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STUDIES ON BIOLOGICAL OXIDATION AND SOME OF ITS CATALYSTS

(C₄ DICARBOXYLIC ACIDS, VITAMIN C AND P ETC.)

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Preface.

For more than a decade my work was devoted to the elucidation of the mechanism of the apparently simple reaction $2\text{H} + \text{O} = \text{H}_2\text{O}$. The experiments have led to most heterogeneous results, as for instance to the discovery, isolation and identification of Vitamins, the discovery of the catalytic nature of C_4 dicarboxylic-acids etc. Some of my observations have found application even in medicine, in the cure of diseases.

In spite of their great heterogeneity these results are intimately connected with the central problem, the mechanism of the reaction $2\text{H} + \text{O} = \text{H}_2\text{O}$. They are but pieces of a mosaic which I cannot hope to complete. This may justify my attempt to overcome my aversion to pen and ink and to sum up my work, showing the connection of its single phases, and coordinating them to the central problem. Most of my original observations are buried under a mass of data and are scattered over a great number of papers. By this book I want to make them more easily accessible to the few who may be interested in them.

It is not my intention, however, simply to sum up my experiments. I want to outline the problem which has occupied me for so many years and still fascinates me.

The special object of this book may justify me if I give undue prominence to my own work and refer to others only as far as will be necessary to make my own story intelligible.

Reference will be given to my own papers or to papers of my collaborators at the end of this book. References to other papers will be found in my original communications or at the bottom of the pages.

When I first contemplated writing this book, my thoughts wandered thankfully to my collaborators whom I will mention in the text. I am also grateful to all those who have helped me on my way which has not been devoid of hardship.

That my ideas could be put to a test unhampered by material difficulties during the last years, I owe to the generous help of the *Josiah Macy Jr. Foundation, New York*.

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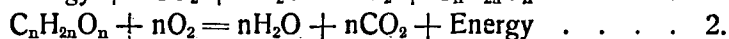
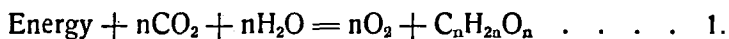
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Introduction.

The Principles of Biological Oxidation.

Whatever a cell does, it has to pay for it and the currency of living systems in which the cell has to pay, is energy. If there is no free energy, there is no life. The sole ultimate source of this energy is the radiation of the sun. This, however, cannot be utilized as such to maintain life, or else life would fail at night. Therefore the radiating energy is packed into small parcels by the chloroplasts of the chlorophyl-containing plants. If the cell needs energy, it does not use radiation but unpacks these parcels of energy, called „foodstuff molecules“. The two fundamental reactions of life are: 1. The making of these packages and 2. their unpacking.



Reaction 2 is the oposite of reaction 1. The first of the two reactions is performed only by chlorophyl-containing plant cells, while reaction 2 is performed by all cells, by the plant, or by the animal, which eats the plant (herbivores) or by the animal, which eats the animal, which ate the plant (carnivores).

This book is concerned with the mechanism of the second reaction.

The analogy of the coal heated steam engine suggests that the essence of this reaction is the coupling (oxidation) of the C with O_2 . C is the backbone of the whole molecule, and its reaction with oxygen is a rich source of energy, while the H_{2n}O_n moiety ($=n\text{H}_2\text{O}$) of the foodstuff molecule represents no energy.

The discovery that this is not so, is one of the greatest events in modern biochemistry and is linked with the name of *H. Wieland*.

According to *Wieland*, whose view is supported by the great bulk of modern literature, the oxidation of the foodstuff molecule consists of the splitting off of H atoms. The whole foodstuff molecule is in essence but a little parcel of Hydrogen, its C atoms are pegs, on which the H atoms are hung. To use *Wieland's* nomenclature, the foodstuff molecule is but an „H Donator“. So in this book I will call the foodstuff simply „Donator“.

The oxidation of the H, given off by the donator, drives the living engine. This reaction is the sole ultimate source of energy in higher organisms, which know but one fuel: Hydrogen. Hydrogen is the fuel of life.

The oxidation of H_2 to H_2O yields 68,000 cal., and is thus one of the reactions, richest in energy, known to the chemist. It is not the object of biological oxidation to liberate this energy. The object is, to liberate it in such a way that it can be transferred to cellular functions which need energy. Energy turned into heat is lost the living machine.

It seems that the quantity of energy, liberated in the oxidation of one single H atom is too big to be dealt with. The cell wants change. The H is thus not coupled at once with Oxygen, which coupling would yield the total energy, but it is linked in succession to a series of substances. At every new step energy is liberated. So the H is oxidised piecemeal and the liberation of energy distributed over a long series of reactions.

