; Nicolet, 1930.]

For an understanding of the problems connected with glutathione we must first turn to those of the substance cysteine,



which is present in glutathione as one of its components. Now free cysteine can exist in the reduced form written here: it can also exist as the disulphide cystine,



Cysteine can pass spontaneously (in the presence of oxygen) to cystine in aqueous solution at pH 7·6, when traces of catalytic metals are present. Metals which are catalytically active at pH 7·6 are iron and copper, and they exert their action at concentrations so low as ca. 10^{-5} *M*. Thus the oxidation of cysteine to cystine by oxygen in aqueous solution is catalysed by metals. This oxidation is sharply to be distinguished from the irreversible oxidation of cysteine which takes place at the surface of iron-containing

charcoal, and which involves the destruction of the whole molecule. The reaction which concerns us is

$$2RSH + \tfrac{1}{2}O_2 \longrightarrow RSSR + H_2O$$

where RSH stands for cysteine and RSSR for cystine. Different metals have different catalytic activities. The most active are iron, copper, and manganese, and their effect is a function of pH. Figs. 9 and 10 illustrate this. In actuality it seems probable that

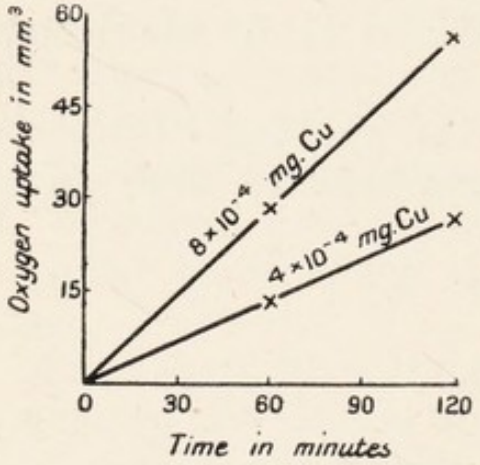


FIG. 9.

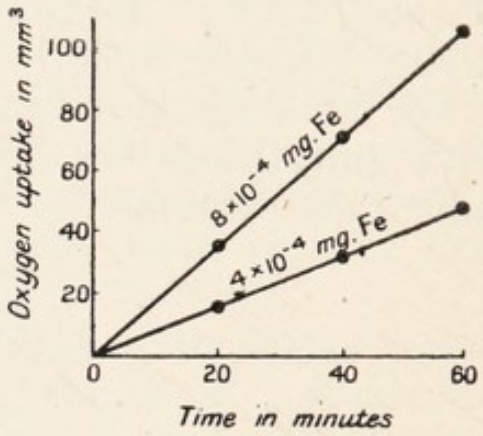


FIG. 10.

FIG. 9.—The catalytic action of Cu on the oxidation of cysteine. 6 mg. cysteine hydrochloride + 0·10 cc. $N/100$ HCl in 2 cc. borate pH 10·3. In air at 20° C.
(WARBURG [1927]. *Biochem. Zeit.* **187,** 255)

FIG. 10.—As fig. 9, but with Fe in place of Cu as catalyst
(WARBURG [1927]. *Biochem. Zeit.* **187,** 255)

hydrogen peroxide is first formed according to the equation

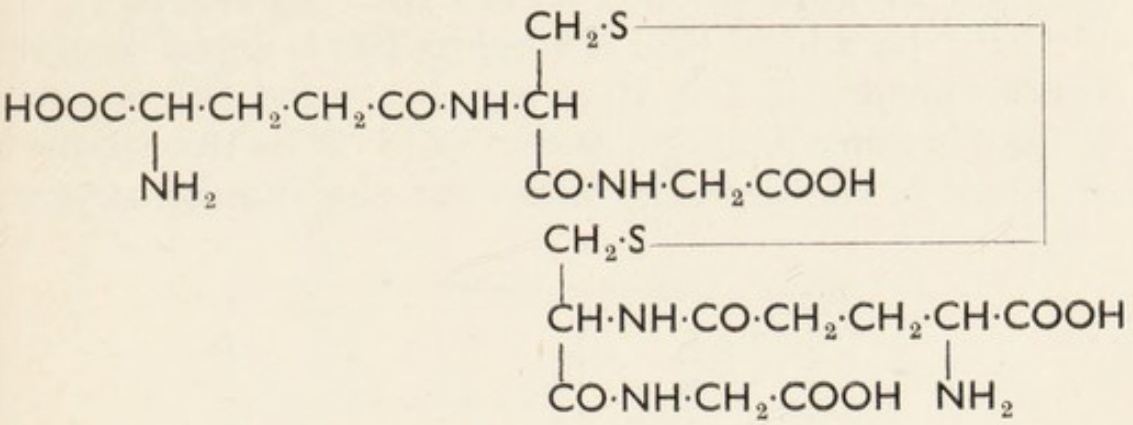
$$2RSH + O_2 \longrightarrow RSSR + H_2O_2$$

and that it then reacts with more cysteine,

$$2RSH + H_2O_2 \longrightarrow RSSR + H_2O$$

Just as cysteine oxidises to cystine so can amorphous glutathione rapidly oxidise in aqueous (aerated) solutions at pH 7·6, and just as with cysteine, so here the presence of metals such as iron or copper, or of iron-porphyrins, is essential for the oxidation [Harri-

son, 1924]. Thus glutathione can oxidise and become diglutamylcystyldiglycine



according to the equations

$$2GSH + O_2 \longrightarrow GSSG + H_2O_2$$
$$2GSH + H_2O_2 \longrightarrow GSSG + H_2O$$

It must be realised that these equations are merely net equations and make no reference to the catalyst or catalysts involved.

Now the action of hydrogen cyanide or of hydrogen sulphide on the copper-catalysed oxidation of cysteine is a marked inhibition. When iron (or an iron-porphyrin) acts as catalyst HCN still inhibits powerfully, but H_2S acts as an accelerant [Krebs, 1929]. Now a precisely similar action occurs with amorphous glutathione. HCN and H_2S inhibit the copper-catalysed oxidation, HCN again inhibits with iron and H_2S accelerates. Again, we have a parallelism between the behaviour of cysteine and of glutathione.

