MULTI-ENZYME SYSTEMS
PREFACE

When Professor F. G. Young was kind enough to send me an invitation from University College, London, to give a course of special lectures on enzymes, it was with no thought that they would be published that I accepted the invitation. At the conclusion of the course, however, a number of the senior members of the audience expressed a desire that they should be published, as they contained a good deal of previously unpublished material. In compliance with that desire it was thought best that the lectures should be printed essentially in the form in which they were delivered. The course was in two parts; the first half dealt with the properties of the catalytic systems, the second half with the energies and equilibrium points of the reactions catalysed by them. References have not been included; the information was derived from so many different sources that it was not practicable to mention them individually.

It is my hope that the publication of these lectures may be of some service to those who heard them as well as to a much wider circle of those who are interested in the subject.

I should like to express my great appreciation of the very kind reception given to me at University College by Professor Young and many others.

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CONTENTS

Lecture I  page 1
Lecture II  34
Lecture III  57
Lecture IV  79
Notes  101
Index  103
LECTURE I

The subject of multi-enzyme systems is perhaps a rather unusual one to choose for a course of lectures. But it is one which has a special interest at the present time, particularly because of the light which the study of such systems is beginning to throw on living matter.

The most effective way of finding out how any complex mechanism works, for example a stop-watch, is first to take it to pieces and to study the isolated parts separately; and after this to see how they fit together, and how they are connected functionally; and finally to reassemble them, building up the trains of wheels and so on, until the mechanism is complete.

This is equally true of the most complex of all mechanisms—living matter. The intracellular enzymes are, so to speak, the works of living matter. For some twenty years past the first phase of the investigation has been proceeding successfully, that is the isolation of separate enzymes in the pure state and the study of their properties singly. A great deal has been learned in this way, but much remains to be done and there is no doubt that this phase will continue for many years yet.

But in recent years there has been a tendency to move on to the synthetic phase, that is to build up systems consisting of two or more enzymes linked together functionally, to study the special phenomena which appear in such systems and the manner in which the constituent enzymes work together in producing them, and to build up trains or chains of enzyme reactions which will reproduce in vitro many of the metabolic transformations which occur in the living cell.
Of course protoplasm, with its network of metabolic reactions, really forms one complex multi-enzyme system (perhaps containing a hundred or more different enzymes), but the systems which have been studied up to the present contain comparatively few enzymes. Many consist of only two enzymes, though in one or two cases systems of up to a dozen or more have been studied. But even the relatively simple systems have been yielding interesting information about the peculiar state of affairs that exists in living matter, and helping us to understand its special biochemical properties.

I should like to start with a brief introduction on enzymes and some of their special properties which are important in this connection.

Enzymes are catalytic proteins. To say that they are all proteins is perhaps a generalisation, but some two dozen have now been isolated in the pure crystalline state, and all without exception were found to be proteins, and the others also show many characteristic protein properties.

If we add up the amounts of the known enzymes which are present in tissues, we find that it comes to a considerable fraction of the total soluble protein, and it has been suggested that the whole of the protein which can be extracted from tissues consists of enzymes.

Be that as it may, the amount of any one enzyme present is usually quite small, varying from 1/10th to 1/10,000th of the total protein in different cases.

Enzymes are very efficient catalysts. They are usually very active and only small amounts are necessary. One molecule of a fairly average enzyme will cause perhaps
300–400 molecules of its substrate to react each second at body temperature, and some are much more active than this. They act under very mild conditions—at room or body temperature in neutral aqueous solutions. This is important, because many of the substances which play an essential part in the reactions in living matter are unstable, and would be destroyed by the drastic conditions often used in organic chemistry.

Enzymes will often very easily bring about reactions which are quite difficult to accomplish by ordinary chemical methods; for instance, in some cases substances which are unaffected by boiling with strong nitric acid are oxidised with extreme ease by enzymes at room temperature.

I think it would be difficult to overestimate the biological importance of the intracellular enzymes. Practically every chemical reaction which occurs in the body is an enzyme reaction, and underlying the complex network of reactions which make up metabolism there is a corresponding system of enzymes which both enables them to take place and controls and directs them.

This directive function of enzymes is of great importance in metabolism, and the transformations which a given organic substance will undergo in the tissues depend not so much on its chemical properties as on what enzymes are present and in what relative amounts they are present.

In these lectures we shall be concerned only with the intracellular enzymes, as the comparatively few enzymes which are found outside cells, for example, the digestive enzymes, usually do not take part in multi-enzyme systems.
Enzymes, as is well known, act by combining with their specific substrates to form a complex. While the substrate is combined with the enzyme it is in a reactive state and capable of undergoing the particular reaction catalysed by that enzyme, either by reacting with another substance or with water, or by undergoing some intramolecular rearrangement. The combination between substrate and enzyme is of a rather special kind. It is freely reversible and in some cases quite weak, but at the same time highly specific. And that brings us to what is probably the most important and fundamental property of enzymes from our point of view, their extraordinarily high specificity, which is quite unparalleled among inorganic catalysts.

It would be going too far to say that each different reaction occurring in the tissues is due to a separate enzyme, or in other words that each enzyme acts on one substance and one substance only. Yet that does almost seem to be true for many of the intracellular enzymes, which have been found to act only on their particular substrate and not on closely related substances. Indeed in cases where the substrate is optically active, as often happens, it is often found that only one optical isomer is acted on by the enzyme and the other is absolutely untouched. It is clear that the 'active centre' of the enzyme (the part which combines with the substrate) has a configuration which fits its substrate quite closely. Some enzymes are less specific, or rather are specific for a particular chemical grouping, for example, one enzyme will oxidise most d-amino-acids, another will oxidise most aldehydes, and so on. But the majority of enzymes seem to be highly specific.
The highly specific nature of enzyme catalysis has some important implications. I should like to quote in this connection some words of my late chief, Sir Frederick Hopkins, spoken in 1933. He said: 'I will ask you to consider whether catalysis on highly specific lines is not among the most fundamental and significant phenomena in nature.' ... 'The organising potentialities inherent in highly specific catalysis have not, I believe, been adequately appraised in chemical thought.... Highly specific catalysts determine just what particular materials, rather than any others, shall undergo change.... I would like to claim that the control of events by intracellular enzymes... by itself secures the status of the cell as a system which can maintain itself in dynamic equilibrium with its environment.... The inter-related activity of highly specific catalysts represents a notable device of Nature which has supported during the course of evolution those dynamic manifestations which characterise living things.' These words might be taken as my text in these lectures.

One consequence connected with enzyme specificity is that enzyme reactions proceed without the formation of by-products. The yield is always 100%; that is to say, for every molecule of substrate which disappears one molecule of the specific product appears. This great advantage in carrying out reactions by means of pure enzymes wherever possible, however, is offset by the fact that enzymes usually occur not singly but in complex mixtures of enzymes, and the isolation of the enzyme desired is not always easy. Nevertheless, purified enzymes have already proved useful to the organic chemist in a number of cases, and it seems likely that they may
have an increasing influence on chemical technique in the future as isolation methods improve.

Another fact which is relevant here is that enzymes, as true catalysts, catalyse the reverse reaction to the same extent as the forward reaction. In other words, they combine with and activate the *products* as well as the starting material.

Finally, enzymes differ greatly in stability. Some are very stable; Professor Keilin has recently opened some tubes of blood, which were sealed up aseptically forty-five years ago and kept at room temperature, and found the enzymes to have almost the same activity as fresh blood, showing a remarkable stability in solution. Other enzymes are very unstable and their survival time at body temperature is measured only in minutes. There is little doubt that in living matter many enzymes are continuously breaking down and having to be resynthesised the whole time.

Proteins are no doubt formed by enzyme action, and since enzymes are proteins it follows that enzymes are formed by enzymes, and this is probably the main reason why living matter is always found to be formed from living matter.

With that brief introduction on enzymes in general let us now turn to the subject of multi-enzyme systems.

In forming, let us say, a two-enzyme system from the separate enzymes it is of no use just to take any two enzymes and mix them. In all probability they will be unconnected; they will not form a *system* and we shall learn nothing more from the mixture than from the separate enzymes. In order that a system may be formed, the enzymes must have some functional connection
which will couple them together. The results will only be of interest if the enzymes form a system which will do things which neither of the two enzymes will do singly. The new properties which emerge when this happens have in many cases thrown a good deal of light on living matter.

The direct coupling of two enzymes together is brought about when the two enzymes possess a common substrate, which acts as a link. This occurs when the product of the reaction catalysed by the first enzyme is the starting-point of the reaction catalysed by the second enzyme. In other words, we have simply the case of successive or consecutive enzyme reactions. Now there are two fundamentally different types of substrate-linked reactions. The first and most obvious type, (a), is where the second enzyme takes the substance which forms the link on to form a new substance, that is to say it takes it a step forward in its metabolism.

Suppose, for instance, we have two enzymes, one converting a substance \( A \) into \( B \), the other converting \( B \) into \( C \). The two enzymes are linked by \( B \) which (enzymes \( E_1 \) \( E_2 \) being reversible) is of course a substrate common to both enzymes. Obviously, if we have the right enzymes we can build up chains of successive enzyme reactions in this way, each enzyme taking the substance a step forward in its metabolism and determining by its specificity the course which the metabolism shall take.

The second type \( (b) \) is where the second enzyme simply undoes what the first enzyme does, as far as the linking substrate is concerned. In other words, it takes this
substrate back to what it was initially. If the first enzyme reaction reduces it, the second reoxidises it; if the first phosphorylates it, the second dephosphorylates it, and so on. Of course this does not occur by a reversal of the first enzyme reaction, but by a different reaction (two different enzymes being involved), which, nevertheless, brings the common substrate back to its starting-point. In the case of an oxidation-reduction reaction the process may take place thus:

\[ \text{AH}_2 + B \rightarrow A + \text{BH}_2, \]
\[ \text{BH}_2 + C \rightarrow B + \text{CH}_2. \]

Here \( B \) again forms the link between the two enzymes. In this system it is reduced by \( E_1 \) and reoxidised by \( E_2 \).

Similarly, with a phosphorylation:

\[ X\text{-Ph} + B \rightarrow X + B\text{-Ph}, \]
\[ B\text{-Ph} + Y \rightarrow B + Y\text{-Ph}. \]

The difference between types (a) and (b) is a fundamental one, because in type (a) the linking substrate is part of the material being metabolised and passes on, whereas in type (b) the linking substrate is part of the catalytic mechanism, is not metabolised and remains behind as a more or less permanent part of the system. The net result of a coupling of type (b) is usually, though not always, a transfer of something from one molecule to another; in the first of these examples, for instance, \( B \) is acting as an intermediate hydrogen-carrier between \( A \) and \( C \), and in the second example as a phosphate-carrier from \( X \) to \( Y \).
In the nature of the case, it is especially enzymes catalysing reactions between two molecules which tend to form coupled systems of this type. Two hydrolytic enzymes cannot form such a system, as a hydrolysis can only be reversed in one way; but a hydrolytic enzyme may be linked with another. For example, in the system

\[
X-\text{Ph} + B \rightarrow X + B-\text{Ph,} \\
B-\text{Ph} + H_2O \rightarrow B + \text{Ph,}
\]

the link $B$ helps the two enzymes to bring about the hydrolysis of $X-\text{Ph}$.

As in the case of systems linked by the type $(a)$ mechanism, there is a tendency to form chains of successive enzyme reactions in which the links are all of the same type, in this case of type $(b)$. The result of this is that the phosphate or hydrogen or other group is passed on from molecule to molecule in a series. For instance, it is common in respiration enzyme systems for the hydrogen from the substrates undergoing oxidation to be passed on by three or four successive intermediate carriers before finally reacting with $O_2$. 

\[
E_1 \quad X-\text{Ph} + B \rightarrow X + B-\text{Ph,} \\
E_1 \quad B-\text{Ph} + H_2O \rightarrow B + \text{Ph,}
\]
For obvious reasons we do not find mixed chains containing both \( (a) \) and \( (b) \) links, we get either one or the other. But a type \( (b) \) chain may start from one of the steps of a type \( (a) \) chain, for example an oxidation step; or it may start from one type \( (a) \) chain and finish on another, thus linking the two type \( (a) \) chains together, where something has to be transferred from one to the other; or it may even start and finish on the same type \( (a) \) chain, thus linking together two different steps of the same chain, as occurs with both phosphate and hydrogen in the glycolysis system, as shown later.

Now when two enzyme reactions are coupled together it means that the second is dependent on the first, and if the first stops the second stops also. This dependence may be due to two quite distinct causes. The second reaction may be dependent on the first for its chemical material, that is to say the first reaction provides the material which is the starting-point of the second. Or it may depend on the first reaction not for its material but for its *energy*. In this case the substances produced by the first reaction may not enter into the second at all, but the latter must have energy in order to take place. This energy is produced by the first reaction and transferred to the second, usually through intermediate systems, so that there is a coupling by energy transfer. This is a special type of system of considerable importance biologically.

As already mentioned, enzymes are true catalysts, which means that they do not affect the equilibrium point of the reaction, but help the system to reach that equilibrium. They do not, so to speak, drive the reaction along. Now in spite of this, enzymes do sometimes
energy transfer

appear to drive the reaction away from the equilibrium point. This does not happen with pure single enzymes, but it may occur in mixtures of enzymes. For example, an aqueous extract of muscle will synthesise phosphoric esters of carbohydrate from free phosphate, in spite of the fact that the equilibrium lies quite in the other direction and indeed phosphatases will hydrolyse these esters practically to completion, giving free phosphate again.

This is an exceedingly important effect. The explanation is that there is another enzyme reaction taking place (in this case the oxidation of carbohydrate) which is a source of free energy, and there is a mechanism whereby some of the free energy is taken from this reaction and transferred to the esterifying enzyme reactions, which are thereby driven away from equilibrium, in the direction of synthesis. The two reactions are then coupled by energy transfer.

The whole system of course is tending towards equilibrium, although that particular reaction is being driven away from the point which it would reach if it were left to itself. The situation is perhaps somewhat analogous to the hydraulic ram, by which some of the water is raised to considerable heights above the starting-point by means of energy taken from the downward flow of the rest of the water.

It seems that it is by multi-enzyme systems of this kind that syntheses of organic substances requiring free energy are performed in living cells. The free energy liberated by one enzyme reaction, usually an oxidation, is transferred to another enzyme reaction, usually a synthesis, which requires it. This kind of process is of fundamental
importance in living matter. For life to exist, it is an absolute essential that some mechanism should be present for trapping some of the free energy of reactions, for storing it and for transferring it so that it can be used for various vital processes.

It used to be customary in elementary physics textbooks to distinguish three kinds of equilibrium—stable, unstable and neutral—usually illustrated by three positions of a top. I should like to add a fourth kind of equilibrium state which seems to me to be of interest, namely that in which a continuous expenditure of free energy is necessary to maintain the system in a steady state. It is not difficult to think of examples of this kind of 'equilibrium'. One would be that of an object placed on an inclined endless belt, on which it slides. It can only be kept in the same position by continuously winding up the belt, thereby expending energy, which is continuously converted into heat. As soon as the energy supply stops the object slides downhill.

This kind of state is especially characteristic of living matter. There must be a continuous supply of free energy merely to keep it alive and in a steady state, even when no external work is being done.

This is mainly due to the fact that many of the essential constituents of living matter are unstable substances, continuously breaking down and having to be maintained by resynthesis, for which free energy is required. This is especially true of a number of components of the catalytic system; not only some of the enzymes, but especially some of the coenzymes, break down very easily. The oxidation reactions, for instance, are catalysed by a number of enzymes in conjunction with two or three coenzymes of
SELF-MAINTAINING SYSTEMS

a nucleotide-like structure, and these coenzymes break down rather rapidly in the tissues, and have to be resynthesised by a phosphorylation mechanism. Now it is the oxidation reactions which they catalyse that yield the free energy required for phosphorylation and resynthesis. So we have the remarkable state of affairs, peculiar to living matter, of a system of unstable catalysts being kept in existence by the occurrence of the reactions which they catalyse.¹

Here we have something which I feel is really fundamental for the proper understanding of the nature of life and death. As long as the metabolism of the cells is proceeding normally the catalytic system is maintained in the fully active state. But let us stop the oxidation reactions, for example by cutting off the oxygen supply. It is true that the tissue can run for a limited time and with diminished efficiency on anaerobic oxidations, but after that the coenzyme concentration starts to fall fairly rapidly. If we readmit oxygen while there is still some coenzyme left, the oxidations will begin again, slowly at first and then more rapidly as more coenzyme is resynthesised. But if we wait until the coenzyme is all broken down before admitting oxygen, then the oxidations cannot start again, owing to lack of coenzyme, and the coenzyme cannot be resynthesised, owing to the lack of energy from the oxidations. This it seems to me is what is really meant by the death of the tissue.