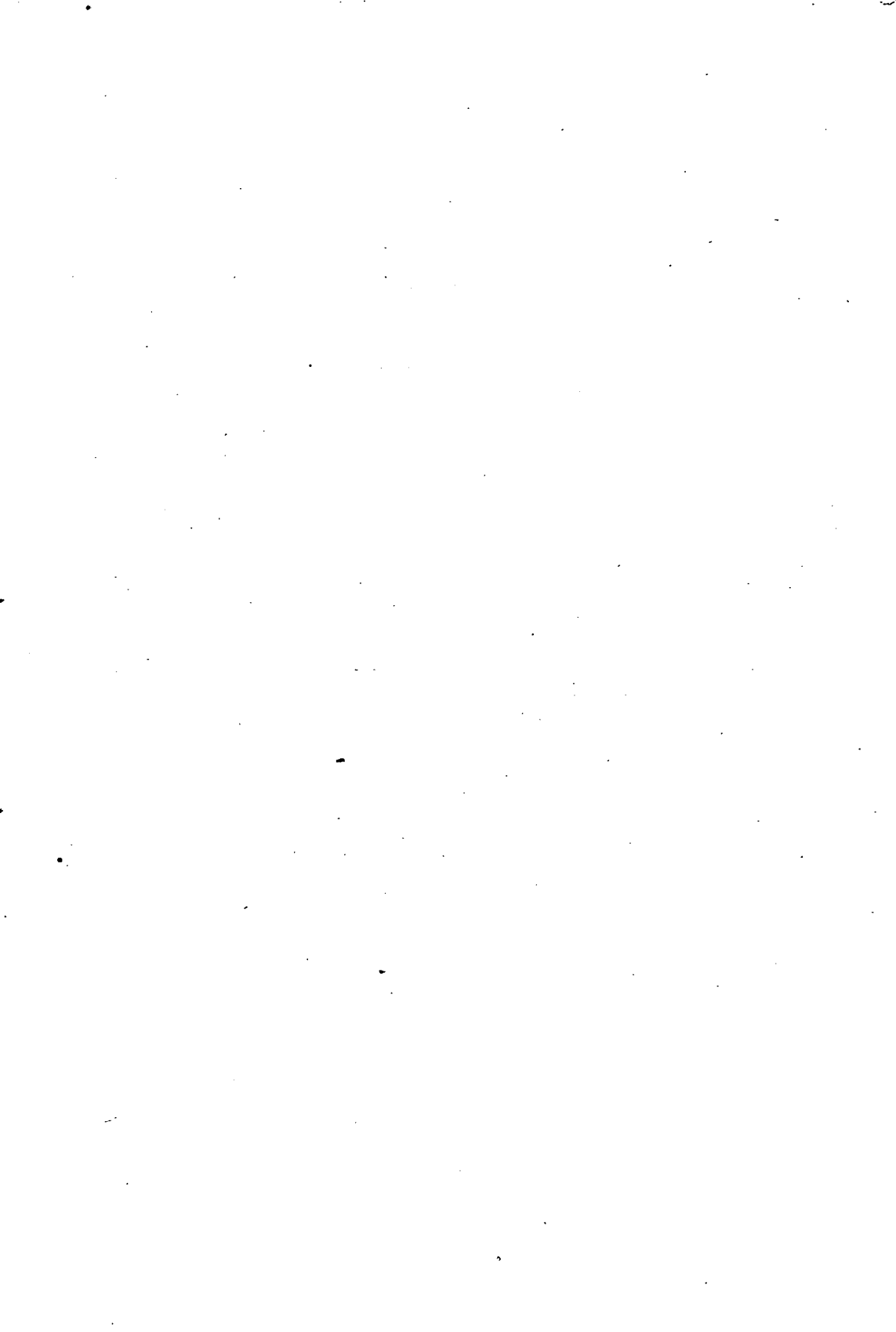
This piecemeal combustion of the H atom is one of the fundamental principles of biological oxidation and it was the object of my work to contribute to its knowledge.

Oxygen was thought of previously as the omnipotent master of oxidation which disintegrates the foodstuff molecule by its oxidising power. By *Wieland's* theory its function became reduced to the more modest rôle of an „H acceptor“. The inspired oxygen does not come in touch with the foodstuff molecule at all. It does not even meet the H split off from the donator. Oxygen acts only in one single reaction, at one end of a

long chain. It disappears from the scene at its first step and leaves behind no more than an electronic change on Fe atoms. Accordingly the O_2 moiety of the expired CO_2 is not derived from the inspired O_2 at all but it represents partly the oxygen contained in the foodstuff molecule, and is partly derived from the water molecules taken up by the donator to fill the place of the lost H atoms.