In all these experiments we have been dealing with cysteine and with amorphous glutathione. Let us now turn to the behaviour of the crystalline material. When dissolved in phosphate buffer at pH 7·6 it can react with molecular oxygen under certain specified conditions. In the absence of metals, or in the presence of HCN, no oxygen uptake is visible. Hence we can conclude that here also metals are required for its oxidation. There is, however, an important difference between cysteine (or amorphous glutathione)

and crystalline tripeptide as regards their behaviour to iron or copper salts. If to metal-free cysteine (or amorphous glutathione) about 10^{-3} mg. of Fe^{++} or of Cu^{++} be added (the pH being 7·6) a rapid oxygen uptake results, if 2×10^{-3} mg. be added the oxygen uptake is twice as large, and so on. Thus the amount of metal is the limiting factor for the rate of oxygen

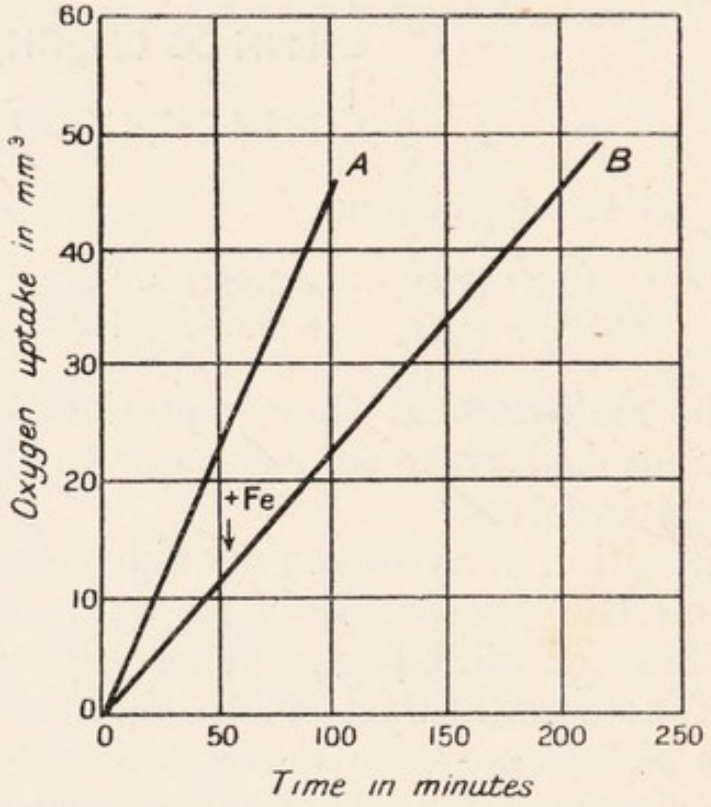


FIG. 11.—Effect of Fe on crystalline glutathione

A : 3·5 mg. crystalline GSH

B : 3·5 mg. recrystallised GSH — 0·0005 mg. Fe added at arrow

In phosphate buffer at 20° C.

(MELDRUM and DIXON [1930]. *Biochem. J.* **24,** 472)

uptake in the case of cysteine or amorphous glutathione. We have already found that HCN inhibits the oxidation of crystalline glutathione, and have deduced therefrom the necessity for iron or copper to catalyse its oxidation. When, however, 10^{-3} mg. Cu^{++} is added to 5 mg. or to 10 mg. of autoxidising glutathione no measurable increase in rate of uptake takes place. Only when so much as 1·0 mg. Cu^{++}

is added does an appreciable effect occur. In other words, though

(*a*) metal is essential for the oxidation of tripeptide glutathione,

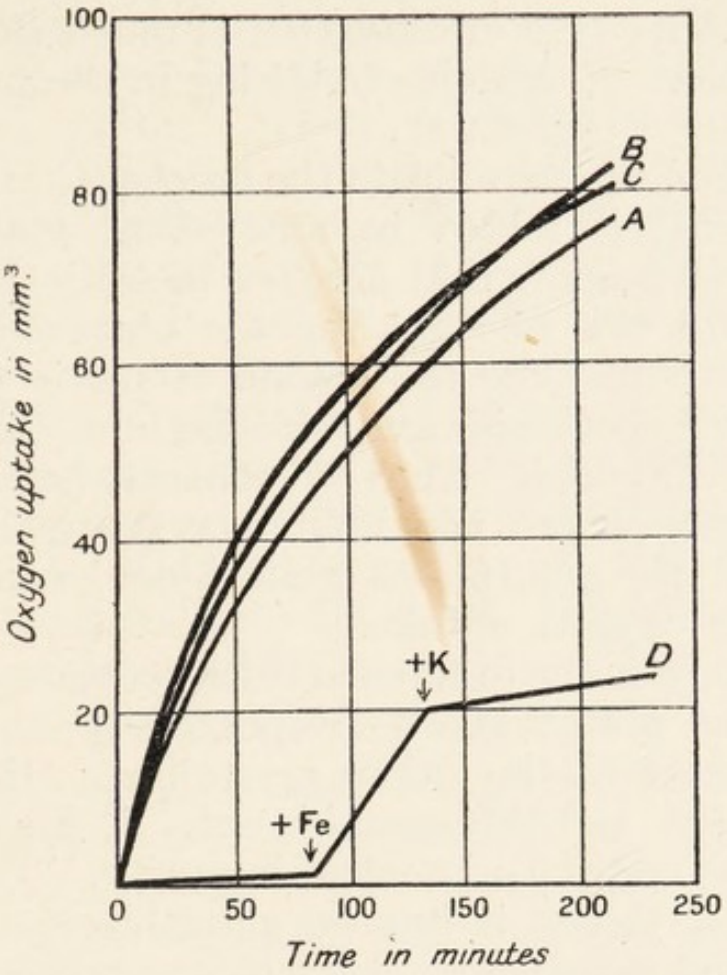


FIG. 12.—Removal of Fe by kaolin

A : 5 mg. crystalline GSH
B : 5 mg. crystalline GSH + 0·1 g. kieselguhr
C : 5 mg. crystalline GSH + 0·1 g. filter paper
D : 5 mg. crystalline GSH + 0·1 g. kaolin; 0·002 mg. Fe (as $FeSO_4$) added to *D* at first arrow, and 0·1 g. kaolin at second arrow. 21° C.

(MELDRUM and DIXON [1930]. *Biochem. J.* **24,** 472)

(*b*) metal is not here the factor which limits the rate of uptake of oxygen.