Now let us consider the various kinds of multi-enzyme systems in more detail. The energy-linked systems form a

¹ This does not mean of course that each enzyme maintains itself; it is a collective effort by a certain minimum number of enzymes which brings about the necessary series of reactions for resynthesis.
special case, and are dealt with more conveniently in the next lecture.

In a single enzyme reaction the rate depends on a number of factors, of which the most important are the specific catalytic activity of the enzyme, the amount of enzyme present, its affinity for the substrate, and the substrate concentration. According to the Michaelis equation, which holds in a large number of cases,

\[ v = \frac{kES}{K_m + S}, \]

where \( E \) is the amount of enzyme, \( k \) represents its catalytic activity, \( S \) is the substrate concentration, and \( K_m \) is the reciprocal of the affinity. Thus, with a constant amount of enzyme, the velocity is related to the substrate concentration by a curve of the following form:

When we have a system of consecutive enzyme reactions, the kinetics become much more complicated. For example, in a system \( A \rightleftharpoons B \rightleftharpoons C \rightleftharpoons D \) we have to consider the amounts of the three enzymes and the affinity of \( E_1 \) for \( A \), \( E_2 \) for \( B \), and so on; but since the reverse reactions are also occurring, we also have to consider the affinity of \( E_1 \) for \( B \), \( E_2 \) for \( C \), and so on. The kinetics are therefore extremely difficult to work out.
But quite apart from all this, we have a new factor coming in, namely the transit time needed for the substrate molecules to migrate from one enzyme to the next, thus: \((E_1)B \rightarrow (E_2)\). This is quite likely to be the limiting factor. Naturally it depends on the distance between the enzyme molecules, and can be shortened if the enzymes are brought close together. There seems to be some evidence that this actually occurs in some cases in the cell. For example, the enzymes connected with the cytochrome system, according to recent work, seem to be situated in the mitochondria of the cell, and therefore all very close together. Other recent work suggests that the group of enzymes catalysing the so-called citric acid cycle are also physically associated. On the other hand, it is probable that the enzymes of the glycolysis system are free in solution.

Although it will be clear that it is scarcely practicable to work out the complete kinetics of a chain of enzyme reactions quantitatively, there are several points which are worth noting in this connection. When the chain has settled down to a steady state, all the successive reactions must obviously be proceeding at the same rate \(v_c\). This means that for each enzyme the substrate concentration must have adjusted itself to that point on its curve which gives this rate. So the various substances will be at very different concentrations, which will be determined by the amounts of the various enzymes present and their affinities.

The amounts of the respective enzymes and substrates will in fact be related inversely. If there is a very large amount of one enzyme the concentration of its substrate will be extremely small. If one of the enzymes is present
only in small amounts its curve, even with excess of substrate, will be comparatively low; this enzyme will then be the limiting factor, or to use the more fashionable term the bottleneck, and it will determine the rate of the whole chain of reactions. The material will then pile up behind it. By the use of selective poisons one can often cause different enzymes in a chain to become limiting and so cause different intermediates to accumulate, which may be useful in studying the system.

Now if a rather slow enzyme is followed by a very active one, the mean life of the intermediate substrate molecules is very short. This means that enzymes in chains can make effective use of very unstable substances. We may have a system in which $F$ is very unstable and decomposes rapidly, but can be converted by $E_5$ into a useful substance $G$. If the enzyme $E_5$ is very active, or if a large amount is present, it will seize the $F$ molecules as fast as they are formed and convert them into $G$ before they have time to decompose. The enzyme reaction competes successfully with the decomposition.
This is a very important effect, because it enables the vital mechanism to make use of certain unstable energy-rich substances which play an essential part in the energy-transfer mechanism of living matter.

A somewhat similar point is that effective use can also be made of substances which, because of equilibrium considerations, can only be present in very small amounts. For instance, we may have a system $L \rightleftharpoons M \rightleftharpoons N$ containing two very active enzymes, but because the equilibrium of $E_6$ is strongly towards $L$ only very small amounts of $M$ can be present. $E_7$ may yet be able to convert the small amounts of $M$ rapidly into $N$, and $E_6$ will rapidly replace the amounts of $M$ used up, so that the whole process may proceed rapidly, even though intermediate formation of $M$ may not be detectable by ordinary methods. A number of systems are known in which this effect plays an important part.

I referred above to the possibility of a competition between reactions. This occurs when there is a branch in the chain, which arises when a substrate may be converted into either of two different substances by two different enzymes, and therefore stands at the parting of the ways, perhaps between two entirely different lines of metabolism. This is of great importance in connection with the directive function of enzymes. Here the whole course of the metabolism is determined by the relative amounts of $E_8$ and $E_9$ and their affinities for $P$. An important example of this lies at the point at which glycolysis and alcoholic fermentation diverge, as we shall see.
Now let us consider some of the more interesting actual multi-enzyme systems, in order to illustrate some of these points.

For the substrate-linked systems of type \( a \) we could theoretically take any of the lines of intermediary metabolism, since all such lines consist of successive enzyme reactions of this type. Unfortunately, very few have yet been fully worked out with regard to the enzymes involved, so that our choice is somewhat restricted. The carbohydrate metabolism system, however, has been worked out fairly completely and we cannot do better than to make use of it for this purpose.

On pp. 22–26 are set out the successive reactions involved in carbohydrate utilisation. They are given in detail as most of the accounts in the literature are incomplete in some respects.

This system illustrates how quite a large number of enzymes can be linked together in a chain—including the aerobic part we have perhaps twenty-five different enzymes all co-operating to bring about the oxidation of glucose or glycogen. Many of these enzymes have been obtained pure and crystalline, those in fact which have a small circle attached to their names, and most of the others have been fairly highly purified. These enzymes co-operate to bring about an organised chain of successive reactions, all directed to accomplishing the oxidation of carbohydrate while retaining much of the free energy in a readily available form through the energy-rich adenosinetriphosphate (ATP).

A system of this kind is rather like a production line in a factory, with the enzymes representing a series of
machines, each of which performs a single operation on the product, which is passed on from one to the other in succession. One must of course have a complete chain—if one machine breaks down, or if one enzyme is missing or poisoned, the whole line is disorganised.

Now in the enzyme system the organisation and coordination is not due to the series of enzymes being spaced out along a conveyor belt; the different enzyme molecules are mixed together in solution. But we have what might be called 'organisation by specificity'. The specificities of the constituent enzymes are so adjusted that the product formed by each enzyme falls within the specificity range of one of the other enzymes present, which is therefore capable of continuing the process and directing the substrate a further step down the right path. This is not an organisation by cellular structural factors but by purely chemical specificity, and I think its importance has not been sufficiently appreciated.

The first part of the table shows how lactic acid is produced in the absence of oxygen, and it forms a good example of the directive power of enzymes, if we compare it with yeast fermentation. The same system occurs both in yeast and in our own tissues (and probably in most other cells) right down to reaction (11). The glucose molecule is phosphorylated first at one end and then at the other, then broken in two, then oxidised by a special mechanism which is coupled with a synthesis of ATP from ADP (adenosinediphosphate), then dehydrated and dephosphorylated to give pyruvic acid. At this point there are two possibilities: lactic dehydrogenase may convert the pyruvic into lactic acid by reaction (12) or carboxylase may remove CO₂ by reaction (12b).
In yeast there is a relatively large amount of carboxylase, which therefore predominates, so that the end product is alcohol; in our own tissues there is little carboxylase and the lactic dehydrogenase predominates and lactic acid is formed. If it were not for this trifling difference in the enzyme system, presumably whenever we took any exercise our muscles would begin to pour alcohol into our blood. This is as good an example as any of the far-reaching consequences of slight changes in enzyme systems, owing to their directive power.

All these reactions are probably reversible, and I would suggest that the whole system is in a kind of floating equilibrium between glucose (or glycogen) at one end and lactic acid at the other. What holds it back, and determines whether it shall go forwards or not, is reaction (8). The whole system hangs, so to speak, on the ATP/ADP ratio, and therefore on the energy requirements of the cell. If the phosphorylation is complete there is no ADP, and so the diphosphoglyceric acid is not removed (apart from some spontaneous decomposition) and effectively prevents the forward direction of reaction (7). But if some ATP is used up by any of the energy-requiring processes which are discussed in the next lecture, ADP is formed, and the whole system can then go forward until the position is restored. By this means the system of enzymes adjusts the carbohydrate metabolism to the energy requirements of the cell.

Incidentally, it may appear at first sight that there is no net synthesis of ATP in this series of reactions, since it shows two molecules being formed and two used up. But since one glucose molecule forms two of triose-phosphate, all the reactions from (6) onwards must be
multiplied by two, so that four ATP molecules are formed and only two used up.

The first part of the table shows the processes occurring in the absence of O₂. The next part, sections A to D, shows some variants, which are self-explanatory. In the presence of O₂, the reduced coenzymes are oxidised by the respiratory enzymes, reaction (12) drops out, and the pyruvic acid from reaction (11) is oxidised by a further group of enzymes, probably through the tricarboxylic acid cycle shown in section E. There is still some controversy about reaction (13) and the enzymic mechanism is unknown, but the other reactions are well established. This aerobic system is coupled with further phosphorylations, but their mechanism is not yet understood.

This is a comparatively complex system; the others which I wish to mention are much simpler.

Before we leave the systems with type (a) coupling, one rather curious two-enzyme system might be mentioned, as illustrating the way in which new properties may arise on mixing enzymes. Xanthine oxidase oxidises the purines hypoxanthine and xanthine to uric acid with uptake of O₂. Catalase decomposes hydrogen peroxide with production of O₂. A mixture of these two enzymes was found by Keilin to have the unexpected property of continuously oxidising ethyl alcohol to acetic acid by O₂, provided that the system was primed with a small amount of acetaldehyde.

Here both enzymes are unusual in having two functions: xanthine oxidase oxidises aldehydes as well as purines, and Keilin found that catalase can cause H₂O₂ to oxidise alcohol. The result is that the two enzymes
The glycolysis system

1. Glucose + ATP ⇌ Glucose-6-phosphate + ADP
   from (8)
   and (11)

2. Glucose-6-phosphate ⇌ Fructose-6-phosphate

3. Fructose-6-phosphate + ATP ⇌ Fructose-1,6-diphosphate + ADP
   from (8)
   and (11)

4. Fructose-1,6-diphosphate ⇌ CH₂O·H₂PO₃ CH₂O·H₂PO₃
   CHOH + CO
   CHO CH₂OH

5. CH₂O·H₂PO₃ CH₂O·H₂PO₃
   CO ⇌ CHOH
   CH₂OH CHO

6. CH₂O·H₂PO₃ CH₂O·H₂PO₃
   CHOH + H₂PO₄ ⇌ CHOH
   CHO CH₂O·H₂PO₃
   OH [not isolated]

7. CH₂O·H₂PO₃ CH₂O·H₂PO₃
   CHOH + CO ⇌ CHOH + CoH₂
   CH₂O·H₂PO₃ CO₂·O·H₂PO₃
   OH
The essential oxido-reduction step is brought about by the enzymes catalysing reactions (7) and (12), which together form a 'coenzyme-linked dehydrogenase system'. Through (6), (7) and (8) the oxidation is coupled with the synthesis of ATP from inorganic phosphate and ADP, the necessary free energy being derived from the oxidation.
**Variants**

A. Starting with glycogen instead of glucose, (1) is replaced by:

\[(1a) \text{Glycogen} + n\text{-H}_3\text{PO}_4 \rightleftharpoons n\text{-Glucose-1-phosphate} \]

\[(1b) \text{Glucose-1-phosphate} \rightleftharpoons \text{Glucose-6-phosphate} \]

**Phosphorylase**

B. In the absence of pyruvic acid, e.g. when the system is just starting up or when (10) is inhibited by fluoride, (12) is replaced by:

\[(12a) \text{CH}_2\text{.O.H}_2\text{PO}_3 \rightleftharpoons \text{CH}_2\text{.O.H}_2\text{PO}_3 \]

\[\text{CHOH} + \text{CoH}_2 \rightleftharpoons \text{CHOH} + \text{Co} \]

\[\text{CHO} \rightarrow \text{CH}_2\text{OH} \]

**α-Glycerophosphate dehydrogenase**

C. In yeast fermentation, (12) is replaced by:

\[(12b) \text{CH}_3 \rightleftharpoons \text{CHO} + \text{CO}_2 \]

**Carboxylase + cocarboxylase**

\[\text{COOH} \]

\[(12c) \text{CH}_3 + \text{CoH}_2 \rightleftharpoons \text{CH}_3 + \text{Co} \]

\[\text{CHO} \rightarrow \text{CH}_2\text{OH} \]

**Alcohol dehydrogenase**

D. When arsenate is added, (6), (7) and (8) are replaced by (6a), (7a) and (8a), with the result that the coupling between oxidation and synthesis is broken, and no net synthesis of ATP by the system occurs:

\[(6a) \text{CH}_2\text{.O.H}_2\text{PO}_3 \rightleftharpoons \text{CH}_2\text{.O.H}_2\text{PO}_3 \]

\[\text{CHOH} + \text{H}_3\text{AsO}_4 \rightleftharpoons \text{CHOH} \]

\[\text{CHO} \rightarrow \text{CH.0.H}_2\text{AsO}_3 \]

**Triosephosphate dehydrogenase**
(7a) $\text{CH}_2\text{O.H}_2\text{PO}_3 \quad \text{CH}_2\text{O.H}_2\text{PO}_3$

$\text{CHOH} + \text{Co} \rightleftharpoons \text{CHOH} + \text{CoH}_2$

$\text{CH}_2\text{O.H}_2\text{AsO}_3$ (from (12)) $\text{CO.O.H}_2\text{AsO}_3$

$\text{OH}$

(8a) $\text{CH}_2\text{O.H}_2\text{PO}_3 \quad \text{CH}_2\text{O.H}_2\text{PO}_3$

$\text{CHOH} \rightleftharpoons \text{CHOH} + \text{H}_3\text{AsO}_4$

$\text{CO.O.H}_2\text{AsO}_3 \quad \text{COOH}$

[Spontaneous]

E. When O$_2$ is present reaction (12) does not occur and the reduced coenzymes from (7), (15) and (20) are probably reoxidised by O$_2$ through the cytochrome-oxidase system. (11) is believed to be followed aerobically in muscle by (13) to (20):

(13) $\text{CH}_3 \quad \text{CH}_2\text{COOH} \quad \text{CH}_2\text{COOH}$

$\text{CO} + \text{CO.O.CO} + \text{X} = \text{C.CO} \quad \text{CO}_2 + \text{XH}_2$

(14) $\text{CH}_2\text{COOH} \quad \text{CH}_2\text{COOH}$

$\text{C.CO} + \text{H}_2\text{O} \rightleftharpoons \text{CH.CO}$

(15) $\text{CH}_2\text{COOH} \quad \text{CH}_2\text{COOH}$

$\text{CH.CO} + \text{Co} \rightleftharpoons \text{CH.CO} + \text{CoH}_2$

$\text{CHOH.CO} \quad \text{CO.CO}$

Aconitate

Isocitric dehydrogenase

Triosephosphate dehydrogenase°
Variants (cont.)

\[(16) \text{CH}_2\text{.COOH} + \text{CH}_2\text{.COOH} \xrightarrow{} \text{CO.CO.OH} \]

\[
\text{Oxaloacetic carboxylase} \\
+ \text{Mn}
\]

\[(17) \text{CH}_2\text{.COOH} + \frac{1}{2}\text{O}_2 \xrightarrow{\text{cytochrome}} \text{CH}_2\text{.COOH} + \text{CO}_2 \\
\text{α-Ketoglutaric dehydrogenase} \\
+ \text{coenzyme}
\]

\[(18) \text{CH}_2\text{.COOH} + \frac{1}{2}\text{O}_2 \xrightarrow{\text{cytochrome}} \text{CH}_2\text{.COOH} + \text{H}_2\text{O} \\
\text{Succinic dehydrogenase}
\]

\[(19) \text{CH.CO.OH} + \text{H}_2\text{O} \xrightarrow{} \text{COH.OH.OOH} \\
\text{Fumarase}^\circ
\]

\[(20) \text{CH}_2\text{.COOH} + \text{CH}_2\text{.COOH} \xrightarrow{} \text{COH.OH.CO.OH} + \text{CO}_2 \xrightarrow{\text{to}} \text{CO}_2 + \text{H}_2 \]

\[\text{Malic dehydrogenase}^\circ
\]

The enzymic mechanism of (13) is unknown and it may take place in stages. Some of these reactions (e.g. (13), (17) and (18)) appear to be coupled with phosphorylations.
are doubly linked and a cyclic mechanism is set up thus:

\[
\begin{align*}
O_2 + CH_3CHO + H_2O & \rightarrow H_2O_2 + CH_3COOH \\
H_2O + CH_3CHO & \leftarrow H_2O_2 + CH_3CH_2OH
\end{align*}
\]

Now let us turn to systems with type (b) coupling. Two main classes of enzymes are involved here: the hydrogen-transferring enzymes or, in other words, enzymes concerned with oxidation and reduction, and the phosphate-transferring enzymes, which are discussed in the next lecture. There are others, for example those which transfer ammonia or methyl groups, but they are not so well understood and perhaps not quite so important. So we will consider now some hydrogen-transferring systems.