Naturally such generalisations hold only for the big bulk of respiration. Here and there a H may find its way to oxygen or an oxygen to some organic molecule. All the same there can be little doubt, that our outlook on oxidation has undergone profound changes. Biological oxidation was thought of before as an interaction of Foodstuff and Oxygen. To-day this process is pictured as a long chain of reactions in which Hydrogen is combusted. The central problem of biological oxidation is the mechanism of this piecemeal oxidation of the single H atoms. At one end of this chain of reactions stands the foodstuff molecule, having no other function than to give off its H atoms. At the other end stands O_2 giving up its electrons. It has been my chief object to contribute to the knowledge of the middle part of the reaction.





PART I.

The Oxidative Mechanism in Animal Tissues.

1. The Activation of O_2 and Cytochrome.

At one end of the system of biological oxidation stands the „Donator“, at the other O_2 . Both the Donator and the O_2 molecule are fairly stable. They both have to be „activated“ in order to react.

It was the great discovery of *Warburg* to show that inspired O_2 was activated by an enzyme-like substance. Nobody has ever seen this enzyme, yet all the same we know even its spectral properties. Its discovery is due to the fact that it is inactivated by mere traces of cyanide. In all probability the prosthetic group of this enzyme is a hemin. As shown by *Hamburger* (7) and myself, this enzyme has a very high affinity for oxygen and is capable of utilizing its last traces. This enzyme probably acts on the O_2 in such a way as to make its four valencies act individually and to avoid formation of H_2O_2 .

With the one-sidedness of a genius *Warburg* called his activator the „Atmungsferment“, „respiratory enzyme“, emphasizing by this name that this enzyme is the only essential catalyst of oxidation. For if it is poisoned by cyanide the whole respiration stops.

For many years violent discussion was going on over the question whether the H or the O_2 activation was the essential part of biological oxidation. The smoke of this battle has blown away. *Fleish* and myself (4) have given the first indication that they are both equally needed and act together. To-day we know that they are both members of a chain, every link of which is equally important.

The result of these studies can thus be summed up by saying: activated oxygen oxidises H.

It was the great discovery of *D. Keilin* to show that the activated oxygen did not act on the system of H activation immediately. Between the two there was cytochrome, equally a hemin. This cytochrome molecule has a Ferrous atom. It is this Fe^{++} which is oxidised by *Warburg's* activated O_2 to Fe^{+++} and reduced again by the activated H.

As *Keilin* has shown, cytochrome is not a single substance. It is composed of three closely related dyes, cytochrome A, B, and C. We do not know the meaning of the existence of three different cytochromes yet and do not know either how they are shunted, whether the three components are alternative ways of oxidation, or are shunted in series, the one getting oxidised only by the „Atmungsferment“ then oxidises the other, which in its turn oxidises the third, which finally oxidises the H. The trend at present is to accept this latter alternative.

The function of the „Atmungsferment“ is to oxidise cytochrome. It is built and is connected with the cytochrome in such a way, that it refuses to oxidise any other substance. *Huszák* in my laboratory is collecting data, which tend to show that even dyes and aromatic amines are oxidised only *via* cytochrome. Accordingly we had better call *Warburg's* enzyme „cytochrome-oxidase“. I will also refer to the complex, consisting of cytochrome and its oxidase, as the Warburg-Keilin System (WKS).

2. The Activation of H. Dehydrogenases and Co-dehydrogenases.

It is easy to demonstrate the activation of H and its catalysts. *Thunberg* has given a simple method for this, which had an important rôle in the development of our ideas. *Thunberg's* experiment consists of the mixing of a suitable dye, such as methylene blue, a „donator“, and the enzymic extract of tissue in absence of O_2 . If the extract contains an activator, the H of the donator will be split off and taken over by the dye, this latter acting as an H acceptor. Giving off H means oxidation, taking up H means reduction. Thus

the donator will be oxidised and the dye reduced. As most dyes lose their colour on reduction, the decolouration of the system indicates the transference of H. Without a catalyst there would be no change. These catalysts, splitting off H from the donator, are called „dehydrogenases“ (or wrongly dehydrases). The H of the donator is in fact not split off as such. It is only shifted over on to a dye and the donator loses its H only in presence of a suitable acceptor.

The dehydrogenases do not change anything of the energetics of this process of H transfer (*H. Borsook*). They just help the H to come off more easily by reducing the energy of activation. The dehydrogenases do not act on the H itself but

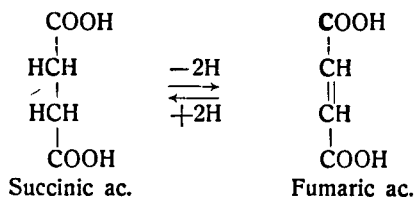


Fig. 1.

on the rest of the molecule. Accordingly they can make their substrate not only give off, but also take up H. For instance, succinic acid, (Fig. 1.) in presence of the succino-dehydrogenase will give off H and reduce methylene blue. The succinic acid, by doing so, turns into fumaric acid. If, however, we mix pure fumaric acid, succino-dehydrase, and reduced methylene blue (leuco-methylene blue), the colourless system will turn blue, indicating that leuco-methyleneblue has given up H to fumarate.