Figs. 11 and 12 illustrate these facts.

Hence it is clear that some other factor besides iron or copper must be concerned in the oxygen uptake of crystalline glutathione, and that this is the limiting

factor in most ordinary circumstances, when 10^{-3} mg. Cu^{++} is present.

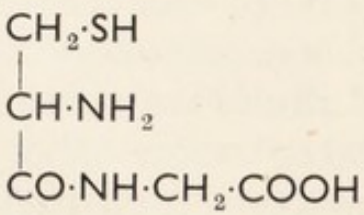
We see then

(*a*) that at least two factors are concerned in the oxidation of crystalline glutathione ;

(*b*) that its rate of oxidation is much slower than that of cysteine oxidising in the presence of 10^{-3} mg. Cu^{++}.

How then can we explain the acceleration of uptake when metal is added to amorphous glutathione ? Suppose we assume that here the metal is the limiting factor, will this account for the observed results ? Clearly it will. For the second factor is present in excess (by hypothesis), and addition of metal increases the rate of uptake. Thus we contrast (*a*) crystalline glutathione, where the unknown factor limits the rate with (*b*) amorphous glutathione, where metal limits the oxygen uptake.

What now is the difference between amorphous and crystalline glutathione ? Superficially the one is amorphous and the other crystalline ; the first is deliquescent, and the second is not. Deliquescence is a frequent accompaniment of impurity. Thus amorphous glutathione is impure glutathione and it has been found possible to convert crystalline material into deliquescent amorphous merely by warming with acid. Thus the second factor which limits the rate of oxidation of crystalline glutathione appears to be a product of the partial decomposition of tripeptide glutathione. Meldrum and Dixon [1930] suggested this substance might be free cysteine. It is perhaps more likely to be, as Mason suggested, cysteinylglycine,



Let us now turn to the properties of cysteine and

of amorphous glutathione and their relationship with tissue proteins. When muscle protein is chopped and washed exhaustively and raised to boiling point in water, a greyish powder results on drying, which we shall call " thermostable muscle powder ". Now such powder reacts with nitroprusside and gives a purplish colour. In other words, it contains the sulphydryl group. When a suspension of such powder is incubated anaerobically with a solution of disulphide glutathione, the glutathione becomes reduced to the tripeptide material [Hopkins and Dixon, 1922; Tunnicliffe, 1925], and the nitroprusside reaction in the powder disappears as the –SH is oxidised to disulphide. Now in incubating such oxidised muscle powder (containing only –S—S–) with a large excess of sulphydryl glutathione (or cysteine) the disulphide groups in the tissue are reduced back to the –SH form. Thus we have to conceive of an equilibrium

$$GSSG + 2TSH \rightleftharpoons 2GSH + TSST$$

and we can estimate the –SH groups in the protein by estimating the total amount of soluble –SH produced in this way. This can readily be done by titrating with iodine in acid solution [Tunnicliffe, 1925, 2] till all the –SH has become oxidised according to the equation

$$2RSH + I_2 \longrightarrow RSSR + 2HI$$

In vivo then there is presumably an equilibrium between tissue –SH and oxidised glutathione. These experiments are anaerobic. Let us now turn to the aerobic behaviour. When muscle powder is freed from fat by extraction with alcohol and ether it still retains its power to reduce glutathione, and when shaken in air with amorphous glutathione or cysteine (the pH being 7·6) a large oxygen uptake results, many times larger than that required to oxidise the total –SH of the system. In other words, the cysteine or amorphous glutathione is catalysing the

oxidation of the protein. But crystalline glutathione not only cannot oxidise muscle protein, but itself loses the power of autoxidising, so that when *crystalline* glutathione is added to muscle powder the total oxygen uptake is almost negligible. On the other hand, muscle powder can reduce disulphide glutathione whether it has been derived from amorphous material or from tripeptide.

Meldrum and Dixon [1930] studied the behaviour of crystalline glutathione and suggested that its autoxidation was due to the combined presence of two factors :

(*a*) metals such as iron or copper (which explains the HCN inhibitions) ;

(*b*) another factor in many ways similar to cysteine which is adsorbed and inactivated by muscle powder when it is present in small amount (as in crystalline tripeptide).

Hopkins [1925] showed that glutathione could catalyse the oxidation of fats and fatty acids. The conditions are, however, rather complex and will not here be discussed.

Let us now turn to the behaviour of glutathione in the cell. We have already found

(1) that tissue residues reduce it ;

(2) that it combines with iron or copper or haematins ;

(3) that it is oxidisable at rates depending on at least two factors.

Our question is—how do these properties concern the cell ? When a nitroprusside test for the sulphydryl group is carried out on almost any aerobic cell a strongly positive reaction is given. In other words, cells maintain their glutathione mainly in the reduced state. Now we have seen that certain protein systems themselves containing –SH can reduce glutathione, but clearly this is a quite inadequate clue to the problem of how the sulphydryl group is kept reduced, as the –SH has to be incorporated into the protein first. Attempts were made

by several workers to cause the reduction of disulphide glutathione by incubating with dehydrogenase systems. No positive results could at first be obtained. Hopkins and Elliott [1931] studied the behaviour of glutathione in liver. They found added

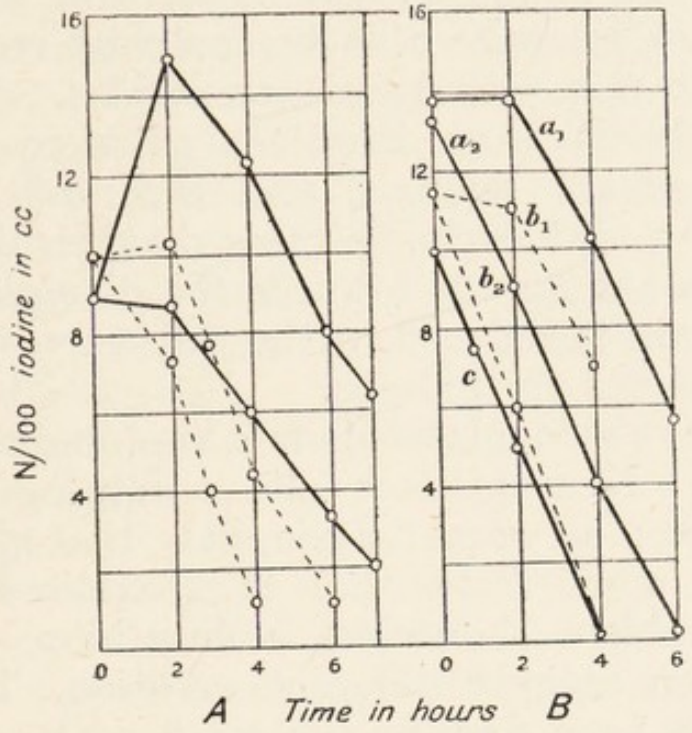


FIG. 13.—The behaviour of GSH in liver, showing effects of added GSSG

A : Continuous lines : lower curve shows normal course of disappearance of GSH from liver of well-fed rabbit ; upper curve given by same liver when 100 mg. GSSG (which does not titrate with I_2) had been added before aeration