On p. 28 is given a list of the best known dehydrogenases. They form a very important class of about thirty enzymes whose function is to oxidise the various organic metabolites. The first column shows the substance oxidised by each enzyme and the second shows the oxidation product. These enzymes are highly specific to their own particular substrates.

The activated substrates do not react directly with molecular oxygen; they react, according to the particular enzyme, with one or other of four intermediate hydrogen-acceptors, namely coenzyme I, coenzyme II, a flavin group within the enzyme molecule, or a cytochrome. Each dehydrogenase can only react with one of these, not more. On the other hand, there may be more than one dehydrogenase acting on the same substrate but using different hydrogen-acceptors. For example, there are two glutamic dehydrogenases, one using coenzyme I.
<table>
<thead>
<tr>
<th>Substrate (H-donator)</th>
<th>Oxidation product</th>
<th>H-acceptor</th>
<th>r'\text{H} at \text{pH} 7.5</th>
<th>dr'H dpH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>CHO, COOH + NH₃</td>
<td>Flavine</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>d-Amino-acids</td>
<td>Ketoacids + NH₃</td>
<td>Flavine</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>l(+)-Glutamic acid</td>
<td>\alpha-Ketoglutaric + NH₃</td>
<td>Coenzyme II</td>
<td>ca. 8</td>
<td>28</td>
</tr>
<tr>
<td>l(+)-Glutamic acid</td>
<td>\alpha-Ketoglutaric + NH₃</td>
<td>Coenzyme I</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>{l-Amino-acids</td>
<td>Ketoacids + NH₃</td>
<td>Flavine</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>{l-Lactic acid, etc.</td>
<td>Pyruvic acid</td>
<td>Cytochrome</td>
<td>8.0</td>
<td>0</td>
</tr>
<tr>
<td>l(+)-Lactic acid (yeast)</td>
<td>Pyruvic acid</td>
<td>Cytochrome</td>
<td>14.0</td>
<td>0</td>
</tr>
<tr>
<td>l(+)-Lactic acid (muscle)</td>
<td>Oxaloacetic acid</td>
<td>Coenzyme I</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>l(-)-Malic acid</td>
<td>\alpha-Ketoglutaric</td>
<td>Coenzyme I</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>l(-)-Malic acid</td>
<td>Acetoacetic acid</td>
<td>Coenzyme I</td>
<td>4.7</td>
<td>0</td>
</tr>
<tr>
<td>d-Triosephosphate + H₃PO₄</td>
<td>Acetic acid + CO₂</td>
<td>Coenzyme II</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>d(-)-\alpha-Glycerol phosphate</td>
<td>Succinic acid + CO₂</td>
<td>Coenzyme I</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>l(+)-\alpha-Glycerol phosphate</td>
<td>Fumaric acid</td>
<td>Cytochrome</td>
<td>14.0</td>
<td>0</td>
</tr>
<tr>
<td>Glucose (moulds)</td>
<td>Glucose acid</td>
<td>Flavine</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Glucose (animal tissues)</td>
<td>Gluconic acid</td>
<td>Coenzyme I</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Glucose-6-phosphate</td>
<td>Phosphogluconic acid</td>
<td>Coenzyme II</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Phosphogluconic acid</td>
<td>Phosphoketohexonic acid</td>
<td>Coenzyme II</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Pentosemonophosphate</td>
<td>Phosphopentonic acid</td>
<td>Coenzyme II</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>CoH₂ [Diaphorase]</td>
<td>Coenzyme I</td>
<td>Flavine</td>
<td>4.0</td>
<td>+1</td>
</tr>
<tr>
<td>[Xanthine</td>
<td>Uric acid</td>
<td>Flavine</td>
<td>2.0</td>
<td>—</td>
</tr>
<tr>
<td>[Aldehydes</td>
<td>Acids</td>
<td>Flavine</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>[Aldehydes</td>
<td>Acids</td>
<td>Flavine</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>[Quinolines</td>
<td>Carbostyrils</td>
<td>Flavine</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Formic acid (bacteria)</td>
<td>CO₂</td>
<td>Cytochrome</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Formic acid (plants)</td>
<td>CO₂</td>
<td>Coenzyme I</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Choline</td>
<td>Betaine aldehyde</td>
<td>Cytochrome</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>
DEHYDROGENASES

and the other using coenzyme II; lactic acid is oxidised to pyruvic by three different dehydrogenases, using coenzyme I, a cytochrome, and flavin respectively.

The dehydrogenases may be said to resemble a set of dominoes, in which one end may bear any one of about thirty or more numbers corresponding to the substrate, and the other end may bear a number from 1 to 4 corresponding with the hydrogen acceptor.

The most important dehydrogenases for our present purpose are those which depend on coenzymes I or II. They act as follows (see diagram):

The substrate and coenzyme combine loosely but specifically with different points on the enzyme. There is then a transfer of hydrogen atoms from one to the other in the direction of the arrow (or for that matter in the reverse direction), after which the reduced coenzyme migrates to another enzyme and there hands the hydrogen on to some other molecule, and so acts as a type (b) link.

The typical respiratory enzyme system can be represented in the following way, as a three-enzyme type (b) chain:

At one side we have the various oxidisable substrates activated by their dehydrogenases; several of these of course react with the same coenzyme. The reduced
coenzyme then passes on its hydrogen to the flavin group of a flavoprotein enzyme (cytochrome reductase or diaphorase) and the reduced flavin then in turn reduces the haematin-protein cytochrome. Finally cytochrome oxidase catalyses the reoxidation of the cytochrome by O\textsubscript{2}. So we have three enzymes—dehydrogenase, flavoprotein, cytochrome oxidase—and the net result is the oxidation of the substrates through three successive intermediate hydrogen carriers.

Incidentally, the structural relationships between these and other carriers are worth noting. If we remove the nicotinamide-ribose group from coenzyme I we obtain ADP, which as we shall see is important as a phosphate-carrier; coenzyme II is similar to coenzyme I in structure, but has one more phosphate group, and so is probably related correspondingly to ATP; the same general type
of structure is shown in the flavin group of diaphorase which forms the next link in the chain. On the other hand, cytochrome has quite a different structure. In reduction of the coenzymes, the two hydrogen atoms are added across the double bond to the nitrogen in the pyridine ring; in the flavin they are added across the quinonoid structure in the alloxazine group; in cytochrome it is the iron atom which becomes oxidised and reduced.

Now if we cut off the supply of O₂, we shall put the respiratory enzyme system out of action as far back as the coenzyme. What happens then is that the various dehydrogenases begin to react with one another through the coenzyme. The coenzyme which is reduced by one dehydrogenase is reoxidised by another working in its reverse direction. These dehydrogenase reactions are all reversible, so that the oxidised form of the substrate can oxidise the reduced coenzyme, as for instance in reaction (12). So we get a series of 'coenzyme-linked dehydrogenase systems', by means of which the coenzyme brings about a distribution of the hydrogen between the different substrates in accordance with their affinities for hydrogen.

These reactions are simply fermentations, and in this way the essential relationship between the enzyme systems of respiration and fermentation is clearly brought out.

The nature of a coenzyme-linked dehydrogenase reaction is more clearly shown thus:
Here the reduced form of the substrate of the first dehydrogenase reduces the oxidised form of the substrate of the second dehydrogenase through the coenzyme. In this and the following diagrams the dotted lines represent combinations between enzyme and substrate and the arrows show the direction of transfer of hydrogen.

Such reactions are important and fairly numerous. The list shows nine dehydrogenases which react with coenzyme I, so that from these thirty-six different systems can be formed, and fifteen more systems can be formed from the coenzyme II-specific enzymes. If we could link coenzyme I with coenzyme II we could get 105 different systems.

The above diagram shows perhaps the best known of these systems, which consist of reactions (7), (12), (12c) and (12a) of the glycolysis-fermentation system. TrPh here stands for triosephosphate (3-glyceraldehydeposphate). The first system forms diphosphoglyceric and lactic acids in animal tissues; the second is the central
reaction of yeast fermentation, and gives diphosphoglyceric acid and alcohol; the third shows the dismutation of two molecules of triosephosphate which occurs when no pyruvate or aldehyde is present, in which case one molecule is oxidised at the expense of the other, which is reduced to glycerolphosphate.

These are very important and well-known reactions, but there are many interesting systems which are less well known, and we may consider some of these in the next lecture.
LECTURE II

Let us begin by looking at a few of the less-known coenzyme-linked dehydrogenase systems. The first is a system which acts when ammonium citrate is added to kidney extract. Citric is in equilibrium with isocitric acid in presence of the enzyme aconitase (by reaction (14)).

\[
i\text{Cit}r \xrightarrow{\text{Citr.}} \text{CoII} \xrightarrow{\text{CoII}} \alpha\text{-kg+NH}_3
\]

overall reaction:

\[
i\text{Cit}r + \alpha\text{-kg+NH}_3 = \alpha\text{-kg+CO}_2 + \text{Gt}
\]

net result: \[i\text{Cit}r + \text{NH}_3 = \text{Gt} + \text{CO}_2\]

The isocitric with its dehydrogenase reduces coenzyme II, which in turn reduces \(\alpha\)-ketoglutaric+NH\(3\) to glutamic acid, by the coenzyme II-specific glutamic dehydrogenase. The isocitric itself, however, is oxidised by this reaction to \(\alpha\)-ketoglutaric+CO\(2\) (by reactions (15) and (16)). The ketoglutaric therefore cancels out, since one molecule is produced and one used up, so that the net result is the formation of glutamic acid and CO\(2\) from ammonium citrate by this four-enzyme system.

Next we have an interesting system found in liver extract which assimilates CO\(2\). The enzyme oxaloacetic carboxylase reversibly adds CO\(2\) to pyruvic to form oxaloacetic acid. Lactic reduces oxaloacetic to malic through coenzyme I with the lactic and malic dehydrogenases, and is itself oxidised to pyruvic. So we get the overall reaction shown, which is freely reversible, and as
the pyruvic cancels out we get finally the formation of malic from lactic and CO$_2$ by these three enzymes.

\[
\text{Pyr} + \text{CO}_2 \xrightarrow{\text{OaCarb.}} \text{Li} \xrightarrow{\text{CoI}} \text{Oxae} \\
\text{L.D.} \quad \text{Mal.D.I}
\]

overall reaction: \( \text{Li} + \text{P} + \text{CO}_2 = \text{P} + \text{M} \)

net result: \( \text{Li} + \text{CO}_2 = \text{M} \)

The next system is rather similar, but involves the other malic dehydrogenase and coenzyme II, linked this time

\[
\text{Pyr} + \text{CO}_2 \xrightarrow{\text{OaCarb.}} \text{G-6-P} \xrightarrow{\text{CoII}} \text{Oxac} \\
\text{Hmp.D} \quad \text{Mal.D.II}
\]

with glucose-6-phosphate and the hexosemonophosphate dehydrogenase. We get therefore a CO$_2$ uptake on the addition of glucose-6-phosphate and pyruvate, with formation of phosphogluconic and malic. Similarly, we

\[
\text{ctkg} + \text{CO}_2 \xrightarrow{\text{OsCarb.}} \text{G-6-P} \xrightarrow{\text{CoII}} \text{Oxsuc} \\
\text{Hmp.D} \quad \text{iCitrD}
\]
MULTI-ENZYME SYSTEMS

may make use of $\alpha$-ketoglutaric with oxalosuccinic carboxylase and isocitric dehydrogenase. We get first oxalosuccinic by reaction (16) and then phosphogluconic and isocitric.

One further system with glucose-6-phosphate may be given; it is of interest as illustrating linking with transaminase.

\[
\begin{align*}
G-6-P & \rightarrow \text{Co II} \rightarrow \alpha\text{-kg} + \text{NH}_3 \\
\text{Hmp.D} & \quad \text{Gt.D.II} \\
\text{Tram} \\
\text{Gt} + \text{Oxac} & \rightleftharpoons \alpha\text{-kg} + \text{Asp}
\end{align*}
\]

net result: $G-6-P + \text{Oxac} + \text{NH}_3 = \text{Ph-Gl} + \text{Asp}$

Glutamic is first formed from $\alpha$-ketoglutaric in the linked dehydrogenase reaction and then in the presence of transaminase it transfers its amino group to oxaloacetic, giving aspartic. The ketoglutaric and glutamic therefore cancel out. In fact the ketoglutaric is acting catalytically as an $\text{NH}_3$ carrier, just as the coenzyme acts as a hydrogen carrier, and the oxidation of the glucose-phosphate brings about the amination of the oxaloacetic with the aid of these three enzymes and two carriers.

All these systems and many others have been shown to act, and the mechanism seems to be quite clear and straightforward. So far we have been linking together only dehydrogenases of the same class, coupling two coenzyme I-specific enzymes or two coenzyme II-specific enzymes. The question now arises whether a coenzyme I enzyme can be linked with a coenzyme II enzyme. So far as I am aware, this has not yet been shown to occur, but I suggest that it could easily take place through malic and its two dehydrogenases in the following way.
Reduced coenzyme II with one malic dehydrogenase first reduces oxaloacetic to malic. The malic then passes to the other dehydrogenase and is reoxidised to oxaloacetic

\[ \text{CoII} \rightarrow \text{M} \rightarrow \text{CoI} \]

by coenzyme I, so that the net result is the reduction of coenzyme I by coenzyme II. One wonders whether the well-known catalytic effects of malic and similar compounds on certain tissue oxidations might not be due in part to such an effect.

Some cases are known where linking occurs with dehydrogenases of other groups than those which react with the two coenzymes. For instance, pyruvate with the lactic dehydrogenase will oxidise xanthine to uric acid with xanthine dehydrogenase, although the latter works through flavin and not coenzymes I or II. The malic dehydrogenase has been shown to link with succinic dehydrogenase—a cytochrome-reducing enzyme—thus:

\[ \text{Fum.} \]

Malic added to muscle is partly converted into fumaric by fumarase (reaction (19)); they then react together, so that the malic is oxidised to oxaloacetic and the fumaric is reduced to succinic, and a dismutation results. The linking mechanism in these cases is unknown.

Finally, the nature of the hydrogen acceptor is unknown in those dehydrogenases which depend on co-
carboxylase, but they can be linked with coenzyme-specific dehydrogenases. For instance, \( \alpha \)-ketoglutaric undergoes a rapid dismutation in animal tissues to form succinic and glutamic as shown. Here again the linking mechanism is unknown.

\[
\alpha\text{kg} \rightarrow ? \rightarrow \alpha\text{kg} + \text{NH}_3
\]

\[
\alpha\text{kgD.} \quad \text{Gt.D.} \quad + \text{coc.}
\]

net result: \( 2\alpha\text{-kg} + \text{NH}_3 = \text{Suc} + \text{CO}_2 + \text{Gt} \)

Now let us leave this part of the subject and turn to the question of energy transfer from one process to another by enzyme systems in living matter. For this purpose we must first consider an important group of enzymes on which work has been done recently, but to which no generic name has yet been given. I refer to the enzymes which put on or remove or transfer phosphoric acid groups. Possibly we might refer to them by some such name as 'the phosphorases'. Much of the work on them is quite recent and I do not think it has yet been collected into any connected account of the subject.

These enzymes are worth very special attention because they play an essential part in that remarkable chemical mechanism which makes the free energy of oxidation processes available for use in various ways in the living cell. When chemical reactions are carried out in the test-tube in the ordinary way, the free energy is generally lost as heat. As I mentioned in the last lecture, an absolute essential in living matter is some mechanism whereby free energy liberated in the oxidation of organic metabolites is so to speak trapped, so that it can be transferred and used for the various vital processes which
PHOSPHORASES

require energy. Among such processes are the synthesis of more complex organic molecules from simple ones for growth and maintenance, the performance of mechanical work in movement and especially in muscle contraction, work against osmotic forces in absorption and secretion, and various other processes.

One such mechanism has been uncovered during the last few years, and there seems to be no doubt that it is the main one and very possibly the only one. This is the mechanism which depends on the formation of what Lipmann has called 'energy-rich phosphate bonds'; that is to say it involves the formation of organic compounds containing phosphate groups which are combined, not by the ordinary ester link which contains little energy, but by an anhydride or other link which contains a great deal more energy.