It is very important to bear in mind this two-way activity of „dehydrogenases“, for not all enzymes of this group have the function of splitting off H from their substrate. Some of them have the function of making their substrate take up H, and some of them again, as will be shown later, have the function of making their substrate take up and give off H alternately and by so doing act as catalytic H transmitters.

At the time I started my studies on oxidation some fifteen years ago, lactic acid was considered to be the main fuel

of the muscle and so I started studying the oxidation of lactic acid. I could convince myself that apart from dehydrogenases, water-soluble, thermostable substances were involved in this oxidation. Many years later I took up this problem again. Led by the study of the poisonous action of arsenious acid (9, 11), I could show in collaboration with *Banga* (14), that the lactic-dehydrogenase had a co-enzyme, which acted as co-dehydrogenase for several other dehydrogenases as well (*Banga*, *Laki*, *Szent-Györgyi* (19), *Göksi* (22). Lactico-dehydrogenase being identical with malico-dehydrogenase (*N. B. Das*) (36) this coenzyme is also the coenzyme of malic acid dehydrogenation.

We were able to show that this co-enzyme belonged to the group of nucleotides (16, 20), without being identical with adenylic acid (14) or adenylyl pyrophosphate (15). At the time of our studies *Euler* and *Nilsson* had already shown that *Euler's* cozymase also activated certain dehydrogenases. Today we know that my lactic co-dehydrase is in fact identical with *Euler's* cozymase.

The whole problem of co-dehydrogenases entered a new phase when *Warburg* showed that his co-ferment I and II (the former being no other than cozymase) contained a pyridine base, nicotinic acid amide, which could take up and give off two H atoms, by means of one of its double bonds.

Without co-dehydrogenase the dehydrogenase does not act at all. The donator, the co-enzyme and the dehydrogenase form one complex in which the H of the donator is shifted over to the co-enzyme, which in its turn gives this over to other acceptors.¹ These co-enzymes are substances of the greatest importance. In all probability they are also instrumental in the transference of energy. According to the latest report of *Meyer-*

¹ The same holds also for the reverse process. *Laki* (27) has shown that codehydrogenase catalyses the opposite reaction also when a substance like pyruvic acid is hydrogenated by the enzyme. In this case, the H necessary for the reduction of the substrate, (e. g. the reduction of pyruvic to lactic acid) is borrowed from the co-ferment. The co-ferment thus transmits H not only from the dehydrogenase to the other acceptors, but transmits H also from an outside source to the enzyme.

hof, the co-enzyme is capable of binding one molecule of phosphoric acid, while it is being reduced. This phosphoric acid is then transferred on to creatine. The splitting of one mol. of creatinephosphate yields 11,000 cal. The energy needed for different activities of the cell can be covered by splitting creatinephosphate. In this way the energy liberated in the dehydrogenation of donators by dehydrogenases is conserved by means of the co-dehydrogenase and made available for cellular functions.

3. The Missing Link.

According to the united theory of *Wieland* and *Warburg*, activated O_2 oxidises activated H. *Keilin* has fitted cytochrome into this system. Cytochrome is oxidised by its oxidase, and oxidises in its turn the activated H of the donator. This mechanism can be clearly demonstrated on the classical example of dehydrogenases, the dehydrogenase of succinic acid.

My early studies on dehydrogenation, however, convinced me that the same did not hold for lactic acid (5). The activated H of lactic acid could reduce dyes, but could not reduce O_2 (cytochrome). My growing experience taught me later that the same was true for several other dehydrogenases and the succinic acid dehydrogenase was unique in its immediate connection with cytochrome.

Ogston and *Green*² studied this problem systematically. They showed that from the eleven donators examined, only succinic acid reduced cytochrome. These dehydrogenases can reduce any odd dye or quinol, but not cytochrome, their natural oxidiser.

It became evident that some important link between dehydrogenases and cytochrome was missing in the system of oxidation and succino-dehydrogenase was evidently an exception. This missing link had to be some sort of a H carrier, which transmitted H from the dehydrogenase to cytochrome (6).