Broken lines show results of similar experiment with liver of fasting animal

B : Effect of inactivating reducing systems in the liver

Curves a_1, b_1, normal course in livers of two well-fed rabbits ; curves a_2, b_2, c, fall in –SH in same tissue previously heated for 1 hour at 50° C.

Temp. 16° C.

(HOPKINS and ELLIOTT [1931]. *Proc. Roy. Soc.* B **109** 58)

disulphide glutathione was rapidly reduced, as indicated by an increased iodine titre (see fig. 13), and that this reduction did not take place when the liver was previously warmed. This indicated that dehydrogenase systems were causing the reduction.

Mann [1932] found that an enzyme preparation made from liver according to the method of Harrison [1931] rapidly reduced glutathione in the presence of glucose. Meldrum [1932] studied the behaviour of glutathione in mammalian erythrocytes and found that glucose, mannose, galactose, and to a lesser extent fructose, were able to cause its reduction in intact (but not with lysed) corpuscles. The hexahydric alcohols were inactive, as were the non-reducing sugars sucrose and raffinose. It thus seems likely that carbohydrates or their derivatives act as reducing agents towards the disulphide group and maintain it reduced in the cell when the appropriate catalysts are present.

As we have seen, glutathione is kept mainly reduced in the cell. Now this fact tells us nothing of the rate of oxidation : all we can say is this—that glutathione is reduced much faster than it is oxidised ; but as for the problem—how fast is glutathione oxidised? —that is an entirely different question. To answer it we shall turn first to the work of Hopkins and Elliott [1931]. They chopped liver and aerated the suspension. When the animal had been starved a rapid decrease in the iodine titre took place from the beginning : when the animal had been well fed previous to killing, there was a latent period of about two hours before the glutathione began to disappear. Then it did so as rapidly as with starved animals. These authors interpreted the latent period as being due to the continual reduction of the glutathione (which was oxidising all the time) by reducing systems, and the sudden drop in iodine titre as being due to disappearance of –SH consequent on the exhaustion of the reducing material. Kidney behaved in the same way. (See fig. 13.) It is thus clear that glutathione plays a marked part in the respiration of liver and of kidney. Little is known of its behaviour in other tissues. Meldrum [1932] studied its behaviour in mammalian erythrocytes. He found glutathione

to be slowly oxidised when a washed suspension of erythrocytes was aerated in the absence of an aldohexose. Addition of an aldohexose to such a suspension he found to cause an increase in the amount of reduced glutathione. We must then picture glutathione in erythrocytes, as in liver, to be undergoing a continual oxidation and a much more rapid reduction : only in the case of erythrocytes the rate of disappearance of glutathione is much slower than in liver. The substance is much more inert. The same author [Meldrum, 1930] studied the behaviour of glutathione in yeast. He altered the respiration of yeast by subjecting it to various influences :

(1) to starvation ;
(2) to narcotics ;
(3) to cooling ;
(4) to potassium cyanide ;
(5) to anaerobiosis ;

and measured the –SH content after such treatment. In no case was the amount of –SH appreciably altered even though the respiration varied from 60,000 mm.^{3} O_2 per g. per hour to 1,300 mm.^{3} O_2 per g. per hour. While this does not exclude glutathione from participating in the respiration of yeast, it does seem unlikely that the tripeptide plays so large a part here as Hopkins and Elliott [1931] have demonstrated for liver. It must thus be realised that while glutathione is maintained reduced in all cells, the rate at which it is oxidised appears to depend very much on the cell concerned. It oxidises rapidly in starved liver or kidney, slowly in erythrocytes or yeast.

Reduced glutathione combines with various metals. This fact has considerable relevance with regard to certain enzyme systems. Waldschmidt-Leitz [1929] and collaborators have studied the activating influence exerted by glutathione (and also by HCN and H_2S) on the hydrolysis of gelatin by bromelin,[1] kathepsin

[1] Bromelin and papain are proteolytic enzymes found in certain plants, and kathepsin is an intracellular peptidase.

and papain. Krebs [1930] has adduced much evidence that metals poison these enzymes and that the glutathione, by combining with the metal, detoxicates the enzyme. We thus have the respiratory mechanisms connected with proteolytic. Perhaps most interesting of all is the recently published work of Lohmann [1932] who claims that glutathione is the co-enzyme of glyoxalase and thus promotes the formation of lactic acid from methylglyoxal

$$CH_3{\cdot}CO{\cdot}CHO + H_2O \longrightarrow CH_3{\cdot}CHOH{\cdot}COOH$$

The action appears to be entirely specific and limited to glutathione; cysteine is without effect, as are HCN and H_2S. In this way is glutathione connected with carbohydrate metabolism.

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CHAPTER VII

MODERN DEVELOPMENTS

THE chemical purification of physiological systems has led to many advances in biochemistry. When thyroxine was obtained crystalline, it became possible to study its reactions, to determine its constitution and synthesise it: the hexuronic acid of von Szent-Györgyi has recently been identified with vitamin C, and it is now possible accurately to determine the properties of the white crystalline powder. Thus it is that in many respects the advancement of biochemistry is essentially an affair of technique—a practical matter—rather than a problem for theoretical development. We have tended in the previous parts of this book rather to neglect the practical side of affairs and to discuss respiratory catalysts from the theoretical aspect. Now we shall see the developments of technique in enzyme chemistry and the views they have led to.