In studying this subject one is often impressed by the variety of different ways in which this system is made use of for biological purposes, and by the interest of some of the chemical mechanisms involved.

The essential parts of this vital system are (1) means of forming the energy-rich bond, the energy coming usually from an oxidation reaction, (2) means of transferring and storing the available free energy in this form, and (3) means of utilising the energy by coupling the dissolution of the energy-rich bond with the various vital processes requiring the energy. Let us then consider the different kinds of phosphorases with this in mind.

The first group, which is really the key to the whole mechanism, might be called the ‘bond-energising-enzymes’. The typical reaction here may be represented as \( X-\text{Ph} = X'\sim\text{Ph} \), where \( \sim \) is Lipmann's symbol to
distinguish the energy-rich bond from the ordinary bond with little energy.

Here we have, to put it very crudely, a molecule $X$, which does not mind whether it is combined with phosphate or not, converted into a molecule $Y$, which has a strong antipathy to phosphate and does its utmost to push it off. The free energy of the above reaction is usually very small, whereas the free energy of the reaction

$$X - \text{Ph} = Y \sim \text{Ph}$$

$X=\sim Y$ is fairly large. This means that the considerable energy liberated when $X$ is converted into $Y$ does not leave the molecule when it is in combination with phosphate during the reaction, but goes into this bond. It is only liberated when the bond is broken; consequently there will be a tendency for any reaction in which this bond is dissolved to have its equilibrium point very far over to that side.

There are perhaps half-a-dozen enzymes which bring

---

**The main groups of phosphorases**

<table>
<thead>
<tr>
<th>Group</th>
<th>Typical reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Bond-energising enzymes</td>
<td>$X - \text{Ph} = Y \sim \text{Ph}$</td>
</tr>
<tr>
<td>(2) Phosphokinases</td>
<td>$\text{ADP} \Leftrightarrow \text{ATP}$</td>
</tr>
<tr>
<td>(3) Phosphotransferases</td>
<td>$X - \text{Ph} \Leftrightarrow Y$</td>
</tr>
<tr>
<td>(4) Phosphomutases</td>
<td>$X - \text{Ph} = \text{Ph} - X$</td>
</tr>
<tr>
<td>(5) Phosphorylases</td>
<td>$X \Leftrightarrow Y$</td>
</tr>
<tr>
<td>(6) Phosphatases</td>
<td>$X - \text{Ph} + \text{H}_2\text{O} \Rightarrow X + \text{Ph}$</td>
</tr>
</tbody>
</table>
about this kind of reaction, but we only know the mechanism in two cases and partially in a third. They are nearly all oxidation reactions; that is to say $X$ is oxidised to $Y$ and the energy of oxidation goes into the bond.

The coupling of oxidation with phosphate-binding was first found with the triosephosphate dehydrogenase by Mrs Needham and by Meyerhof, simultaneously and independently, in 1937, and they must be regarded as the pioneers in this subject, which was later developed to a large extent by Lipmann. The mechanism is shown in reactions (6) and (7) of the glycolysis system. The aldehyde group of the triosephosphate (phosphoglyceraldehyde) when in combination with the enzyme, can freely and reversibly take on a molecule of inorganic phosphate. It is then oxidised by coenzyme I to a carbonyl with the phosphate still attached. The link is thereby converted into a kind of anhydride link, which is energy-rich and has a very strong tendency to get rid of its phosphate. It is in fact unstable and breaks down spontaneously fairly rapidly, but before it has time to break down and lose its energy, its phosphate is transferred to ADP by the enzyme catalysing reaction (8), which is among the most active enzymes known. In this way an energy-rich ATP bond is formed at the expense of the triosephosphate oxidation by this two-enzyme system.

The free energy of reactions (6) plus (7) taken together is very small, which means that almost the whole energy of the oxidation goes into the bond. The heat of the reaction is zero.

Another reaction of this class is reaction (10), where the phosphate bond is made energy-rich by the energy
of conversion of glyceric to pyruvic acid. Reaction (10) also has only a very small free energy.

Another case is the oxidation of pyruvate by the pyruvic dehydrogenase, where according to Lipmann the product is acetyl-phosphate.

The second group of enzymes is composed of what Mrs Needham and I have called in 1943 the 'phosphokinases'. The first of these, hexokinase, was known before the war, but the others have come into prominence more recently. They are phosphate-transferring enzymes which transfer phosphate groups from one molecule to another, but without the intermediate formation of free phosphate. The typical reaction is that shown in the list. One of the molecules is always ADP, the other $X$ may be any one of a number of organic compounds, according to the particular phosphokinase. Examples are reactions (1), (3), (8) and (11) of the glycolysis system.

The mechanism of the reaction has been investigated by Bücher in the case of the phosphoglyceric enzyme which catalyses reaction (8), and it appears to be as represented in the following diagram:

```
\[
\begin{array}{c}
\text{ADP} \\
\text{Ph} \\
\text{OO.O.C.G.P.} \\
\end{array}
\]
```

\[
\downarrow
\]

```
\begin{array}{c}
\text{ADP} \\
\text{Ph} \\
\text{OO.O.C.G.P.} \\
\end{array}
```
$G$ represents the glyceric acid molecule. The adenine end of the ADP molecule combines with the enzyme; the other end can then come over on to the phosphate and displace the phosphoglyceric from its combination (or conversely). At no time is the phosphate free—it is always combined with either one molecule or the other; and in this case, where both of the links are energy-rich, it is important to note that the energy can be transferred from molecule to molecule along with the phosphate by such enzymes.

In some cases the $X$-$\text{Ph}$ link is energy-rich; in others it is the ordinary energy-poor link, and in these cases the free energy of the reaction is rather large and consequently the equilibrium point is far over to the left. This is the case, for example, with hexokinase (reaction (1)), which in itself proves the large energy difference which exists between the two phosphate bonds.

All the phosphokinases apparently depend on $\text{-SH}$ groups within their molecules for their activity and all require the addition of Mg. There is some reason to think that the enzyme-substrate combination may involve the formation of a $\text{-SH-Mg-phosphate}$ complex.

At the moment some ten phosphokinases are known, as shown in the following list, but no doubt there are others and a search is now being made for new ones. The sign $\sim$ means that the $X$-$\text{Ph}$ link is energy-rich. Hexokinase phosphorylates the three sugars named, but apparently no others. The phosphoglyceric and pyruvic enzymes help to form ATP in glycolysis, as we have seen. The creatine enzyme is important for the storage of phosphate bond energy. There is only a rather small amount of ATP in the cells, so that not very much
MULTI-ENZYME SYSTEMS

Phosphokinases

\[
\begin{array}{ll}
\text{Ox} & \text{Glucose, fructose, mannose} \\
\text{Galactose} & \text{Galactokinase} \\
\text{Fructose-6-phosphate} & \text{Phosphohexokinase} \\
\text{Pyruvic acid} & \\
\text{Creatine} & \\
\text{Adenyllic acid} & \text{Myokinase} \\
\text{Glycerol} & \\
\text{Acetic acid} & \\
\text{Glutamic acid} & \\
\end{array}
\]

Energy can be stored in this form at any instant. But there is much more creatine. Creatine-phosphate has an energy-rich link and through this enzyme it is in free equilibrium with ATP, so that much larger amounts of free energy can be stored in the form of creatine-phosphate.

Myokinase is of interest because it is itself adenylic acid (adenosinemonophosphate), so that the reaction is a dismutation of ADP: \( ADP + ADP \rightleftharpoons AMP + ATP \). Under certain conditions it therefore enables the second pyrophosphate link in ATP to be used, for example when it is added to hexokinase.

The last of the enzymes was found recently during our search for phosphokinases (it was discovered also independently in America). It transfers phosphate from ATP to the side-chain carboxyl group of \( L \)-glutamic acid,
and the phosphate can then be replaced by NH₃. Glutamine is thus formed and an equivalent amount of inorganic phosphate is simultaneously split off from ATP, which supplies the energy for the formation of the amide.

Other phosphokinases are probably involved in the formation of the coenzymes, as in the synthesis of co-carboxylase from vitamin B₃ and in the formation of coenzymes I and II.

Now there is quite a close analogy between hydrogen-transfer by the dehydrogenases through the coenzyme and phosphate-transfer by the phosphokinases through ADP.

![Diagram](https://via.placeholder.com/150)

Just as the coenzyme forms a kind of central exchange depot for hydrogen, between many dehydrogenase substrates, so does ADP form a central exchange depot of phosphate groups, between many phosphokinase substrates.

As we get hydrogen-transfer by 'coenzyme-linked dehydrogenase reactions', so we can get phosphate-transfer from one substrate to another by 'ADP-linked phosphokinase reactions', of which each involves two phosphokinases linked together by ADP. These systems also are very important. We have already seen two examples in reactions (8) and (1) or (11) and (9). With
the phosphokinases given in the list, sixty-six different systems of this kind could be formed.

The next group of enzymes, which have been termed 'phosphotransferases', was discovered only very recently, in fruits by Axelrod in America and in animal tissues by Mrs Appleyard in Cambridge. They transfer phosphate groups from one molecule to another, but not through ADP, and as far as we know they involve only phosphate ester linkages and are not concerned with energy-rich bonds. It is not yet known what natural reactions they catalyse in cells.

The next group of enzymes, the 'phosphomutases', transfer phosphate from one position in a molecule to another position in the same molecule, which perhaps we may write symbolically as $X$-Ph $\rightarrow$ Ph-$X$. ADP is not involved here, nor is free phosphate formed, as shown by isotope experiments. I think it is not impossible that they may be really enzymes of the preceding class, and that the real reaction is a transfer of phosphate to a new position, not in the same molecule, but in another molecule of the same kind, thus: $X$-Ph $+ X \rightleftharpoons X +$ Ph-$X$ or, let us say, glucose-1-phosphate $+ $glucose $\rightleftharpoons$ glucose $+ $glucose-6-phosphate. So far there is no definite evidence of this.\(^1\)

At present only two of these enzymes are known, namely phosphoglyceromutase and phosphoglucomutase, catalysing reactions (9) and (1b) respectively.

Next we have the 'phosphorylases', which transfer an organic molecule from phosphate to another molecule, as shown in the table. In contrast to the phosphokinases and phosphotransferases, which transfer phosphate from $X$ to $Y$, these transfer $X$ from phosphate to $Y$.

\(^1\) But see note (1), p. 101.
The net result is that the energy in the $X$-$Ph$ link goes to supply energy for the synthesis of a new molecule $X$-$r$. If $X$-$Ph$ contains a high-energy bond, the equilibrium point of this reaction will be well over towards the side of synthesis. To put it crudely again, the phosphate tries to get rid of $X$ and when the presence of the phosphorylase gives it an opportunity it repels $X$ on to $r$.

So we arrive at the important result that the synthesis of a molecule $X$-$r$, using the oxidation of some substance $S$ to supply the necessary energy, can be accomplished by a mixture of four enzymes: one to oxidise $S$ by a phosphorylating mechanism, a phosphokinase to transfer the phosphate from the product to ADP, another phosphokinase to transfer phosphate from ATP to $X$, and a phosphorylase to transfer $X$ from phosphate to $r$. The living cell is known to synthesise a number of substances in this way and probably it will be found to be a fairly general mechanism.

There is need for further work on phosphorylases. There are probably quite a number, but only four have been studied and only one really thoroughly investigated. This is the enzyme which makes starch in plants and glycogen in animals, and is usually called simply 'phosphorylase' or sometimes 'polysaccharide phosphorylase'. In this case $X$ is glucose and $r$ is the end of a polysaccharide chain, so that the reaction may be written:

\[
\begin{align*}
\text{Ph} & \quad \text{Ph} \\
\text{Gl} & \quad \text{Gl} \\
\text{Gl-Gl-Gl-} & \quad \text{Gl-Gl-Gl-} \\
\ldots & \quad \ldots
\end{align*}
\]
This enzyme has been used to synthesise starch \textit{in vitro} from glucose-1-phosphate in quantities of 100 g. or more.

It will not start a new chain; it needs the end of a pre-existing chain, and it will then go on adding glucose molecules indefinitely. When pure, it builds up straight unbranched chains and the product approximates to the amylose type of structure, but in animal tissues there is another enzyme which causes a glucose molecule to be added on to the side of the chain here and there, and then by lengthening these side-chains the phosphorylase builds up the highly branched glycogen.

The phosphorylase reaction is freely reversible, and with inorganic phosphate it will break down starch and glycogen by splitting off glucose-1-phosphate molecules one after the other from the ends of the chains, as in reaction (1a).

This enzyme contains a phosphorylated prosthetic group of unknown chemical nature, and it is interesting in view of what was said in the first lecture that in a fatigued muscle this group breaks off, but the enzyme is completely resynthesised after a short rest.

Another phosphorylase forms and splits cane sugar. Here $X$ is glucose and $Y$ is fructose:

\[
\begin{array}{c}
\text{Ph} \\
\text{Gl}
\end{array} \xrightarrow{Y} \begin{array}{c}
\text{Fr} \\
\text{Fr}
\end{array} \quad \begin{array}{c}
\text{Ph} \\
\text{Gl}
\end{array}
\]

Recent work has shown that the specificity is not strictly limited to this reaction, and this enzyme has been used to synthesise some hitherto unknown disaccharides \textit{in vitro}. 
Another phosphorylase, recently discovered, forms and splits nucleosides. Here \( X \) is ribose and \( R \) is the purine base hypoxanthine:

\[
\begin{array}{c}
\text{Ph} \\
| \\
\text{Rib} \\
\end{array}
\rightleftharpoons
\begin{array}{c}
\text{Ph} \\
| \\
\text{Rib} \\
\end{array}
\]

\[
\begin{array}{c}
\text{Rib} \\
\text{Hx} \\
\end{array}
\rightleftharpoons
\begin{array}{c}
\text{Rib} \\
\text{Hx} \\
\end{array}
\]

There seems to be a similar enzyme for guanine, and it is possible that the nucleosidases are all of this type. There is little doubt that this is the way in which nucleosides are formed and that these enzymes play an important part in building up nuclear material and coenzymes.

There is some evidence also that acetyl choline, which of course is important in the mechanism of nerve, is formed by a similar system.

There are probably many other enzymes in this group. What one would like to know above all is whether peptide links are formed in such a way from phosphorylated amino-acids, in fact whether this mechanism is responsible for protein synthesis. So far as I am aware, the nearest case to peptide formation is hippuric acid, which has recently been shown to be formed by such a phosphorylating mechanism involving ATP.

It is worth pointing out that the enzymes which hydrolyse the links in these substances are quite different from those which synthesise them. Starch is built up by phosphorylase and broken down by amylases, sugar is built up by another phosphorylase and broken down by saccharase, ATP is formed by a phosphokinase and hydrolysed by ATP-ase, glutamine is also formed by a
phosphokinase and split by glutaminase and so on. The synthesis does not take place simply by a reversal of the hydrolysis reaction but by a different reaction. In each case there is a kind of cycle of synthesis by one path and breakdown by another:

![Diagram](attachment:image.png)

Actually, however, synthesis can occur in both ways, because the hydrolysis is reversible, and if one adds high concentrations of $A$ and $B$, the hydrolysing enzyme will form a small equilibrium amount of $A - B$ in accordance with the law of mass action. This amount, however, is generally insignificant in comparison with that formed by the phosphorylating mechanism, in which the ATP energy sends the equilibrium right over to the side of synthesis.

The last group of these enzymes, the 'phosphatases', are simply hydrolysing enzymes, which split off inorganic phosphate, but without any associated synthetic reaction. Some split simple monoesters $R\text{-}\text{Ph}$, others act only on diesters $R\text{-}\text{Ph}\text{-}R'$, others split the pyrophosphate link $\text{Ph}\text{-}\text{Ph}$ or $R\text{-}\text{Ph}\text{-}\text{Ph}\text{-}R$ (the enzymes which split dinucleotides may be of this type) or $R\text{-}\text{Ph}\text{-}\text{Ph}$ (for example ATP-ase).

Now having considered the various kinds of phosphorases, we are in a position to examine some examples of the many different ways in which the phosphate mechanism is used for carrying out vital processes.
Among such energy-requiring processes I have already mentioned chemical synthesis, work against osmotic forces, and mechanical work.

I have already said something about the part played by this system in chemical synthesis. The mechanism of formation of glycogen from glucose in muscle will serve as a simple example.

\[
\begin{align*}
\text{Gl} + \text{ATP} & \xrightarrow{\text{Hk}} \text{Gl-6-Ph} \\
\text{Gl-6-Ph} & \xrightarrow{\text{PGM}} \text{Gl-1-Ph} \\
\text{Gl-1-Ph} & \xrightarrow{\text{Phl}} \text{Glycogen}
\end{align*}
\]

Assuming that ATP has already been formed, we need three further enzymes: hexokinase forms glucose-6-phosphate from glucose, phosphoglucomutase converts this into glucose-1-phosphate and phosphorylase converts this into glycogen.