But had this succino-dehydrase nothing to do with the missing link and was not succinic acid the substance trans-

² *F. I. Ogston and D. E. Green, Biochem. J. 29. 1933, 2005, 1935.*

mitting H from other dehydrogenases to cytochrome? Experience has shown that succinic acid, activated by succino-dehydrogenase, can give its H to cytochrome. In fact it is the only substance which can do so, and moreover, it can do so at an enormous rate. It can give over more H to cytochrome, than is ever mobilised in the total respiration. If we add succinate to the tissue, more Oxygen will be taken up than the tissue ever consumes in normal respiration. Supposing that the H mobilised in respiration could be transmitted to fumaric acid fast enough, and would reduce it to succinate, mere traces of this dicarboxylic acid would be sufficient to carry the whole respiration and transmit its whole H to cytochrome. The dehydrogenation of succinic acid is a reaction of zero order. That is to say that the smallest quantities of this acid will be dehydrogenated at a maximum rate. A very

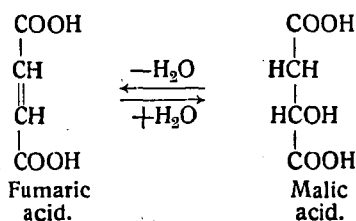


Fig. 2.

active succino-dehydrogenase is present in all animal tissues hitherto examined and is present also in yeast and bacteria.

The substance which had such an exceptional dehydrogenase, also had to have an exceptional function and importance.

Succino-dehydrogenase is not the only enzyme which indicates a special function of C_4 dicarboxylic acids. Equally unique and unintelligible is the function of another enzyme acting on these acids: fumarase. This enzyme, discovered by *H. Einbeck*, makes fumaric acid take up one molecule of water and become malic acid. It also makes malic give off water. (Fig. 2.) If either one of these two acids is added, it is turned into an equilibrium mixture of both substances. ($\text{F/M} = 1/3$). This enzyme is equally ubiquitous as succino-dehydrogenase and its activity is paralleled only by catalase.

It is equally hard to believe, that these enzymes, succino-

dehydrogenase and fumarase should be able to act on the C_4 dicarboxylic acids only by accident or that substances, which have such powerful enzymes should have no special importance. The C_4 dicarboxylic acids are not known to be substances of first rate metabolic importance and to lie on the route of the intermediary metabolism of one of our principal foodstuffs. Theories supposing such a rôle have no experimental foundation.

In the subsequent chapters I will occupy myself a great deal with these four C atoms containing dicarboxylic acids. I will use for them the short symbol of C_4 .

4. The Succinate-Fumarate Theory.

The theory suggested itself that succinate, together with its dehydrogenase, acts as catalytic H transmitter between the

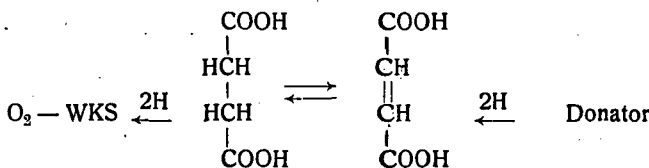


Fig. 3.

Warburg-Keilin System (WKS) and other dehydrogenases. This theory is represented in Fig. 3.

According to this conception cytochrome oxidises only the succinic acid activated by its dehydrogenase directly, while the foodstuff gives up its H only to the fumaric acid, formed by the oxidation of succinic acid, and activated on the same enzyme.

To test this theory one could try to find out what added fumaric acid would do to respiration, or how the specific inactivation of the succino-dehydrogenase would affect O_2 uptake.

That fumaric acid, added to the tissue, increases respiration, was known from *Thunberg's* experiments twenty five years ago and has been corroborated since by *Grönwall* and several other workers. This oxygen uptake was explained by the fact that the C_4 are oxidised by tissue and used as fuel. *Görsy* (18) and myself, however, showed that this explanation

could not be correct, at least not if only small quantities of fumarate were added. Fumaric acid greatly increased respiration, but could be recovered unchanged after the experiment.

I. Banga (27) made the interesting observation that it was not exactly on „increase“ of respiration we observed, but was rather a „stabilisation“ of the respiration.

It is known that animal tissues, like the pigeon breast muscle, if suspended in physiological solutions such as phosphate or Ringer-Phosphate, show a vigorous respiration at first. The O_2 uptake, however, very soon begins to fall off, and becomes reduced to small values in the later period of the experiment. The first curve on the left in Fig. 4. shows the oxygen uptake of 0,5 g. muscle, suspended in phosphate.

If a small quantity of fumarate is added to the system, (second curve), this decrease in oxygen uptake will not be observed, and respiration goes on undisturbed for a long period. The measurement of the respiratory quotient shows that this respiration, stabilised by fumaric acid, is a normal one. The RQ. of intact muscle is round unity. The falling off of respiration in the respirometer goes hand in hand with a fall of RQ., which decreases to 0,8. By the addition of small quantities of fumaric acid the RQ. again goes up to unity. (*Stare and Baumann*,³ *Annau and Straub*) (33).

These results can be explained in a simple way. In the respirometer the minced tissue is always suspended in a relatively big volume of fluid. Now some of the C_4 present will diffuse out into the fluid, whereby the succino-dehydrogenase becomes desaturated and unable to exert its function. If fumaric acid is added from without, this loss is compensated, and respiration can go on.