As an example of how enzymes are purified, we shall consider the researches of Willstätter [1928] and his school on the peroxidase which is prepared from horseradish roots. To discuss Willstätter's researches in detail is beyond the limits of this book, and the steps described illustrate the *principles* of enzyme purification. (Each different stage has been given a number, and the explanation of this process has to that number the suffix A attached.)

(1) Horseradish roots are washed and cut into slices $\frac{1}{2}$–1 mm. in thickness, and dialysed in running water for 4–6 days.

(1A) The peroxidase being colloidal in nature cannot pass through the cell wall while the dialysable salts, sugars, etc., are readily lost. The process is simply dialysis with cell wall as membrane in place of parchment or collodion.

(2) The roots are filtered and suspended in 10 l. water containing 40 g. $(COOH)_2.2H_2O$ for 5 kg. roots, for 4–6 hours.

(2A) The oxalic acid alters the pH and

(*a*) thus increases permeability,

(*b*) causes adsorption of peroxidase on to the solid cell structures.

(3) The dialysate is sucked off and the roots ground and washed with water (using certain precautions), and the enzyme extracted by half-saturated $Ba(OH)_2$ added in small amounts at a time to the ground roots.

(3A) The alkaline solution alters the pH of the acid roots and then the enzyme comes off the cell walls. This is the process of " elution ", i.e. change of pH changes the extent of adsorption.

(4) The alkaline extract is neutralised with CO_2.

(4A) The reason for saturating the solution with CO_2 is gradually to neutralise the alkali. The use of strong acid such as H_2SO_4 may lead to intense local acidity and consequent destruction of the neighbouring enzyme.

(5) The solution contains much protein matter and this is precipitated by adding $\frac{9}{10}$ the volume of 96 % alcohol, the enzyme remaining in solution.

(5A) This simply makes use of the greater solubility in alcohol of peroxidase.

(6) The solution is evaporated (after filtering) at low pressure and temperature, and the enzyme is precipitated by five times the volume of absolute alcohol, washed with absolute alcohol, and dried *in vacuo*.

(6A) The reason for preparing solid peroxidase is the greater stability of enzymes in the solid state.

We have now obtained a solid preparation of peroxidase which retains its activity for many months, especially *in vacuo* at 0°.

To follow the subsequent purification it is now necessary to define the activity of a peroxidase preparation and the enzyme unit. The *activity* of an enzyme preparation is obtained thus : an amount of enzyme is used which forms 15–25 mg. purpurogallin in 5 minutes under certain conditions (in a solution of 5 g. pyrogallol and 50 mg. H_2O_2 dissolved in 2 l. water at 20°). The result of this determination expressed as mg. purpurogallin per 1 mg. enzyme dry weight is the " Purpurogallinzahl " (P.Z.). The *unit* of enzyme (" Peroxydaseeinheit ", P.E.), is 1 g. enzyme of P.Z. = 1, or 1 mg. peroxidase of P.Z. = 1,000.

The following example—taken from Willstätter—illustrates the method employed for further purification.

The peroxidase solution (0·818 g.) was dissolved in 1 l. water and made to $N/50$ $[H^+]$ with $CH_3 \cdot COOH$ and 20 g. pure kaolin added in small portions to the ice-cold solution. The adsorbate was sucked off and suspended in 600 cc. ice-cold water, N NH_3 added with shaking till the solution contained 0·1 % NH_3. The yellow-gold solution of peroxidase was sucked off, and the elution repeated. Such a procedure was repeated with Al_2O_3 (A) as adsorbent and again with kaolin. Finally, the enzyme was precipitated with tannin, the precipitate dissolved in acidified aqueous alcohol, and the enzyme removed with kaolin.

The following table shows the amount (P.E.) and activity (P.Z.) of the enzyme.

Process applied	P.E.	P.Z.
Original material	401	490
Elutriate from kaolin adsorption . .	397	—
Elutriate from first $Al(OH)_3$ adsorption	302	1950
Elutriate from second $Al(OH)_3$ adsorption	239	1910
Elutriate from kaolin adsorbate from tannin ppt.	88·8	3070

A highly purified preparation of peroxidase has thus been obtained. What conclusions can be drawn therefrom ?

It is found that all peroxidase preparations contain iron : is it possible that the metal is in some way associated with the enzyme ? Suppose we estimate the iron content of various peroxidase preparations—fig. 14 is obtained. There is no very constant ratio between activity and iron content. It is however possible that the ratio P.Z./Fe falls off because of

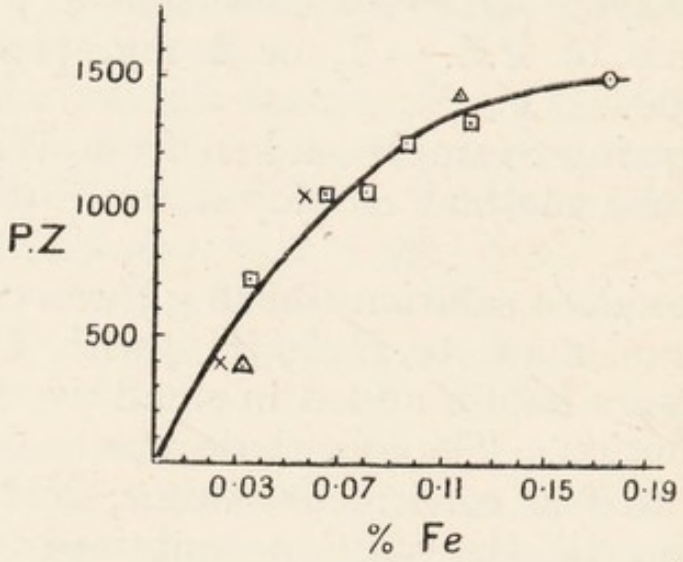

FIG. 14.—Fe content of various peroxidase preparations
⊙ Purified preparation from horseradish
⊡ Another preparation as above
△ Purified preparation from turnips
× Another preparation as above
(WILLSTÄTTER and POLLINGER [1923]. *Ann.* **430,** 269)

inactivation of the enzyme or because of some accompanying inactive impurity. Though there is no very constant ratio P.Z./Fe, yet there is always some iron in peroxidase preparations.