But the phosphate mechanism is used in living matter for other chemical transformations besides synthesis. For instance, the reverse conversion of glycogen in the liver into glucose when needed to keep up the blood sugar level involves phosphorylase to form glucose-phosphate, followed by a phosphatase, which hydrolyses this to free glucose, which then passes into the blood.
An analogous mechanism, used in a somewhat different way, seems to be responsible for calcification in bone and tooth formation. According to the phosphatase theory of calcification, some soluble phosphate compound is hydrolysed by a very active phosphatase in these tissues so as to give a local concentration of free phosphate which is sufficiently high to cause deposition of calcium phosphate. I do not think this theory can be taken as proved, but the process can be imitated \textit{in vitro} and it is strongly supported by the fact that there is a quite exceptionally high concentration of phosphatase in bones and teeth.

One further example of the use of ATP for metabolic transformations is the conversion of fructose into glucose in the liver by a three-enzyme system. Hexokinase first forms fructose-phosphate from fructose, this is converted into glucose-phosphate by oxoisomerase, and a phosphatase hydrolyses this to give free glucose.

\[
\begin{align*}
\text{Fr} + \text{ATP} & \quad \Downarrow \text{Hk} \\
\text{Fr-Ph} & \quad \Downarrow \text{O1} \\
\text{Gl-Ph} & \quad \Downarrow \text{Ph} \\
\text{Gl} + \text{Ph} &
\end{align*}
\]

The ATP mechanism is believed to be involved in biological transmethylations and several other reactions which we cannot now discuss, as well as metabolic processes as diverse as sulphur assimilation in autotrophic bacteria and, according to a recent theory, photosynthesis in the green plant.

Now coming to the question of work against osmotic forces, let us consider the following mechanism. Suppose
we have a membrane which is permeable to a substance $B$ but not permeable to its phosphorylated form. There is, in fact, a marked tendency for many cell membranes to be comparatively impermeable to phosphorylated compounds. Let us suppose further that we have an enzyme mechanism for phosphorylating $B$, say ATP and a phosphokinase, but that this is present only on one side of the membrane. Then the $B$ present on this side of the membrane will be phosphorylated, and since it will then be unable to get back it will be trapped on this side of the membrane. The concentration of free $B$ on this side will be very small, owing to the action of the phosphokinase, and more $B$ will therefore diffuse through the membrane and in turn become phosphorylated. This will continue until the concentration of $B$ on the left side of the membrane has fallen to the equilibrium concentration of free $B$ on the right side. This will be very small because of the energy difference between the ATP and $B$-Ph bonds, so that practically the whole of the $B$ will be drawn over to the right side of the membrane and concentrated there. This of course means that work has been done against the concentration gradient, and this has come from the bond energy of the ATP. Thus a three-enzyme system consisting of say the triosephosphate dehydrogenase, phosphoglyceric phosphokinase and $B$ phosphokinase can do osmotic work, using energy from the carbohydrate oxidation. It is a fairly common thing in biology to get a concentration difference between the two sides of a membrane maintained by the occurrence
of oxidation processes. If the oxidations are stopped, the concentration difference disappears.

The phosphorylating mechanisms are essentially intracellular, so that the right side will generally represent the cell interior. This mechanism has been clearly shown in the case of vitamin $B_1$. The blood contains some $B_1$; in the tissues $B_1$ is phosphorylated to form cocarboxylase (its pyrophosphate). The $B_1$ is therefore drawn into the tissues and trapped there as cocarboxylase.

In fact this seems to be a fairly general mechanism. The blood tends therefore to contain non-phosphorylated, and the tissues the corresponding phosphorylated, compounds.

These effects probably play an important part in other absorption processes. It has been claimed that glucose is absorbed by the intestine by phosphorylation, though this does not seem to be fully proved yet, and fat absorption also seems to involve phosphorylation of the fat. In the kidney glucose is reabsorbed from the urine in the tubules apparently by being phosphorylated in the cells. Phlorrhizin interferes with phosphorylation and the glucose then passes on and appears in the urine.

If we picture a sheet consisting of cells in which the phosphokinase is located near one face and the phosphatase near the other, and suppose further that, while the cell membranes on both sides are permeable to $B$ but not to $B$-Ph, the central layer is permeable to $B$-Ph but not $B$, then it is clear that we have a mechanism by which ATP energy would cause $B$ to be secreted right through the cell sheet from one side to the other. There is no evidence, however, that this is the actual mechanism of secretion, and in fact it seems that phosphatase has the opposite distribution within the cells.
Now with regard to mechanical work, and especially muscle contraction, a great deal of work has been done recently on the biochemical mechanism and considerable progress has been made. Nevertheless, I doubt whether one can yet say just how this mechanism acts. But there are some extremely interesting and suggestive facts. Much evidence has now been produced that the free energy comes, directly or indirectly, from the ATP system. Further, it is very striking that the contractile protein of muscle, myosin, seems to be identical with ATP-ase, the enzyme which catalyses the reaction ATP → ADP + Ph, and therefore the enzyme which releases the ATP phosphate bond energy.

This at once suggests some most interesting possibilities, if the catalyst of the reaction which liberates the energy is also the machine which does the work. One thinks at once of a contractile enzyme with a fibril-like molecule which changes its shape when the substrate with which it is combined reacts, so that the free energy of the reaction it catalyses appears as mechanical work. In fact, several workers have shown that the myosin molecule undergoes a profound change of form during its catalysis of ATP hydrolysis, and a number of theories have recently been based on these effects. In the present state of knowledge I do not think it would be profitable to go into them here, but the subject is a fascinating one, and one hopes that before long it may be possible to determine the molecular mechanism of contraction.

Finally, ATP energy has been shown to be converted into certain other forms of energy in biological systems, for instance into light in luminescent cells and into electrical energy in the special organ of the electric eel.
Those are a few of the ways in which this remarkable and ubiquitous mechanism can be used. It is employed in such a variety of different ways in living matter, and is involved in so many different vital processes, that one becomes more and more convinced (especially in the absence of any indications of an alternative system) that here we have the main, and perhaps the only, biochemical mechanism by which energy is made available in living matter.
LECTURE III

In the two preceding lectures, in dealing with multi-enzyme systems, I have pointed out the special importance of two classes of reactions in living matter, the hydrogen-transfer reactions and the phosphate-transfer reactions. I drew an analogy between the dehydrogenase reactions in which hydrogen is transferred from molecule to molecule through coenzyme and the phosphokinase reactions in which phosphoric groups are transferred from molecule to molecule through ADP.

The two remaining lectures will deal with these systems from a rather different point of view. Hitherto I have been concentrating on the enzymes and considering the systems from the point of view of the catalytic mechanisms. Catalysts are essentially concerned with reaction rates. Now I shall pay more attention to an aspect which is determined by the substrates rather than the enzymes, namely the equilibrium points of the reactions, which of course are closely linked with the free energies of the reactions. Now the question will be, assuming that we have the necessary catalysts, will the reaction go, which way will it go and how far will it go? For example, if we take alcohol and pyruvate with their two dehydrogenases, will the alcohol reduce the pyruvate, or must we start from the opposite end with lactate and aldehyde in order to get any reaction? In other words, how will the hydrogen finally distribute itself among the various dehydrogenase substrates?

In the case of hydrogen-transfer reactions we obtain the necessary information from oxidation-reduction
potential data, and I should like now to give an account of some new methods which I have been developing for conveniently handling such potential data. By the hydrogen-potential scale we can deal with these fermentation reactions, and not only with them but also with the respiration enzymes. I should like also to give a preliminary account of a new analogous scale of 'phosphate potential' for dealing with the phosphorylation systems. Much of the material of these two lectures, therefore, has not previously been published.

The present lecture deals principally with the theory of the hydrogen scale. Let us start by considering how we can set up a scale which will indicate how intensely reducing or oxidising a given system is, in fact how we can arrange the various systems on a quantitative scale of oxidising or reducing properties.

The word 'system' will now be used with the special meaning of a mixture of the oxidised and reduced forms of a given substance, for instance a mixture of quinone and hydroquinone, of reduced and oxidised flavin, of lactate and pyruvate, or of succinate and fumarate.

On such a scale the strongly reducing systems will be situated near one end, the strongly oxidising ones at the other. The more strongly reducing a system is, the lower it will come on the scale, and any system (given the necessary catalysts) will be capable of reducing all the systems lying above it, and of being reduced by (or of oxidising) all those below it.

The distance between any two systems on the scale should give a measure of the driving force of the reaction between them, and two systems lying at the same point on the scale should be in equilibrium with one another.
This indicates that what we require is a scale based on
the free energy of the systems, since the free energy
change is the driving force of a reaction and is zero at
equilibrium.

First we have to find a common reference standard to
which all the systems can be related. This is a simple
matter—molecular $H_2$ gives us just what is needed. Any
ordinary oxidation-reduction system, for which we may
write $AH_2$ and $A$ for the reduced and oxidised forms
respectively, can be related to $H_2$ by the simple equili­
brum

$$AH_2 \rightleftharpoons A + H_2,$$

which can be brought about by a platinum catalyst in a
great many cases.

Now the free energy of $H_2$ gas at one atmosphere is
always taken as zero, so that all we have to do is to take
$H_2$ at one atmosphere for the zero of our scale, and to
place each system at such a position on the scale that its
distance from the zero is proportional to the free energy
change of the above reaction.

This can be done very simply in the following way.
This equilibrium which is set up at a Pt surface means
that a definite pressure of molecular $H_2$ in solution exists
at this surface. The pressure is usually extremely small,
but quite definite. It is in equilibrium with the oxidation-
reduction system, and is a measure of the reducing
intensity of the system, being very low if the system is
strongly oxidising and relatively high if it is strongly
reducing. It is convenient to base our scale on this $H_2$
pressure, but as we want a linear free energy scale we
must take the logarithm of the pressure and not the
pressure itself.
The connection is easily seen if we carry out the reaction of the system with \( \text{H}_2 \) at one atmosphere in two stages. First, take one mole of \( \text{H}_2 \) and allow it to expand reversibly until its pressure is reduced from one atmosphere to the pressure with which the system is in equilibrium. The free energy of this step is proportional to the logarithm of this equilibrium pressure, being actually \( 2.3RT \) times the logarithm. Now let the \( \text{H}_2 \) at this pressure react with the system reversibly, according to reaction (a). Since it is in equilibrium with the system, the free energy change of this step will be zero. So the free energy of the reaction of the system with \( \text{H}_2 \) at one atmosphere is proportional to the logarithm of the \( \text{H}_2 \) pressure (in atmospheres) with which the system is in equilibrium, and if we take this quantity for our scale (or, as is more convenient, minus this quantity, since the pressures are less than one atmosphere) it will fulfil all the requirements we have laid down. This quantity is known as the rH of a system: \( \text{rH} = -\log [\text{H}_2] \).

So we get the rH scale which was originally suggested for other reasons by Mansfield Clark in 1923. Unfortunately, the very great advantages of this scale do not seem to have been appreciated, and after a brief period of use it was dropped and the majority of workers now express their results in the less satisfactory form of electrode potentials. My aim in this lecture is to show that if the data are expressed in the form of rH a series of simple and most convenient rules emerges to which there is no counterpart when they are expressed on any other scale. In the next lecture it will be shown that an analogous scale of phosphate energies can be constructed to which the same set of rules applies.
The rH scale extends from rH$_0$, which of course corresponds to one atmosphere of H$_2$, at one end to one atmosphere of O$_2$ at the other, which is at rH 41. Beyond these limits are regions of H$_2$ or O$_2$ overvoltage, in which the pressure of H$_2$ or O$_2$ is greater than one atmosphere. Most of the biological systems lie within the lower half of the range, roughly from rH 0 to 25.

We can place a scale of calories at the side of our rH scale, so that we can read off directly the free energy $-\Delta F$ of the reaction of any system with H$_2$. But as this is given by $2 \cdot 3RT$ times the rH it will only be valid for one particular temperature, though a change of 10° will only make a 3% difference. Most of the measurements have been carried out at 30°C, and at this temperature each rH unit corresponds to 1380 calories.

There is quite a close analogy between the rH scale of oxidation-reduction and the pH scale of acidity-alkalinity. Not only are the definitions of rH and pH quite analogous, but many of the laws governing the behaviour of acids and bases on the pH scale are similar to those for oxidising and reducing substances on the rH scale.

But first let us see how rH is measured. Just as with pH, there are three methods: (a) electrode measurements, (b) indicators, and (c) calculation from thermodynamical data.
We may consider (b) first, as the simplest. A series of rH indicators is available covering the more important range. These are reducible dyes whose rH has been determined by electrode measurements. A small amount is added to the system, with an enzyme if necessary, in the absence of O\textsubscript{2} and the colour shows whether or not it is reduced. One in the right range is soon found; it is then allowed to come to equilibrium with the system, and from the colour the percentage reduction and hence the rH are estimated. This is a convenient but not a very exact method, and just as with pH indicators it depends indirectly on electrode measurements.

The main method is the use of the electrode. It is really a kind of hydrogen-electrode, used to determine not the pH but the H\textsubscript{2} pressure. Let us recall the mechanism of the hydrogen-electrode. Molecular H\textsubscript{2} (in solution) can combine with the Pt surface in a form something like that represented in the diagram. These H atoms can come off the surface not only as H\textsubscript{2}, but also as H\textsuperscript{+} ions leaving a negative charge on the Pt. There is a balance between three factors: the pressure of molecular H\textsubscript{2}, the concentration of H\textsuperscript{+} ions in solution and the electrical potential of the Pt. Increasing the H\textsuperscript{+} concentration will drive more H\textsuperscript{+} ions on to the Pt, which will become more positive. Increasing the molecular H\textsubscript{2} pressure will increase the concentration of H atoms on the surface, so that more will be driven off as ions and the Pt will become more negative. This
balance is expressed in the usual well-known hydrogen-electrode equation

\[ E_h = \frac{2 \cdot 3RT}{F} \log \frac{H^*}{\sqrt{H_2}}, \]

where \( E_h \) is the potential of the Pt in volts with respect to the usual electrode zero (the hydrogen-electrode at pH 0), \( R \) is the gas constant, \( T \) the absolute temperature, \( F \) the Faraday or charge on 1 g. of H*, the logarithm is \( \log_{10} \) (which accounts for the factor of 2.3, because the theoretical derivation gives \( \log_e \)), \( H^* \) is the molar H* concentration, \( H_2 \) is the H* pressure in atmospheres, and the root sign is because 1 H* comes from \( \frac{1}{2} \) a H* molecule. For brevity we shall write \( C = 2 \cdot 3RT/F \). \( C \) has a value of 61.5 mV. at body temperature or 57 mV. at \( 15^\circ C \).

Now, as already mentioned, Pt can catalyse the reaction \( A + H_2 = A + H_2 \) in many cases. This is due to the existence of the third equilibrium shown in the diagram, between the oxidation-reduction system in solution and the H atoms on the Pt, and through them with the molecular H*. So the rH of a system is measured, not by passing H* gas to fix the H* pressure at one atmosphere, but simply by immersing the electrode in the solution of the system, which then sets up a H* pressure corresponding to its own rH, and the electrode potential reveals what that pressure is, if the pH is known.

Thus in measuring pH the H* pressure is set at a known value and the potential is measured to determine the pH; in measuring rH the pH is set at a known value and the potential is measured to determine the H* pressure with which the system is in equilibrium.
MULTI-ENZYME SYSTEMS

From the definitions of \( rH \) and \( pH \) the equation can obviously be rewritten

\[
\frac{E_h}{C} = \frac{rH}{2} - pH
\]

so that the \( rH \) is given by

\[
rH = 2\left(\frac{E_h}{C} + pH\right).
\]

This is easy to work out from the value of \( C \), or a nomogram may be used as shown. Here the \( rH \) is found by placing a straight edge, or a piece of black thread, across from the potential to the \( pH \) values.

The method of carrying out the electrode measurements is extremely simple. A piece of Pt foil is simply dipped into a solution of the system at known temperature and \( pH \) and its potential measured against any convenient half-cell, for example a calomel electrode, with an ordinary potentiometer. For obvious reasons, \( O_2 \) should generally be absent, but this is not necessary if the system reacts very rapidly with the electrode and slowly with \( O_2 \). A familiar case of this kind is the quinhydrone electrode, which is really a hydrogen-electrode with the \( H_2 \) pressure fixed at \( 10^{-23} \) atmospheres by a mixture of hydroquinone and quinone in equal amounts.