Muscle contains about 10 mg. % C_4 and if it contains 50 % free water, this makes 20 mg. %. The experiment shows that this is just about the concentration in which fumaric acid, if added to the outside fluid, would maintain maximal respiration in the respirometer. The normal concentration of C_4 in the tissue is thus the concentration at which the enzyme is saturated.

³ *F. I. Stare and G. A. Baumann*. Proc. Roy. Soc. Ser. B. 121, 338, 1936.

These experiments seem to be interesting not only as evidence of the catalytic function of C_4 , but also as an explanation of the behaviour of minced tissues in the respirometer. They show that it is the loss of C_4 which causes the rapid falling off of respiration. The failing respiration in its turn induces irreversible damage to the tissue, the respiration of which can never be restored again if fumarate is added to the tissue at later periods of the experiment.

*J. H. Quastel's*⁴ and his collaborator's experiments have also opened the way for us to find out what respiration does, if the succino-dehydrogenase is inactivated.

If the succino-dehydrogenase is a member of the chain of biological oxidation, its poisoning must entail a corresponding decrease in respiration. With regard to the low normal concentration of C_4 in the tissue, even small quantities of malonate should have such an effect.

The authors mentioned have shown that the succinodehydrogenase could be poisoned in a specific way by malonic acid. This C_3 dicarboxylic acid, owing to its close relation to succinate is also absorbed by the succino-dehydrogenase. The affinity of malonate to the enzyme is even somewhat greater than that of succinate.

So if both substances are offered simultaneously to the same enzyme, malonate competes successfully with succinate and inhibits its oxidation.

The last curve on Fig. 4. shows the effect of 4 mg. of malonate on respiration (Vol. 4 ml) which is depressed by this poison in much the same way as by cyanide.

Our experiments with fumarate and malonate were repeated by *Boyland* and *Boyland*,⁵ *Greville*,⁶ *Stare* and *Baumann*,⁷ and were corroborated. *Stare* and *Baumann* were

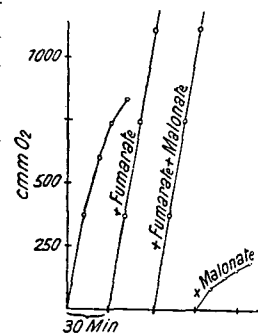


Fig. 4.

⁴ *J. H. Quastel*, *Biochem. J.* 20, 166, 1926; *Qu.* and *Wooldridge*, *Ibid.* 22, 689, 1928.

⁵ *Boyland* and *Boyland*, *Biochem. J.* 30, 224, 1936.

⁶ *G. D. Greville*, *Ibid.* 30, 877, 1936.

⁷ *s. p.* 20.

also able to convince themselves of the „stabilising effect“ and catalytic nature of fumaric acid.

5. The Malate-Oxaloacetate Theory.

The succinic-fumaric theory was thus strongly supported by the experiment. At the same time, however, it had one grave shortcoming: it did not explain the meaning of fumarase. Furthermore there was one experiment, which seemed to prove distinctly that the theory was wrong. This experiment was as follows: malonate was added to the tissue, and so the respiration ceased, owing to the poisoning of the succino-dehydrogenase. If to this system some fumaric acid was simultaneously given, the respiration went on, in spite of the presence of malonate. (Curve 3 in Fig. 4.). Later *N. B. Das* (37) showed that this experiment did not disprove the succinate-fumarate theory, but at that time I thought it did.⁸ Not loosing faith in the C_4 however, I wondered whether these acids could not transmit H by a change between two other forms, corresponding to a higher degree of oxidation. This problem involved a very great deal of labour. *Banga, Laki* (27) carefully studied all the possible oxidation products of succinate

⁸ The explanation is this: malonate inhibits the adsorption of succinate on the succino-dehydrogenase very strongly. It inhibits fumaric acid much less in its adsorption, since fumarate has a greater affinity to the enzymes. Thus malonate is unable to compete with the added fumarate, the quantity of which is mostly very great compared with the quantity of the C_4 originally present in the tissue. The adsorbed fumarate molecules take up the H coming from the donator and transmit the same to cytochrome exerting hereby their catalytic function unhampered by malonate. Here and there a molecule of the C_4 will be left loose by the enzyme at the moment it holds the two H atoms, and is thus present as succinate. The readsorption and reoxidation of this succinate will be inhibited by malonate. In this way the whole added fumarate will be stabilized in presence of malonate in the form of succinate and respiration will stop. This transformation of fumarate into succinate is a slow process. (27) The quantity of C_4 originally present in tissue, however, is very small. Added malonate will compete more successfully with this small quantity of C_4 and will convert it into succinate in a very short time. So if no fumarate is added to the tissue, the inhibiting action of malonate will be immediate.

in regard to their oxidation, reduction and redox potentials. In order to investigate this problem, specific micro methods had to be worked out, a very hard task. I will come back to these later.