Now Willstätter and Pollinger [1923] invariably found that their peroxidase preparations were highly coloured and, indeed, contained much of some haematin. In view of the already known fact that haematins (to a greater degree when combined with bases to form parahaematins) exert a peroxidase-like activity (in that they cause the oxidation of guaiacol

and of benzidine in presence of H_2O_2) the suggestion is self-evident that the haematin portion of peroxidase preparations is in some way connected with their specific function. Kuhn, Hand and Florkin [1931] have estimated the haematin content of peroxidases spectrophotometrically. They found a fairly close relationship between the amount of haematin and the activity of the enzyme. Their data have been tabulated below :

Peroxidase preparation	P.Z.	Haematin iron %	$\frac{\text{P.Z.} \times 10^{-3}}{\text{Haematin iron \%}}$
6	3400	0·009	378
10	1710	0·0052	329
14	1080	0·0033	327

Zeile and Hellström [1930] have obtained a similar relationship for the haematin content of various catalases and their activities. It would appear then to be not improbable that both peroxidase and catalase are haematin compounds

(*a*) because they are inhibited by HCN and H_2S ;

(*b*) because there is a fairly constant ratio between their contents of haematin and their activities.

Now while it is true that *any* haematin will act as a (thermostable) peroxidase and blue guaiacum in the presence of H_2O_2, the activity of peroxidase (per mole haematin) isolated from plant or animal source is very much greater than that of any haematin solution (by this is meant an alkaline aqueous solution of crystalline haemin). The activity is several thousand times greater in the case of the enzyme [Willstätter and Pollinger, 1923]. Solutions of peroxidase and of catalase exhibit the spectrum of parahaematin, yet no parahaematin of known constitution possesses activity to a remotely comparable degree. There must be some very *specific property*

involved in peroxidase action. The haematin of peroxidase preparations is only slowly reduced by hydrosulphite, and not at all in the absence of alkali and pyridine. In this respect it differs markedly from all other known parahaematins which, with the exception of catalase, are universally reduced with ease by hydrosulphite [Kuhn, Hand and Florkin, 1931; Zeile and Hellström, 1930].

Again, let us consider cytochrome: it can exist as a parahaematin, or reduced as a haemochromogen. This is shown by its spectrum. But its properties are distinct from all other known substances of related chemical nature in that it is

(1) only slowly autoxidisable;

(2) apart from helicorubin, the only haematin derivative to be oxidised by indophenol oxidase.

Thus the combination of a definite and unique haematin with a probably equally unique nitrogen-containing compound shows properties of an unparalleled nature. Here again we have the idea of "specificity". *It is now clear that a very slight structural alteration in an organic molecule may produce biological effects of the most profound nature.* It must of course be realised that the concept is not new, and the idea of organic structure affecting physiological properties originates perhaps with the work of Crum Brown and Fraser on the effect of methylating natural bases. Within recent years the importance of molecular constitution has become apparent in respiration. To say that iron catalyses biological oxidations is without meaning: to estimate (as is often done) the amount of iron in a tissue and deduce therefrom its respiration is like explaining the mechanism of a motor car by the weight of iron in the derelict chassis. The structure of the catalyst is of decisive importance.

We must then qualify the statement that the Atmungsferment and cytochrome, together with catalase and peroxidase, are haematin compounds,

by emphasising the fact that they each have a *definite* organic structure which confers on each its *highly specific* properties. They have (with the exception of cytochrome) the power of combining with HCN and with H_2S, and it seems likely that these poisons inhibit cell respiration by forming compounds with such haematins.

When emulsions of unsaturated fats or fatty acids are shaken with haematins, a rapid oxygen uptake results. The haematin catalyses the oxidation of the ethylenic linkages in the fats [Robinson, 1924]. Now this oxygen uptake is *not* inhibited by cyanides. The question has been further developed by Kuhn and Meyer [1930]. They measured oxidation of fats and of substances containing unsaturated linkages such as bixin [1] in the presence of haematin catalysts with, and without, cyanide. In no case was inhibition observed.

Thus we come to the view that all haematin catalyses are not inhibited by cyanide, but that the presence or absence of an inhibition depends quite as much on the substance oxidised as on the type of catalyst. When haematin is catalyst the oxidation of cysteine is stopped by HCN, but not the oxidation of linolenic acid.

What relevance has this to cell respiration ? We shall see later. For the moment let us consider the observed facts. The classic work of Dixon and Elliott [1929] tabulated the respiration of various tissues in presence of cyanide. The following table illustrates their results. It is clear that while the respiration of yeast is entirely, or almost completely, stopped by cyanide, that of most mammalian tissues is only very partially affected.

[1] Bixin is

$$CH_3{\cdot}OOC{\cdot}CH : (CH{\cdot}C(CH_3) : CH{\cdot}CH :)_4 CH{\cdot}COOH$$

Tissue	Normal rate of O_2 uptake (mm.³ per g. per hour)	Inhibition produced by KCN (% of normal)			Uptake not inhibited by KCN (mm.³ per g. per hour)
		M/3000	*M*/300	*M*/30	
Ox kidney .	320	42	52	50	160
Sheep kidney .	1700	75	80	82	310
Ox liver . .	310	16	25	54	140
Sheep liver .	360	43	62	61	140
Yeast . . .	4000	—	—	85	600
Yeast " fed "	36000	78	92	92	2800

How are we to interpret these results ? There are two ways of regarding the facts. It may be said that the normal respiration of the tissue concerned consists of two parts,

(*a*) cyanide-sensitive,

(*b*) cyanide-insensitive,

and that the presence of cyanide eliminates the former, leaving the latter unaffected. Again it may be said that the *normal* respiration is entirely cyanide-sensitive and that the presence of this reagent

(*a*) inhibits the *normal* respiration, and

(*b*) calls into being a *new* (cyanide-stable) respiration.

It is, of course, possible to compromise between these two views, which, stated as they are, represent the extreme positions. Whichever view is most correct, it may well be that the cyanide-stable respiration results from the oxidation of fats. Thus the respiration of Chlorella is not sensitive to cyanide unless in the presence of glucose [Emerson, 1927].

In brief the modern study of respiration has led

(1) to the emphasis of molecular specificity and the dominance of organic structure in determining catalytic properties ;

(2) to the discovery of the widespread occurrence of haematin catalysis.

It has further become probable that the sensitivity of respiration to cyanide and sulphide can be explained by the latter combining with haematin catalysts.