The electrode method of measuring \( rH \) is certainly the best, but it cannot be used for all systems because some do not react with the electrode. Most hydrogen-carriers such as flavins, dyes, cytochrome, etc., react readily, as well as polyphenols, but coenzymes I and II and the substrates of the dehydrogenases do not. With these the indicator method can often be used, or better a combination of this and the electrode method. If both
indicator and electrode are present, the indicator takes the rH of the system and the electrode measures the rH of the indicator; in other words, the indicator acts as a hydrogen-carrier between the system and the electrode.
Alternatively, one can use the third method—calculation—if the substances can be found in the free energy tables. Since the free energy of H₂ at one atmosphere is zero, the free energy change of the reaction \( AH_2 = A + H_2 \) is simply the difference between the free energies of \( AH_2 \) and \( A \), and as this is the free energy of the reaction of the system containing equal amounts of \( AH_2 \) and \( A \) with one atmosphere of H₂, we can plot it directly on our calorie scale.

Let us take the succinic-fumaric system as a simple example. In neutral solution both are present as the ions and the reaction is

\[
\text{Succ}^- = \text{Fum}^- + H_2
\]

The free energies given in the tables for the ions at 25° are as shown, so that the difference is -20470 calories, which is the position of the system on our scale, corresponding to about pH 14. This agrees with electrode measurements in presence of the dehydrogenase.

So much for methods of measurement.

The pH of a system depends on four things:

(a) The nature of the system, that is to say whether it is a strong or weak reducing agent, in other words its affinity for hydrogen or the value of the equilibrium constant \( K \) of the reaction \( AH_2 = A + H_2 \).

(b) The degree of reduction of the system, that is the ratio of the concentration of the reduced form to that of the oxidised form \( AH_2/A \).

(c) It may depend on the pH. Normally (that is when the effects are not complicated by the ionisation of either \( AH_2 \) or \( A \) as acid or base) the pH does not change with
alteration of pH. But if the state of ionisation of the system varies with the pH, the rH depends on the pH in a simple way which is described below.

(d) The temperature $T$, though changes due to this cause are usually not large.

Of these, I should like to consider especially (b) and (c).

The percentage reduction of the system affects the rH in the following way. The mass action equation corresponding to the above reaction is of course

$$\frac{[A][H_2]}{[AH_2]} = K,$$

from which by taking logarithms we get

$$rH = -\log K - \log \frac{AH_2}{A}.$$

This is exactly analogous to the familiar titration curve of an acid, for which $pH = -\log k_a - \log AH/A'$, where $AH$ now represents an acid. So that if we plot the rH against the percentage reduction we get the same S-shaped curve which is so familiar in pH work. The different systems of this type of course will have different values of $K$; this means that they will give curves lying at different positions on the scale, but of the same form, just as a series of acids will give titration curves at different positions on the pH scale according to their $pK$'s, but all of the same shape.
The mid-point is given by \(-\log K\), and I propose to call this \(rH_0\), so that the equation of the curve becomes \(rH = rH_0 - \log AH_2/A\).

Now let us take a pair of oxidation-reduction systems and mix them; for example we may take succinate + fumarate and a dye methylene blue + its reduced form, in presence of the succinic dehydrogenase to catalyse the reaction between the two systems. If we take equal amounts of all four substances we shall start at the mid-points of the two curves. Then a reaction \(S + MB = F + MBH_2\) will occur, and the points representing the states of the systems will move along the two curves equally in opposite directions as the \(S/F\) and \(MBH_2/MB\) ratios change, until both systems arrive at the same \(rH\), when the free energy change becomes zero and the reaction stops at the equilibrium point. If we had taken ten times as much of the succinic system as of the dye system, the succinic point will only move horizontally one-tenth as much as the methylene blue point and we shall reach a different equilibrium point. If we start at any other points on the curves the same kind of treatment applies. It is very like mixing two buffers, where the resultant pH depends on the pK’s, the percentage neutralisations and the relative amounts of the two buffers.

It should be noted that the \(rH\) of a simple system is independent of dilution; it depends on the ratio \(AH_2/A\).
and if the concentrations of both reduced and oxidised forms are halved the rH remains unchanged.

It will be best to leave the effects of pH on the rH of systems until we come to consider the rule which deals with them.

It was mentioned earlier that when the data are expressed in the form of rH a set of simple rules emerges by which we can obtain useful information from them. These rules will now be stated.

Rule 1 tells us how many equivalents of hydrogen are needed for the conversion of the oxidised into the reduced form of the substance. We may call the number of equivalents \( n \); for the reaction \( A H_2 = A + H_2 \), \( n = 2 \), as in most cases, but in some systems, for example cytochrome, \( n = 1 \). Now if we determine the curve, let us say by titration with an oxidising agent, and mark off on it the two points where \( A H_2/A \) is \( 1/10 \) and \( 10/1 \) respectively, we define the ‘span’ of the curve as the difference in rH units between these two points. The rule states that if \( n = 2 \) the span = 2 rH units, if \( n = 1 \) the span = 4 units.

Rule 2, which is related to the previous rule, states that for systems where \( n = 2 \), a tenfold change of \( A H_2/A \) changes the rH by one unit. So that if one has a chart
giving the rH's of systems at 50% reduction (equal amounts of oxidised and reduced forms), such as the one which is given later, one can see at a glance what would be the rH of the systems at other degrees of reduction. If there is 1000 times as much of the reduced as of the oxidised form, the rH will be three units below the value shown; if there is 100 times as much of the oxidised as of the reduced form, the rH will be two units higher, and so on. For the few systems where \( n = 1 \), the displacement will be twice this.

Rule 3 gives the free energy of the reaction of any mixture \( AH_2 + A \) with \( H_2 \) at one atmosphere, which we may call \( -\Delta F_{\text{H}_2} \). As we have seen already

\[
-\Delta F_{\text{H}_2} = 2.3RT \cdot rH = 1380 \times rH \text{ calories at 30°.}
\]

This can be read off directly from the calorie scale at the side of the rH chart. It should be noted that this is the energy per \( H_2 \) mole (or per 2H atoms) transferred. This is also the energy per mole of \( A \) reduced, except where \( n = 1 \), in which case it is the energy per two moles of the substance. As will be seen later, this is a useful feature.

Rule 4 is the corresponding rule for the reaction of the system with \( O_2 \). It states that \( -\Delta F_{O_2} = 1380 \times (41 - rH) \) calories at 30°, and is simply the distance between the system and \( O_2 \) on the chart. It is useful because it gives the free energy when the substance in question is oxidised in respiration.

Rule 5 is for the reaction of any system with any other, and it states that \( -\Delta F = 1380 \Delta rH \), where \( \Delta rH \) is simply the difference between the rH values of the two systems. This applies whatever the percentage reduction of the two systems.
Now $\Delta F$ changes as the reaction proceeds and the concentrations alter, and it goes down to zero as the mixture reaches equilibrium. But the standard free energy $\Delta F^\circ$, which is a property of the reaction itself, is the free energy change when all the reactants are at unit concentration (or more strictly unit activity). It is the value of $\Delta F$ when all the components in solution are 1 m.

If the concentrations are all unity obviously the concentrations of the reduced and oxidised forms of both systems are equal and both systems are at the midpoints of their curves ($rH_0$). So we get—

Rule 6 which states that

$$-\Delta F^\circ = 1380 \Delta rH_0 = 2.3RT \Delta rH_0.$$  

Incidentally if $rH_0$ is substituted for $rH$ in rules 3 and 4, they give the standard free energies of the respective reactions.

Now this leads to a remarkably simple and useful rule. The $\Delta F^\circ$ is related to the equilibrium constant of a reaction by the well-known equation

$$-\Delta F^\circ = 2.3RT \log K.$$  

So from the previous rule we get—

Rule 7, namely \(\log K = \Delta rH_0\), which is perhaps the most useful of all the rules. It means that on a chart giving the positions of the various systems in their half-reduced states, such as that on p. 73, the distance between any two systems is equal to the logarithm of the equilibrium constant of the reaction between them. For example, let us take the succinic system reacting with methylene blue, for which the constant is defined by the equilibrium concentrations as

$$K = \frac{[F][MBH_2]}{[S][MB]}.$$  

At pH 7.6
there is a difference of 1 rH unit between them, so that log $K = 1$ and $K = 10$. If the difference had been 3, $K$ would have been $10^3$ or 1000.

This simple rule is most valuable, especially in connection with linked dehydrogenase reactions and fermentations. From a chart showing the positions of the various dehydrogenase systems, one can see at a glance the value of the equilibrium constant of the reaction between any pair of dehydrogenases at any pH. Some are shown on the chart given here, and the rH values of the others, so far as they are known, are given in the list of dehydrogenases on p. 28.

Rule 8 gives the heat of the reaction if the rate of change of the rH with temperature is known. By applying the rH notation to the Gibbs-Helmholtz equation we get

$$\Delta H = 2.3RT^2 \frac{drH_0}{dT} = 418100 \frac{drH_0}{dT} \text{ calories at } 30^\circ.$$

This has been found to give quite good results.

Rule 9 tells us how the rH varies with pH, and it enables us to obtain the acid and basic dissociation constants of the components from rH measurements. If, as has been done on the chart, we plot the rH of the half-reduced systems against the pH, the rule states that if $n = 2$ the slope of the curve is equal to the change of electric charge on the molecule which occurs when the reduced form is oxidised. If $n = 1$, the slope is twice this. If the reduced and oxidised forms carry the same charge the rH does not vary with pH; if the oxidised form has one more positive (or one less negative) charge than the reduced form we get a '1-unit slope', that is to say an increase of 1 rH unit for each pH unit, and so on. It is
O$_2$ overvoltage

FCy ferro-ferricyanide

Adr adrenalin
Fe Fe$^-$ Fe$^{2+}$
Cat catechol

QH quinhydrone

Ip indophenol (dibrom)
C cytochrome $c$
PyH pyridine-haemochromogen
Hb haemoglobin-methaemoglobin
CNH cyanide-haemochromogen
A ascorbic
MB methylene blue
S succinic-tumaric
FP flavoprotein (Warburg)
P pyocyanin
H haem
I indigo
L lactic-pyruvic
Al alcohol-aldehyde
F riboflavin
Aa acetoacetic-$\beta$OH butyric
Co cozymase
X xanthine-uric acid

H$_2$ overvoltage
easy to pick out on the chart many regions of zero, 1-unit, 2-unit, and occasionally a \(-1\)-unit slope.

This important rule requires further explanation, and for this we must look into the reasons for a change of \(rH\) with change of \(pH\). It arises from the fact that when the components of the system ionise as acids or bases, the amounts of the undissociated molecules are no longer equal to the total amounts of the substances added.

Take a simple case like quinhydrone. The oxidised form, quinone, does not ionise, so that we can write

\[ O_{xt} = Q, \]

where \(O_{xt}\) represents the total oxidised form. The reduced form, hydroquinone, does not ionise appreciably between \(pH 0\) and 8, but in more alkaline solution first one phenolic group and then the other ionises as an acid, so that the total reduced form \(R_{et}\) is the sum of the three forms

\[ R_{et} = QH_2 + QH' + Q''. \]

If \(k_1\) and \(k_2\) are the first and second dissociation constants, the ordinary laws of acidic dissociation give us

\[ R_{et} = QH_2 \left( 1 + \frac{k_1}{H^+} + \frac{k_1 k_2}{H^{2+}} \right). \]

So we have to replace \(QH_2\) and \(Q\) in our earlier \(rH\) equation \(rH = rH_0 - \log \frac{QH_2}{Q}\) by these values and we obtain

\[ rH = rH_0 - \log \frac{R_{et}}{O_{xt}} + \log \left( 1 + \frac{k_1}{H^+} + \frac{k_1 k_2}{H^{2+}} \right), \]

where the last logarithm term is new and represents the effect of dissociation.
The effect is that the whole S-shaped curve moves as the pH changes. The mid-point is displaced from $rH_0$ (its position when the system is unionised) by the amount of the last term.

We now require a new symbol to represent the $rH$ of the half-reduced *ionising* system, and for a reason which will be clear later I propose to call it $r'H$. It is the sum of $rH_0$ and the last term in the above equation, and gives the $rH$ of the half-reduced system under any circumstances.

This equation refers to the particular case of the quinhydrone system; the form of the last term will be different for different systems, according to the particular way in which they dissociate.

Rules 6, 7 and 8 still apply to ionising systems, if one continues to take the mid-points (in that case $r'H$); the results then automatically take into account all the ionic adjustments occurring when the reaction takes place at the pH in question, which is very convenient and simplifies calculation greatly.

Now to return to the quinhydrone system, as the pH increases we get three regions where the three forms of hydroquinone predominate in turn and the three corresponding terms within the last bracket become dominant one after the other. The $r'H$-pH curve therefore approximates to three straight lines. Up to pH 8 the last two terms can be neglected, and as log $1 = 0$ we have a zero slope. From about pH 9 to 12 the first and third terms are
much smaller than the second, which gives a 1-unit slope. Above pH 12 the third is the largest and we get a 2-unit slope. This illustrates the rule that the slope is equal to the change in the number of charges, as shown in the diagram.

The corners are rounded off because over a small range of pH two of the terms are making contributions, but if the straight parts are produced they cross at a pH equal to the pK of the particular dissociation responsible for the change of slope. The curve will pass through a point 0.3 rH units above (or below) the point of intersection. This is easily seen from the last term of the rH equation. If in the expression \( \log (1 + k_1/H^-) \) we put \( pH = pK_1 \), as it is at the intersection point, it becomes \( \log 2 \) or 0.3.

**Rule 10**, which follows from rule 9, states that any bend in the curve which has its concave side upwards (e.g. \( \ominus \) or \( \ominus \)) represents a dissociation constant of the reduced form, and every bend with its convex side upwards (e.g. \( \ominus \) or \( \ominus \)) represents a dissociation of the oxidised form. Each constant causes a change of 1 unit in the slope. The dissociation may be either acid or basic; both have the same effect and the rH data do not distinguish between them.

The quinhydrone system is a comparatively simple one. The equations which have been given for pH effects in other systems are often extremely complicated. But rules 9 and 10 enable us to dispense with all these equations and to draw the rH-pH curve at once if we know the dissociation constants. For example, the dye indophenol blue has an rH in acid solution of 22, and the oxidised
EFFECT OF IONISATION

\( rH \) Rules

1. If span = 2, \( n = 2 \); if span = 4, \( n = 1 \).
2. A tenfold change in \( \Delta H / \Delta \equiv 1 \) \( rH \) unit if \( n = 2 \).
   \( \equiv 2 \) \( rH \) units if \( n = 1 \).
3. \( -\Delta F_k = 2 \cdot 3 R T \cdot rH = 1980 \cdot rH \) calories at 30°.
4. \( -\Delta F_0 = 1980(41 - rH) \) calories.
5. \( -\Delta F = 1980 \cdot \Delta rH_0 \).
6. \( -\Delta F^o = 1980 \cdot \Delta rH_0 \).
7. \( \log K = \Delta rH_0 \).
8. \( \Delta H = 2 \cdot 3 R T^2 \frac{d\Delta H_0}{dT} = 418100 \frac{d\Delta H_0}{dT} \) calories at 30°.
9. \( pH \) slope = charge change on oxidation if \( n = 2 \).
   = twice charge change if \( n = 1 \).
10. Bend \( k_R \equiv k_B \), bend \( \gamma \equiv k_0 \).

form has a dissociation constant at \( pH \) 6.4 and the
reduced form two, at 7.2 and 10.3. We draw a straight
line at \( rH \) 22 until we reach \( pH \)
6.4. As this constant belongs to the
oxidised form we continue down­
wards with a 45° slope until we
reach \( pH \) 7.2. This next constant
belongs to the reduced form, so
that we continue horizontally to
\( pH \) 10.3 and thereafter upwards
at an angle of 45°. After rounding
off the angles slightly in accordance
with the rule the curve is complete, and will be found
in the chart.

Acid or basic groups which ionise equally strongly in
the oxidised and reduced forms of a substance do not
show up on the \( rH \) curves. In the indophenol system it is
easy to see that if the constants at 6.4 and 7.2 were
made to become equal they would cancel one another
out; the small downward-sloping part would disappear
and there would be no trace of them on the curve. It is
only those groups whose state of ionisation is affected
by the oxidation which show up on the rH curves, which
of course is exactly what we should expect.

The application of these ten rules to the rH chart
yields a great deal of information about the systems
shown on it, and about the free energies and equilibrium
constants of reactions between them. I shall refer to some
of this information more specifically in the next lecture.
With a little practice it becomes extremely easy to apply
the rules, and the chart then becomes a most useful tool.