This work clearly showed, that if the C_4 were involved in transport by some more highly oxidised form, this could be no other than malate-oxaloacetate. In this case, instead of fumarate, oxalacetate had to be the acceptor of H, while the malate formed on reduction of oxaloacetate had to be reoxidised by the WKS. This theory is summed up in Fig. 5.

It is natural, that if these reactions, the oxidation of malate and the reduction of oxaloacetate were a link in respiration, both these reactions should be very fast and should be able to proceed at a rate equal to the rate of the total respiration.

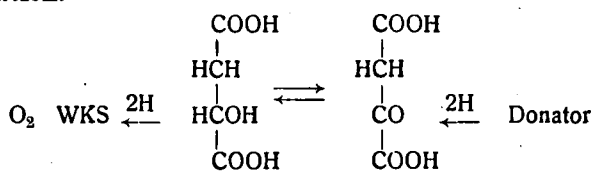


Fig. 5.

The muscle was known to contain a very powerful dehydrogenase of malate.⁹ *Laki* (27) found that the kinetics of this enzyme were such as to allow us to suppose that the enzyme could transmit the whole mobilized H by means of the small concentration of malate present in the tissue. The concentration-action curve is not quite as favourable as in the case of succinate, but for this the fumarase maintains a concentration of malate three times as high as that of fumarate.

Banga (28) showed by her „Abfang“-method, that on this enzyme malate was actually oxidised to oxaloacetate, and that the rate of this oxidation could be of the order of the rate of total respiration. Equally, oxaloacetate, added to the tissue, was reduced at a strikingly fast rate. The minced muscle could

⁹ There was some doubt as to whether this enzyme acted on malate, or on fumarate. *D. E. Green* held the former view, *Laki* and myself the latter. In the end we had to give in having found definite evidence for *Green's* contention. (38).

reduce about twice or thrice as much oxaloacetate as would be necessary to carry the whole respiration. This reduction of oxaloacetate is such a striking phenomenon that it is impossible to suppose that it has no physiological meaning. It is one of the basic facts which any theory of respiration has to explain before it can be accepted.

Banga (28) has shown also, that oxaloacetate, added to the tissue is reduced by the same H which is burned in respiration. Thus the reduction of oxaloacetate allows us to measure a new magnitude: the *quantity* of H activated by the tissue.

According to the theory presented here, the H of the foodstuff is transferred first to oxaloacetate. Now if we incubate the tissue in presence of an excess of oxaloacetate, and measure the reduction of the latter, we use the physiological acceptor and actually measure the quantity of H activated and mobilised from foodstuff in respiration, provided that the activation of oxaloacetate and the transfer of H from donator to oxaloacetate can keep pace with the H mobilisation. In *Thunberg's* experiments, working with dyes as artificial H acceptors, we do not measure the quantity of H activated, for the quantity of dyestuff reduced in these experiments is very small compared with the O_2 taken up by the same tissue in respiration. Oxaloacetate actually represents *the* physiological H acceptor, and its quantity reduced is actually a measure of the quantity of H activated.

6. The United Theory.

Both the alternative theories, outlined in the two previous chapters, had much in their favour but both had also their weaknesses. There were three fundamental facts to explain: the existence of the succino-dehydrogenase, the existence of fumarase, and the rapid reduction of oxaloacetate. The succinate-fumarate theory explained only the first, the malate-oxaloacetate only the last, while the meaning of fumarase remained unexplained by both theories.

Thus the problem suggested itself, whether both theories were not correct and the H transport was actually effected over both steps, as summed up in Fig. 6. In this double theory the H of the donator is taken over by oxaloacetate, which latter hereby becomes malate. Both these substances are activated on the malico-dehydrogenase. The malate gives over its

newly acquired two H-s to fumaric acid, activated on the „succino-dehydrogenase“. The succinate hereby formed is the substance acted on by cytochrome. This united theory would not only give the explanation of the two facts explained by the single theories, but would also give an adequate explanation for the existence of the fumarase, the function of which would be to maintain the right proportion between the different forms of C_4 .