Such generality as can be ascribed to the results of recent research would appear to lie in these ideas.

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GLOSSARY

Active centre . .	A term used to connote an insoluble centre of catalytic activity characterised by a strong unbalanced field of force and by a high degree of specificity.
Ascorbic acid . .	See Hexuronic acid.
Atmungsferment .	See Respiratory ferment.
Autoxidation . .	The spontaneous oxidation of a substance oxidising in the presence of oxygen, without any action exerted by other molecules.
Catalase . . .	An enzyme which catalyses the decomposition of hydrogen peroxide to water and molecular oxygen. It cannot perform oxidations.
Catalysis . . .	Catalysis is characterised by three criteria. (*a*) A catalyst cannot initiate a new reaction : it merely accelerates one already taking place, though immeasurably slowly. (*b*) It modifies the rate of opposite reactions to the same extent. (*c*) As it is unchanged in the process it is clear that a small amount of catalyst can effect a change in indefinitely large amounts of reactants. It must be understood that these criteria of catalysis are ideal, and will not bear rigid criticism when applied to actual systems. They are true only when applied to small amounts of catalyst acting as a reversible system at equilibrium, a condition which can but rarely exist *in vivo*.
Cytochrome . .	A coloured substance found in most aerobic cells. It contains three

closely allied molecules which are metal porphyrins united with unknown (and probably specific) nitrogen-containing substance or substances. These molecules have been named cytochrome *a*, *b*, *c*. Cytochromes *a* and *c* are iron-porphyrins, and are oxidised by the indophenol oxidase. Cytochrome *b* has not yet been extracted and is autoxidisable to some extent, though it, too, is probably oxidised *in vivo* by indophenol oxidase.

Dehydrogenase . . The name given to an enzyme which catalyses oxidation so that the net result is as though hydrogen had been transferred from the substance oxidised to that reduced. They are subdivided into anaerobic dehydrogenases which reduce cytochrome and cannot reduce oxygen in the presence of their substrates, and aerobic dehydrogenases which can directly reduce oxygen to hydrogen peroxide.

Dehydrogenase systems A convenient term used here by the author for the complete system of dehydrogenase enzyme plus its reducing substrate, e.g. for succinic dehydrogenase and succinate.

Enzyme . . . The name used for a heat-labile catalyst occurring *in vivo*. All oxidases and dehydrogenases are enzymes, but the converse is untrue—many enzymes perform hydrolyses and not oxidations.

Glutathione . . A tripeptide of glycine, glutamic acid and cysteine present in most aerobic cells. It probably acts as an oxygen carrier, being reversibly oxidised and reduced.

Haemocyanin . . A respiratory pigment found in some crustacea. It cannot catalyse oxidations, and acts as an oxygen transporter by combining with it in the lung and giving it up to the tissues.

Haemoglobin . .	The red respiratory pigment of mammalian blood. It combines with oxygen reversibly, absorbing it at high pressure and giving it off in the capillaries and arterioles.
Hexuronic acid. .	An acid (now named ascorbic acid) isolated from plants and suprarenal glands by Szent-Györgyi in crystalline form. It cures scurvy and appears to be either vitamin C, or a precursor. It can be oxidised and reduced reversibly, and may act as an intermediate carrier in the cell.
Hydrogen acceptor .	A term used for a molecule which is reduced by taking up hydrogen. Oxidised cytochrome is a naturally occurring hydrogen acceptor and is reduced by anaerobic dehydrogenase systems.
Hydrogen donator .	A molecule such as lactate or succinate is said to be a hydrogen donator when it is oxidised under the influence of its specific enzyme and yields its hydrogen to cytochrome or methylene blue.
Indophenol oxidase .	The oxidase which oxidises cytochrome. It is sensitive to HCN, H_2S and to CO.
Oxidase . . .	An enzyme which performs oxidations in the presence of molecular oxygen.
Oxygen acceptor .	A substance which becomes oxidised by taking up oxygen or its equivalent. Thus cytochrome accepts oxygen in the sense that it becomes oxidised by indophenol oxidase.
Oxygenase . .	In its original meaning (that of Bach and Chodat) a system which catalytically produced hydrogen peroxide ; in the sense of Onslow the system contains an enzyme and substrate ; and in the later (and erroneous) sense of Cook, Haldane and Mapson it means cytochrome oxidase plus cytochrome, i.e. a system similar to the Atmungsferment.

Peroxidase . .	An enzyme which performs oxidations of (e.g.) tyrosine or histidine or pyrogallol in the presence of hydrogen peroxide.
Porphyrin . .	The name given to the nucleus of pyrrole groups left when iron is removed from haematin. The removal of magnesium from chlorophyll likewise yields porphyrins.
Respiratory ferment .	The oxygen-activating ferment studied by Warburg, who considers it to be an iron porphyrin, because of its spectrum, its sensitivity to HCN and to CO.
Specificity . .	The specificity of an enzyme limits the number of substances it will attack. Thus uricase is specific to uric acid and will oxidise it alone to allantoin.
Substrate . .	The substance oxidised or hydrolysed by an enzyme is said to be its substrate. Thus uric acid is the substrate of uricase.

APPENDIX

IN studying cell respiration two methods of attack have become widespread and seem worthy of description. In dealing with dehydrogenase systems Thunberg has evolved a method which is very widely used and which is easy and rapid to effect. We have seen how dehydrogenase systems reduce dyes such as methylene blue to colourless leucomethylene blue, and we have seen how the leucomethylene blue becomes oxidised back to methylene blue in presence of air. It is accordingly necessary, in studying dehydrogenase systems, to use an anaerobic technique. For this purpose Thunberg makes use of small tubes which can be evacuated. Two of these " Thunberg " tubes are shown in figs. 15 and 16. Suppose we wish to study the reduction of methylene blue by lactic acid in presence of lactic dehydrogenase. The method would be as follows. About 2 cc. of enzyme preparation are pipetted into the tube, about 0·3 cc. of methylene blue solution (1/5000 in water), and (say) 0·5 cc. of $M/100$ sodium lactate. Then the tube is evacuated at the water-pump, the tube tapped sharply several times to ensure complete removal of oxygen, and the residual air washed out with pure nitrogen. A second evacuation is then carried out, and nitrogen again run in. The time of reduction of the dye is then measured. This description gives the essentials of the method, but it has to be modified to suit different objects, and many refinements have been omitted. (Ahlgren has described the method in detail in Abderhalden's *Handbuch.*[1])