It should be emphasised that these rules only emerge
when the data are expressed on the rH scale, which is
perhaps the reason why they have been unnoticed for so
long. So far as I know, they have not been published
previously.

As already mentioned, scarcely any data are published
in the form of rH, and most workers give their results
as direct electrode potential readings in volts ($E_h$). It
might be thought that corresponding simple rules should
exist which could be applied to data expressed in this
way, but actually this is not the case, as a few trials will
quickly show. There is not even a simple relationship
with the free energy; for instance the $E_h$ of the succinic-
fumaric system in neutral solution is about zero, although
the free energy is considerable.

Data in the literature are usually given in the form of
two quantities, $E_0$ and $E'_0$. $E_0$, the so-called normal
potential, is simply related to rH by the equation
$$rH_0 = 2E_0/C.$$ $E'_0$, which is the voltage at half-reduction,
is related to r'H by the equation
$$r'H = 2E'_0/C + 2pH.$$ These two equations enable one to compare data in the
literature with the chart very easily.
LECTURE IV

Let us now consider some illustrations of the application of rH data to biological systems, and especially to some of the multi-enzyme systems which bring about hydrogen-transfer processes such as respiration and fermentation reactions.

First let us make a rapid survey of the chart. In the upper part we have a region rather empty of biological systems, but containing a few inorganic ones such as the ferrous-ferric, ferro-ferricyanide and iodide-iodine systems. Then we come to a zone (23–30) where most of the polyphenols lie. Below this we have a region containing the various iron-porphyrin compounds. Below this again (6–12) are the flavins and flavoproteins. Finally the various dehydrogenase systems extend down, past the coenzymes, to the lower end of the scale. The succinic system is exceptional in lying outside the usual dehydrogenase zone. Then we have a number of dyes which are used as hydrogen-acceptors, at various points throughout the lower half of the scale.

Before we examine these systems in detail there is one point that we should consider. Suppose that we have two systems \(A\) and \(B\), and that they are to be linked through an intermediate hydrogen-carrier \(C\), so that hydrogen is transferred from \(A\) to \(B\) through \(C\). What should be the position of \(C\) on the rH scale if it is to work efficiently?
The obvious place is position $C_1$, where it can be readily reduced by $A$ and oxidised by $B$. If it lies very far away, in position $C_2$, it will not be able to act, because $A$ will only be able to reduce it to a minute extent and $B$ will then have such a minute concentration of the reduced form with which to react that the reaction is bound to be very slow. It is found in fact in non-enzymic systems that it is only substances which lie in the same $\text{pH}$ region as the systems which make efficient hydrogen-carriers.

But enzyme systems have rather more latitude in this respect. If the enzyme catalysing the reaction of reduced $C$ with $B$ has a very high affinity for reduced $C$, or if a very large amount of this enzyme is present, the reaction may proceed at a reasonable rate, even with very small concentrations of reduced $C$. Consequently, it is sometimes found that linked dehydrogenase reactions are catalysed by a carrier lying perhaps 3 or 4 units outside the range (position $C_3$). It must be remembered also that in the living cell the dehydrogenase systems may be very far removed from their half-reduced states. In this system the oxidised $A$ may be removed by another enzyme reaction so completely that the system is brought down by 3 or 4 units to position $A_2$, so that $C$ may in fact be brought within the range.

Let us now examine some of the systems in more detail. The ferrous-ferric system of course has a charge-difference of 1, but as $n=1$ we have a 2-unit slope in accordance with rule 9. The same is true for the ferro-ferricyanide system.

Coming to the iron-porphyrin systems, cytochrome $c$ also has a 2-unit slope. Application of rule 1 to the
reduction curve shows that \( n = 1 \), so that the slope shows that there is a charge change of 1 on oxidation. It has been recently shown that the oxidised form has a dissociation constant at pH 7.7, so that there is no change of charge on oxidation above this pH and the curve becomes horizontal. Cytochrome \( c \) lies at a strategic position in the centre of the diagram. These iron systems lie well above the dehydrogenases and similar biological systems, between them and \( O_2 \). It is therefore easy to understand why in respiration reactions the \( O_2 \) first reacts with iron compounds, and also why these compounds play little or no part in fermentation processes.

Continuing with the haematin compounds, haemoglobin has a curve similar to cytochrome, with a dissociation of methaemoglobin at about 6.7 which is linked with the oxidation. This curve of course is for the haemoglobin-methaemoglobin transformation; the oxygenation to oxyhaemoglobin is not an oxidation and naturally will not appear on an rH chart. Ferricyanide lies well above haemoglobin, and so can easily bring about the well-known oxidation to methaemoglobin. This reaction goes to completion; there is a difference of nine units and therefore an equilibrium constant of one thousand million, though of course this is far too large to measure directly.

Most of the haematin compounds which are not combined with proteins lie much lower. Haem itself lies at 10 with a zero slope. The haemochromogens show great differences according to the nature of the nitrogenous base. When haem is combined with pyridine it rises to about 20, also with zero slope. Cyanide-haemochromogen, however, is like ferricyanide and has a
2-unit slope. Most other bases give haemochromogens near the pyridine one, but histidine and pilocarpine haemochromogens, on the other hand, lie about one pH unit to the left of cyanide-haemochromogen and more or less parallel to it. We cannot discuss these differences in detail here, but they can be correlated with what is known of the structure of these compounds.

Of the polyphenols we have already considered quinhydrone. Adrenalin can only be measured in acid solution, owing to the great instability of its o-quinone. Perhaps it is of some interest that the polyphenol and catechol oxidases, which catalyse the oxidation of these substances by O\textsubscript{2}, are copper-containing proteins, and the only copper-protein which, as far as I know, has been measured, namely haemocyanin, was found to be at about 33. It is possible therefore that (though they have not been measured) these enzymes may be almost the only biological systems lying between the polyphenols and O\textsubscript{2}, and so able to act as effective carriers in bringing about this reaction.

The dyes need not be discussed in detail. We have already considered indophenol blue. A similar indophenol is used as an oxidising agent for titrating ascorbic acid, and it is easy to see from the chart why this is satisfactory and methylene blue is not. The ascorbic acid curve in acid solution coincides with that of pyocyanin. Pyocyanin shows a new phenomenon, namely that the curve divides into two branches below about pH 5. The meaning of this appears from the reduction curve. In neutral solution it shows the normal curve for a system with \( n = 2 \). But in acid solution it becomes a 2-stage curve, and for each of the stages \( n = 1 \). As the
solution is made more acid, the left half remains stationary but the right half moves downwards, and the two mid-
points correspond to the two branches of the pH curve.

This can only mean that the two hydrogen atoms, instead of coming off together as in most other systems, are coming off successively, so that there is an inter-
mediate form between $AH_2$ and $A$ which we may write $\cdot AH$, with a free bond. This kind of free radical is
known as a 'semiquinone'. Here the oxidised form $A$ must have a dissociation at pH 5 which is not possessed by either of the other forms, so that the curves for the two steps diverge and the effect is clearly revealed.

Semiquinone formation, which occurs in a few other systems, for example, flavins, is of interest to us because there seems to be a connection between this property and the ability to act as a link or hydrogen-carrier between the coenzymes and cytochrome. Possibly this is connected with the fact that for coenzyme $n = 2$ and for
cytochrome \( n = 1 \). A direct reaction would require two molecules of cytochrome and one of coenzyme to react together simultaneously; whereas a coenzyme molecule can transfer its two H atoms to the carrier in one step, and the carrier can then be reoxidised in two independent steps involving one cytochrome molecule each, so that the necessity for a trimolecular reaction is avoided.

Flavins in the free state all seem to have the same \( \mathrm{pH} \) value, including flavin dinucleotide, according to our recent unpublished results. It is somewhat remarkable how little has been done on flavoproteins, when we consider how much work has been done on them in other respects. One measurement at a single \( \mathrm{pH} \) has been published on Warburg's flavoprotein, shown by the point at \( \mathrm{pH} 12 \), and we have carried out a preliminary measurement on diaphorase, which gave the same result. It is interesting that combination with protein produces a change of more than five units.

The position of coenzyme I is important because so many systems react with it, and from its \( \mathrm{pH} \) one could deduce the \( \mathrm{pH} \) of a number of dehydrogenase systems and hence the free energies of many metabolic reactions. Unfortunately there is an uncertainty of about one unit either way in its position. The value calculated by Borsook would give a position slightly higher, but the equilibrium with crystalline alcohol dehydrogenase would give about that shown in the chart. The position of the alcohol system is known from thermodynamical data. The position of coenzyme II is unknown, but on chemical grounds would probably be in the same region.
Now we come to the dehydrogenase systems. The succinic system has been measured in presence of the dehydrogenase as far down as pH 4.5, at which point the enzyme becomes inactivated. The measurements agreed with calculated values. The continuation into acid solution has been deduced from the known values of the two succinic and two fumaric dissociation constants.

The oxidation of succinate by $O_2$ is brought about by a two-enzyme system linked by cytochrome $c$

$$\text{Succ} \rightarrow \text{Cyt}c \rightarrow O_2$$

![Diagram](image)

and it is of some interest to know how the free energy output of 37,000 calories is divided between the two enzymes succinic dehydrogenase and cytochrome oxidase. The chart tells us at once that at pH 7.5 about one-third of the total is produced at the dehydrogenase and two-thirds at the oxidase, but that this partition changes rapidly with pH. Actually, the free energy of the reaction of one molecule of cytochrome with $O_2$ is only about 12,000 calories—half the amount shown on the chart, but two molecules of cytochrome are required to oxidise one of succinate; as already mentioned, the scale gives the energy per 2 H atoms transferred, and it therefore gives the correct partition of the energy between the two enzyme reactions. It is a convenient feature of the rH chart that it automatically makes the correction.

This procedure of course can be extended to a whole type (b) chain, and one can see at a glance what proportion of the energy appears at each step.
We have seen that there is evidence that the succinic dehydrogenase can be linked with other dehydrogenases, although the mechanism is not clear. As it lies well above the other dehydrogenases it is always the oxidising enzyme in such coupled systems. This agrees well with Szent-Györgyi’s theory of respiration, which postulates that succinic with its dehydrogenase is part of the catalytic mechanism, and that it acts as an intermediate hydrogen-carrier by being reduced by other dehydrogenases and reoxidised by the cytochrome system.

A group of several dehydrogenase systems is situated close together at pH 8, including the lactic, glutamic, malic and glycerolphosphate systems, and these are all in equilibrium with one another. It cannot be said that their positions are all precisely known, because not all of the enzymes have been obtained pure, and in equilibrium measurements with impure enzymes the possibility of side reactions is not always excluded. But they are not likely to be much in error. The alcohol system is accurately fixed by thermo-dynamical data. Towards the lower end of the scale we have the xanthine-uric acid system; the hypoxanthine-xanthine system is slightly below this. The acetaldehyde-acetic acid system has not been directly measured, but can be calculated\(^1\) to be in the position shown in the next diagram, with a \(-1\) unit slope because the oxidised form (acetic acid) is ionised and the reduced form is not. This diagram shows further details of the lower end of the pH scale.

Before considering the difficult triosephosphate system, I should like for the moment to treat the oxidation of triosephosphate in carbohydrate respiration simply as

\(^1\) See note (2), p. 101.
the oxidation of an aldehyde to a carboxyl group. That is to say we will neglect for the moment the locking up of some of the free energy by the formation of a phosphate bond. Without the phosphorylation we might suppose that the triosephosphate system would not be very far removed from the acetaldehyde system, possibly a little lower. This is only a guess; unfortunately we do not yet know the actual position, though perhaps it might be measurable in the presence of arsenate to eliminate the phosphorylation. If the unionised triosephosphate system has about the same $rH$ as the acetaldehyde system, the $rH$ will in fact become slightly more
negative in neutral solution since phosphoglyceric is a stronger acid than acetic ($\text{pK} = 3.42$). The system will then be represented by the curve marked 'Tp?'. There is no reason why the system should not be negative in the absence of a Pt surface.

To return to the respiration process; if we can regard this respiration reaction as essentially an oxidation of an aldehyde by $O_2$ it is interesting to note that the two reactants are separated by almost 50 $\text{rH}$ units, and therefore the free energy is exceptionally large for a single oxidation reaction.

It is also interesting to compare this aerobic reaction with the anaerobic process—glycolysis or fermentation. Instead of about fifty units we have only about fifteen, so that very much less energy is liberated. Whether the cell will derive correspondingly more benefit from the aerobic process depends on whether it can make use of the energy of the cytochrome oxidase reaction, which accounts for about a third of the whole aerobic energy. So far we have no certain knowledge on this point, though it has been thought, on somewhat inadequate evidence, that the oxidation of succinate is coupled with a phosphorylation, and other aerobic processes involving cytochrome are known to involve phosphorylations. It seems rather likely, therefore, that this energy is not wasted.

According to Szent-Györgyi's theory the aerobic process would be brought about by a four-enzyme system thus:

$$
\text{R.CHO} \rightarrow \text{Ca} \rightarrow \text{Mal.} \rightarrow \text{Suc.} \rightarrow \text{Cyt e} \rightarrow O_2
$$

|-------|--------|--------|---------|

<table>
<thead>
<tr>
<th>$\text{R.CHO}$</th>
<th>$\text{Ca}$</th>
<th>$\text{Mal.}$</th>
<th>$\text{Suc.}$</th>
<th>$\text{Cyt e}$</th>
<th>$O_2$</th>
</tr>
</thead>
</table>

|-------|--------|--------|---------|


If we examine the chart we find that the aldehyde, coenzyme, malic, succinic, cytochrome and $O_2$ actually do occur in that order, in good agreement with the theory.

Now let us return to the triosephosphate dehydrogenase system, and take into account the coupling of the oxidation with phosphorylation. The reaction is now $Tpter + Ph + Co = diphG + CoH_2$ and is shown fully as reactions (6) plus (7) of the glycolysis system. The effects of coupling with the phosphorylation are threefold. First, the tilt of the pH curve disappears and it becomes horizontal. At first sight this is unexpected for a system oxidising an aldehyde to a carboxyl group, but actually no acid group is now produced on oxidation, for the carboxyl is linked to phosphate in an anhydride linkage as diphosphoglyceric and cannot ionise. Secondly, the system becomes less reducing, or in other words is displaced upwards on the chart to the position shown as $'Tp+P'$, which has been determined by measurements of the equilibrium with coenzyme I. This effect is to be expected, for if some of the energy of conversion of aldehyde to carboxyl is used in forming the phosphate bond, that available for reducing other systems will be less by that amount. Thirdly, the position of the half-reduced system moves up and down on the chart as the inorganic phosphate concentration is altered, because phosphate now enters into the equilibrium. All this makes the system a rather difficult one to deal with.

If we require the standard free energy $-\Delta F^\circ$, for calculation of equilibrium constants, we ought obviously to set the phosphate concentration at 1 $M$, as has been done in plotting the $'Tp+P'$ line. Then it is found that
the system is exactly in equilibrium with coenzyme I at pH 7.4. If our position for coenzyme I is correct its rH will be 4, if Borsook's value is correct it will be about 5.

Of course in the cell the free phosphate concentration is much less than 1 M, and the apparent position will be perhaps 2 or 3 units higher, in the position of the dotted line. This means that there will be no reduction of the coenzyme by triosephosphate unless the diphosphoglyceric is fairly completely removed, in agreement with experience.

Now if we add arsenate to break the coupling with phosphorylation, the reaction becomes \( Tp + Co = PhG + CoH_2 \), no phosphate bond is formed, the whole energy of the conversion of aldehyde to carboxyl is available for reduction; the system then becomes very strongly reducing, falling perhaps to the position already marked as 'Tp?'. The difference between 'Tp+P' and 'Tp?' obviously gives the energy of the phosphate bond in diphosphoglyceric, and if we knew the position of the system in the presence of arsenate we could read this off from the chart. The figure as drawn would indicate a value of some 17,000 calories at pH 7.5, but this should not be taken as anything more than a very rough estimate.

Now if instead of adding arsenate, we add to the triosephosphate dehydrogenase the ADP-ATP system and the necessary phosphokinase to transfer the phosphate from diphosphoglyceric to ADP, the reaction becomes \( Tp + Ph + ADP + Co = PhG + ATP + CoH_2 \). As the diphosphoglyceric concentration is greatly reduced by the ADP system, the rH now drops to a new value, shown by 'Tp+P+ADP'. The difference between this and 'Tp+P' is clearly the energy of the phosphokinase
reaction (reaction (8)) and is simply the difference between the phosphate bond energies in diphosphoglyceric and in ATP. The value for this difference (about 3.5 units) has been taken from Bücher's recent measurement of the equilibrium constant of this phosphokinase, which was crystallised by him.