When it is desired to measure the oxygen uptake of tissue preparations, or of enzymes, an entirely different

[1] Ahlgren, Abderhalden's *Handb. d. biol. Arbt. Abt.*, IV, 671.

technique has to be used. Many modifications have been produced, but all essentials are included in the following description. Suppose we wish to study the oxygen uptake when succinate is added to a brain preparation. The tissue is washed and sliced or ground with sand. A known amount is placed into the right-hand cup *A* of the Barcroft apparatus shown

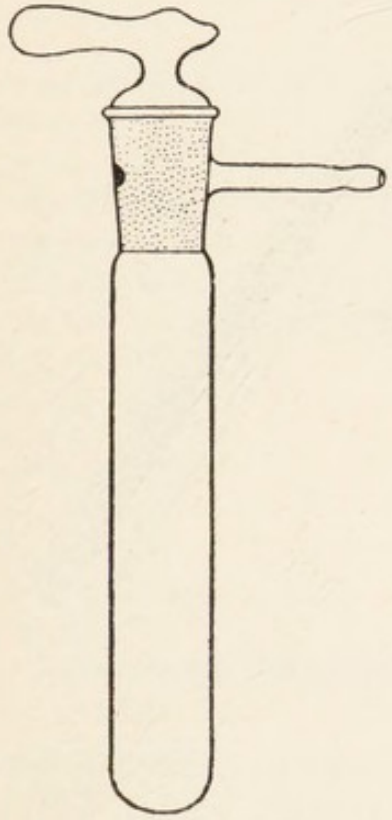

FIG. 15

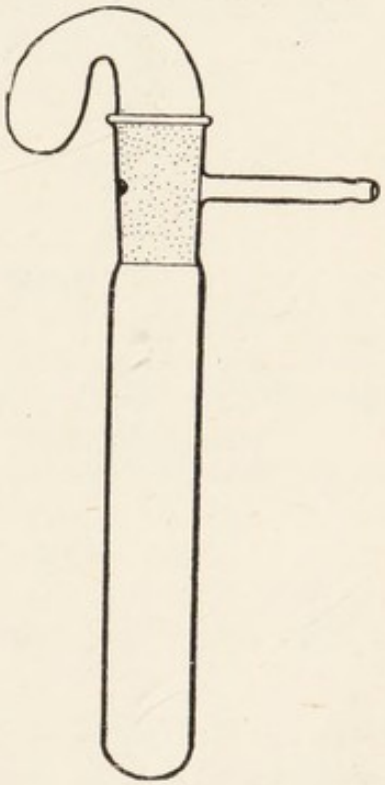

FIG. 16

FIG. 15.—The common type of Thunberg tube

FIG. 16.—Another form of Thunberg tube, devised by Keilin. A small quantity of fluid can be placed in the hollow stopper, and when the tube is inverted after evacuation the fluids are mixed

in fig. 17. (This apparatus was devised in a slightly different form by Barcroft, who applied it to the analysis of blood gases.) An equal amount of tissue is placed in the left-hand cup, and buffer added till the total volume is 3 cc. The right-hand cup is placed in position as shown after succinate has been run in and the volume made up to 3 cc. Both cups being in position, the whole apparatus is placed in a bath at constant temperature, say 37°, and shaken in a horizontal direction, oscillating

about 120 times a minute. After the cups have been in the bath for about 1 minute, they are gently ground into position till the two glass surfaces grate. Then the whole is shaken for about 5 minutes more and the taps are closed. The oxygen uptake is indicated by the movement of paraffin, coloured with Sudan III, in the

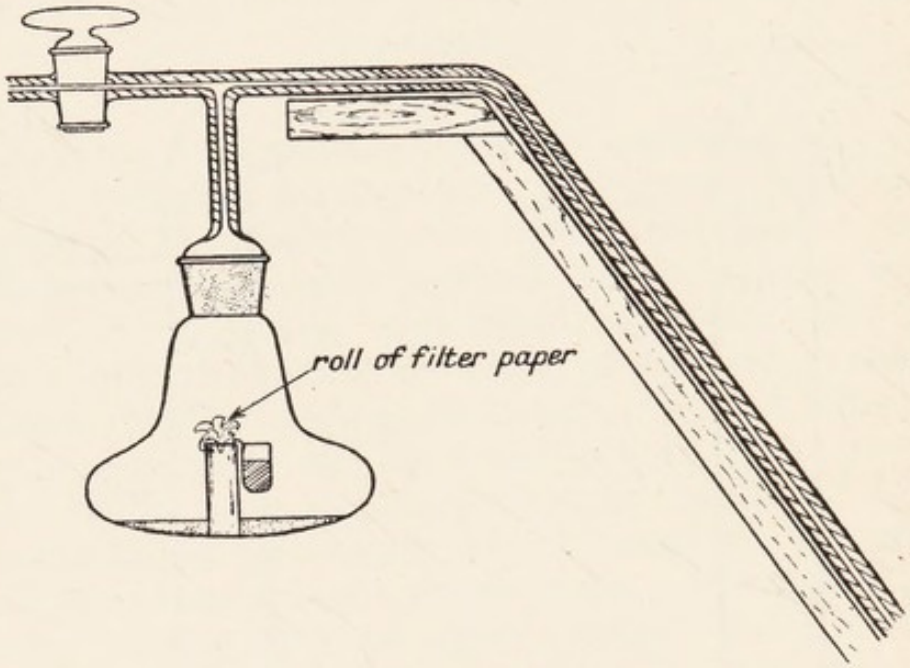

FIG. 17.—The Barcroft differential manometer

capillary. If the tissue evolves carbon dioxide during the experiment it is necessary to place about 0·2 cc. of strong NaOH in the small pot in the centre of each cup. For a detailed description of the Barcroft apparatus and its limitations see Dixon and Elliott [1929, 1930] [1] and Dixon [1934].[2]

[1] Dixon and Elliott, (1929) *Biochem. J.* **23**, 812. Dixon and Elliott, (1930) *Biochem. J.* **25**, 296.

[2] Dixon, *Manometric Methods*, Cambridge (1934).

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