The line representing the system remains horizontal in the presence of the ADP system, although an acid carboxyl group is formed when the phosphate is removed from diphosphoglyceric, because another acid group disappears when the phosphate is added to ADP. On the other hand, it is not generally realised that the energy of the energy-rich phosphate bond varies greatly with the pH, because an acid group is formed when it is hydrolysed. This is clearly shown in the diagram.

In this diagram it is easy to see that the distance between ‘Tp?’ and ‘Tp+P+ADP’ gives the energy of the phosphate bond in ATP, for we have seen that the distance between ‘Tp?’ and ‘Tp+P’ gives the diphosphoglyceric bond energy and the distance between ‘Tp+P’ and ‘Tp+P+ADP’ gives the difference between the diphosphoglyceric and ATP bonds. The value of the ATP bond energy shown in the diagram is about 12,500 calories at pH 7.5. This is in agreement with the value of 12,900 deduced by Lipmann from the hydrolysis of phosphopyruvate, and the value of 12,000 deduced by Meyerhof from the triosephosphate-cozymase equilibrium. None of these values, however, should be regarded as more than a rough approximation. In the absence of adequate free-energy data for the compounds involved, they have had to be based on somewhat indirect calculations from not-too-accurate data, and
they may require some modification in the light of later work.

Hitherto we have been dealing with hydrogen-transfer systems. Now, as mentioned earlier, there is a very close analogy between hydrogen-transfer and phosphate-transfer systems, and since phosphate-transfer reactions play an important part in energy transfer in living matter, it seemed to me that it would be helpful if one could construct a scale of phosphorylation intensity, which would be analogous to the rH scale of reducing intensity. One could then set out the various systems on a chart like the rH chart, and if the scale were properly chosen the same rules (or at any rate closely analogous rules) would apply as in the case of rH. We could then read off the free energies and equilibrium constants of the various phosphorylation and phosphate-transfer reactions. Every system would be capable of phosphorylating any system lying above it, or of accepting phosphate from any lying below it.

As already pointed out, a compound with a high-energy bond will tend to drive off its phosphate much more strongly than one with an energy-poor bond, so that one can imagine a kind of phosphate pressure, rather analogous to the hydrogen pressure in reducing systems. It is really measured by the concentration of inorganic phosphate with which the system is in equilibrium: the higher the phosphorylating power of a system, the higher this equilibrium concentration of free phosphate will be.

The basis of the treatment is the reaction $XP \rightleftharpoons X + P$, which is exactly analogous to the reaction $AH_2 \rightleftharpoons A + H_2$. 

of the oxidation-reduction system. So if we base our new scale on this phosphate concentration and plot \(-\log P\), where \(P\) is the molar concentration of inorganic phosphate in equilibrium with the system, we shall have a scale which will obey all the laws of the \(rH\) scale. We might provisionally call this quantity \(rP\), by analogy with \(rH\),

\[ rP = -\log P. \]

Now precisely as in the case of \(rH\) we have

\[ K = \frac{[X][P]}{[XP]} \]

and in logarithmic form

\[ rP = -\log K - \log \frac{XP}{X}. \]

Therefore if we plot the \(rP\) against the percentage phosphorylation we shall get the familiar S-shaped curve once more. The span of the curve will be two \(rP\) units; there does not seem to be any analogy to the difference between the 1- and 2-equivalent systems on the \(rH\) scale.

Rule 2 is the same: a tenfold change in the \(XP/X\) ratio corresponds to an \(rP\) change of 1 unit. Rule 3 is the same: the free energy of the reaction of the system with 1 m phosphate is given by \(-\Delta F_x = 2\cdot3RT\cdot rP\). There seems to be nothing to correspond to rule 4, which related to the reaction with \(O_2\). Rule 5 is the same: the free energy of the transfer of phosphate from one system to another is given by \(-\Delta F = 2\cdot3RT\cdot \Delta rP = 1380\cdot \Delta rP\) calories at 30\(^\circ\), where \(\Delta rP\) is the difference between the \(rP\) values of the two systems. Rule 6 is the same:

\[ -\Delta F^o = 1380\cdot \Delta rP_0. \]
Rule 7 is also the same: \( \log K = \Delta rP_0 \), where \( K \) is the equilibrium constant of the phosphate transfer reaction, so that one can read off the equilibrium points in exactly the same way as with \( rH \). Rule 8 is the same:

\[
\Delta H = 2.3RT^2 \frac{drP_0}{dT}.
\]

With rules 9 and 10, relating to the effect of pH on the rP, we meet with a real difference between rH and rP, namely that, unlike the \( H_2 \) molecule, phosphate ionises in solution and has three dissociation constants. This introduces a complication, though it can be dealt with fairly easily. Actually the third constant is very weak and can be neglected at pH values below 12. It is this group which links with \( X \), so that the first and second groups remain acidic:

\[
X \text{HO--PO--O} \stackrel{\text{OH}}{\text{(pH 2)}},
\]

\[
X \text{HO--PO--O} \stackrel{\text{OH}}{\text{(pH 6.8)}},
\]

though on combination with \( X \) their dissociation con-
THE pH SCALE

95

stants are usually altered somewhat. If they remained the same of course they would have no effect on the rP.

We can work out the effects of pH precisely as we did for rH. The total free phosphate $P_t$ will be made up of three forms

$$P_t = P + P' + P''$$

and if $k_1$ and $k_2$ are its two dissociation constants we have

$$P_t = P \left(1 + \frac{k_1}{H^+} + \frac{k_1k_2}{H^+2}\right).$$

Similarly, for the total $X$-phosphate

$$X_{Pt} = XP + XP' + XP''$$

$$= XP \left(1 + \frac{k_1'}{H^+} + \frac{k_1'k_2'}{H^+2}\right),$$

$X$ will generally be a sugar or some similar substance which will not be ionised, but in the case of an energy rich compound the phosphate may be linked to an acidic group such as a carboxyl, whose ionisation must be included:

$$X_t = X + X'$$

$$= X \left(1 + \frac{k''}{H^+}\right).$$

We now substitute these values in the rP equation and get the complete equation

$$rP = rP_0 - \log \frac{X_{Pt}}{X_t} + \log \left(1 + \frac{k_1'}{H^+} + \frac{k_1'k_2'}{H^+2}\right)$$

$$- \log \left(1 + \frac{k_1}{H^+} + \frac{k_1k_2}{H^+2}\right) - \log \left(1 + \frac{k''}{H^+}\right).$$

Actually this is exactly the same as the rH equation for an analogous system, with one extra term to allow for the ionisation of the phosphate. Here rP is written for $-\log P_t$, referring to the total inorganic phosphate and not to any particular phosphate ion; this is what is always
measured and used in expressing equilibrium constants in such reactions.

This equation simply means that rule 9 applies to the pH curves just as before: the slope is equal to the change of charge on dephosphorylation or hydrolysis, but the charge after the hydrolysis is the sum of the charges on $X$ and free phosphate. For instance a reaction $XP'' \rightarrow X + P'$ has a charge change of $+1$ and a reaction $XP'' \rightarrow X' + P'$ has a charge of zero and a zero slope. Rule 10 also follows: a bend with the concave side upwards is due to a dissociation of $XP$, but one with its convex side upwards is due to a dissociation of either $P$ or $X$. Having got these rules we can dispense with the equation for most purposes.

$rP$ Rules

1. $\text{Span} = 2$.
2. A tenfold change in $XP/X \equiv 1$ $rP$ unit.
3. $-\Delta F_p = 2.3RT \cdot rP = 1380. rP$ calories at $30^\circ$.
4. $-\Delta F = 1380. \Delta rP$.
5. $-\Delta F^\circ = 1380. \Delta rP^\circ$.
6. $\log K = \Delta rP^\circ$.
7. $\Delta H = 2.3 RT \frac{dP_0}{dT}$.
8. pH slope = charge change on dephosphorylation.
9. Bend $\downarrow \uparrow = k_{XP}$, bend $(\downarrow \uparrow) = k_P$ or $k_X$.

The $rP$ values we shall deal with will all be negative, because all the systems are in equilibrium with phosphate concentrations greater than $1 \text{ M}$. Most of the phosphate concentrations in fact are unattainably high, but this need not disconcert us; they are the concentrations that would be in equilibrium with the systems if they could be attained. By reducing the $XP/X$ ratio we may bring the equilibrium phosphate concentration down to attainable values for purposes of measurement.
The treatment which I have given here for rP is quite analogous to that given for rH on pp. 74-7. Like rH₀, rP₀ gives the mid-point of the curve when the system is unionised, for example in strongly acid solution. We can use the symbol r'P, like r'H, for the mid-point when ionisation occurs. Therefore r'P is the sum of rP₀ and the last three terms of the complete equation, and gives the rP of the half-phosphorylated system at any pH.

It is easy to see that the r'P value of any system is also the logarithm of the equilibrium constant of the hydrolysis of the system. Since 'phosphate-bond energy' is defined as the $-\Delta F^\circ$ of hydrolysis of the bond, it follows that (at 30°) the bond energy is $1380 \times r'P$. The scale is therefore a direct scale of bond energies, which is a most useful feature and enables us to read off the bond energies immediately from the calorie scale at the side.

When we come to plot the various systems on an rP chart we realise how very few reliable measurements have actually been made on the free energies of organic phosphate compounds and how great is the need for such measurements. Unfortunately, we have no phosphate electrode, and we have to work mainly from equilibria determined by chemical estimations.

The final figure represents a very tentative attempt to construct an rP chart. It should not be regarded as anything more than a rough approximation. The forms of the curves are probably correct, as they are based on the dissociation constants of the systems, which are fairly accurately known in most cases. But the relative positions of the different systems may require some modification when better measurements become available.

The glycerol-glycerolphosphate system ought to be the
starting-point, as it is almost the only system whose equilibrium with inorganic phosphate has been directly measured, using phosphatase as catalyst.\(^1\) This system is shown as 'GP'. Unfortunately the glycerol phosphokinase has not yet been purified, so that this line leads no further at present. Lipmann has given a rough estimate of the bond energy of glucose-1-phosphate ('G-1-P') from the phosphorylase reaction. Possibly a good estimate could be made from the sucrose phosphorylase reaction, if the equilibrium constant for cane sugar inversion were available. The conversion of glucose-1-phosphate into glucose-6-phosphate ('G-6-P') has an equilibrium constant of 20 and there is therefore a difference of 1.3 units between them. Fructose-6-phosphate (not shown) lies very close to the latter.

The two waves in all these curves are due to the fact that the two dissociation constants of phosphate become slightly stronger on combination with the sugar. Each wave is therefore due to a slight separation of $k_{XP}$ from $k_F$.

For the ADP–ATP system I have assumed, in the light of the previous discussion, about 12000 calories at pH 7.5, but this may have to be modified. Its position in fact is the chief uncertainty in the chart. It is shown as 'ADP', and we know from the myokinase equilibrium that the AMP–ADP system is almost coincident with it. It has a $-1$ unit slope because the reaction is

$$\text{ATP}'''' = \text{ADP}'''' + P''',$$

with a charge change of $-1$. At pH 6.8 both ATP and ADP have a dissociation constant, which therefore cancels out, but so also has phosphate, so that the curve turns over and becomes horizontal, corresponding to the reaction $\text{ATP}'''' = \text{ADP}'''' + P'$. 

As we have seen, the phosphoglyceric-diphosphoglyceric system ('diPG') is situated about 3 units beyond the ATP system, and the $-1$ unit slope continues down to the point where the phosphoglyceric carboxyl group dissociates ($pK$ 3.42). The pyruvic-phosphopyruvic system ('PPy') probably lies within 1 unit of the position indicated. The existing data on the equilibrium between phosphocreatine and ATP suggest about the position shown as 'CrP', but the equilibrium needs redetermining with purified enzyme in the absence of myokinase. In phosphocreatine the second phosphate ionisation is much stronger than in the other systems ($pK$ 4.6), so that the central wave is prolonged. All these high-level systems are placed in relation to ATP, and if the latter has to be moved up or down they must be moved similarly.
This diagram, rough as it is, seems likely to be helpful in explaining the behaviour of phosphate-transfer systems. For instance, it shows clearly why the creatine phosphokinase equilibrium depends so strongly on the pH, while the phosphoglyceric phosphokinase equilibrium does not. It brings out also the large effect of pH on phosphate bond energy. It is interesting that in slightly acid solution the ATP energy falls to only about thrice that of an ester bond, while creatine-phosphate becomes a very high-energy system.

In conclusion, let me recapitulate and sum up very briefly the substance of these lectures. We have seen how enzymes can be coupled together functionally into systems of enzymes, and we have seen some of the processes which such systems can bring about. For life to exist, we must have a certain minimum number of enzymes linked together in a system, which can both bring about energy-yielding reactions and also transfer and use this energy for other processes. We have seen the special importance within this system of two kinds of reactions brought about by coupled enzymes, namely hydrogen-transfer reactions, for generating the energy, and phosphate-transfer reactions, for transferring and using it. And lastly we have seen how energy data for these reactions can be conveniently handled; for hydrogen-transfer reactions by the use of the rH scale and chart, and for phosphate-transfer reactions by means of the rP scale. It is my hope that these lectures will do something to show that the study of multi-enzyme systems is one which has a special importance and interest of its own.
Notes (1) (see p. 46). Since this book went to press the mechanism of the phosphomutase reactions has been elucidated by Caputto and Leloir and by Cori. The suggestion made on p. 46 that the process consists in a phosphate transfer from one molecule to another has proved to be correct, but the actual reaction differs somewhat from that proposed. In each case the phosphomutase reaction can only occur if there is present a small amount of the corresponding diphospho-compound (glucose-1,6-diphosphate or 2,3-diphosphoglyceric acid respectively). The process then takes place as follows:

\[
\text{6-P-Glucose-1-P} + \text{Glucose-1-P} = \text{6-P-Glucose + 6-P-Glucose-1-P},
\]

\[
\text{2-P-Glyceric-3-P} + \text{Glyceric-3-P} = \text{2-P-Glyceric + 2-P-Glyceric-3-P},
\]

so that the amount of the diphospho-compound remains constant.

Notes (2) (see p. 86). The free energy of acetaldehyde (aq.) is about \(-34,300\), that of acetic acid (aq.) is \(-96,200\) and that of water is \(-56,690\). The free energy of the reaction \(\text{Ac} + \text{H}_2 = \text{Ald} + \text{H}_2\text{O}\) (that is \(\Delta F_{\text{H}_2}\)) is \(+5200\). The \(\text{rH}\) is therefore negative by Rule 3, and the system is in equilibrium with a \(\text{H}_2\) pressure greater than atmospheric. The slope is zero until the \(\text{pK}\) of acetic acid at about \(\text{pH} 4.6\) is reached and thereafter becomes \(-1\).

The system has not been measured directly as it does not react with the electrode, and all the \(\text{rH}\) indicators tried have been completely reduced. No evidence of reversibility could be obtained, as was to be expected in view of these figures.

Notes (3) (see p. 98). After this book was in proof a paper by Meyerhof appeared giving determinations of the equilibrium constants of hydrolysis of several phosphate esters. Phosphatase was used at \(\text{pH} 5.8\) and 8.5, and the esters included glycerol-phosphate and glucose-6-phosphate. The results are in quite good agreement with the curves given in the \(\text{rP}\) chart. Meyerhof’s announcement that he is carrying out a series of determinations of phosphate-bond energies will be welcomed.
INDEX

ADP-linked systems, 45
ATP as source of energy, 52–56
ATP bond energy, 91, 99
ATP, functions of, 49–56

Bond-energising enzymes, 39–41

Coenzyme-linked systems, 31–38
Consecutive enzyme reactions, 15–17

Dehydrogenase systems, 31–38
Dehydrogenases, 27–29

Energy transfer, 10–12, 38, 41
Enzyme properties, 2–6
Enzyme specificity, 4–6
Enzymes, linking of, 7–9

Fermentations, 31

Glycolysis system, 18–26

Linking of enzymes, 7–9

Organisation by specificity, 19

pH, effect on rH, 72–77
pH, effect on rP, 95–97

Phosphatases, 50
Phosphate bond energy, 39–41, 90–92, 97–100
Phosphate transfer, 41–46, 92
Phosphokinases, 41–46
Phosphomutases, 46, 101
Phosphorases, 38–50
Phosphorylases, 46–49
Phosphorylation systems, 50–52
Phosphotransferases, 46
Properties of enzymes, 2–6

Respiration systems, 29–31

rH chart, 73
rH measurements, 61–66
rH nomogram, 65
rH of biological systems, 79–91
rH rules, 69–77
rH scale, 58–61
rH, theory of, 66–78

rP chart, 97
rP of biological systems, 98–100
rP rules, 93–97
rP scale, 92–93
rP, theory of, 93–97

Semiquinones, 83

Triosephosphate system, 87–91