The first question is whether the theory is thermo-dynamically possible. In this special case the problem was, whether it was possible for malic acid to give over H to fumaric acid, in other words, whether the system malate-oxaloacetate would reduce the system fumarate-succinate. The redox potential of the succinate-fumarate system was known, ($E'_0 = -0,018$ V;

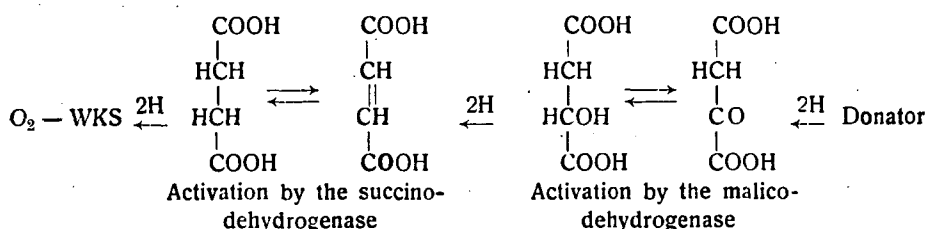


Fig. 6.

pH 7.2, 37°C) the potential of the system malate-oxaloacetate had to be measured. The difficulties of this measurement are described in *Laki's* (39) paper in which this potential is given ($E'_0 = -0,169 \pm 0,009$ V; pH 7, 37°C).

These potentials show that the process is possible and *Green*¹⁰ showed that such an oxido-reduction between both systems actually occur if the necessary electroactive H transmitters are present.

After having shown that the process can occur, the question as to whether it really does occur had to be answered. *F. B. Straub* (43) working with cytochrome, demonstrated that it actually did occur and even malate reduced cytochrome only *via* fumarate-succinate. The transfer of H from malate to fumarate could be demonstrated also by means of dyes. *Straub* showed that the H coming from the donator could reach

¹⁰ *D. E. Green. Biochem. J. 30, 2095, 1936.*

cytochrome only by this double system of H transfer. He found the same to be true also for dyes, applied as acceptors in place of cytochrome. *Banga* (44) showed the same was true also for systems composed of purified enzymes and known donators. I will speak of these experiments of *Straub* and *Banga* later more in detail.

7. Remarks on H Transfer.

On the first approach I pictured the succino- and malico-dehydrogenases as transmitting H by oxidising and reducing the C_4 store of the tissue alternately. This picture, however, is wrong. *I. Banga* (44) washed her enzyme preparations repeatedly with a great excess of water. They could still transmit H from the donator to cytochrome. The enzymes retained the traces of C_4 , necessary for their function with great tenacity and had to be dialyzed for hours, in order to be freed from C_4 . Without these acids the enzymes naturally transmitted no more H. They could transmit H again only if they were supplied with a trace of these C_4 .

The enzymes thus do not transmit H by oxidising and reducing a certain quantity of C_4 , but exert their function by firmly holding single molecules of C_4 in an activated condition. They just hold these molecules, and hold them in such a way, that they should be able to give off their H and temporarily bind new H atoms, if such are coming from the donator. The function of these enzymes is not to oxidise succinate or malate, but to transfer H. Accordingly they are not dehydrogenases at all, but are H transportases. These H transportases consist, as most other enzymes, of a protein and a prosthetic group. This latter is no other than our C_4 which rather answers to the definition of a prosthetic group than to that of a substrate.

By „activated“ succinate or malate we mean, that it is neither exactly succinate nor malate we find on the enzymic surface. The „activated“ fumarate is a structure of 4 C atoms, which is capable of binding temporarily two H atoms, thus having two more or less free valencies. Probably it can bind and transmit H atoms also one by one. (Cytochrome as oxidising

agent is monovalent). If we split off this prosthetic C_4 , its two free valencies close as a double bond and we get fumarate. If we split it off at the moment it holds two H atoms, the resulting substance will be succinate. Probably the dehydrogenated malate, activated on the enzyme, is rather oxyfumarate than ocaloacetate. Oxyfumarate is very unstable in the free condition and rearranges at neutral reaction into oxaloacetate.

Accordingly the theory of H transfer could be represented better by Fig. 7 than Fig. 6. The black semicircles represent the protein.

It is quite a different proposition to ask, what happens if we suddenly place the succino-dehydrogenase in a strong solution of succinate. In this case a rapid oxidation of the succinate ensues, the excess of this substance pushing off the fumarate molecules from the enzyme. This, however, is

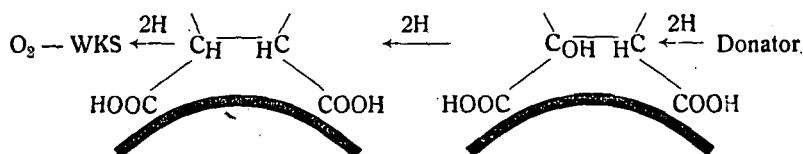


Fig. 7.

an artificial experiment, and has little to do with physiological conditions and functions.

The reader will probably discover a contradiction in my statements. I showed before, that even suspending the tissue in fluid removes enough C_4 to interfere with respiration. In this chapter I have stated, that even repeated washing does not remove these substances. The contradiction is only an apparent one and all depends on quantitative relations. The H transportases are just saturated at the normal concentration of 10 mg. % of C_4 . The whole of this saturation is needed to transport the great quantity of H mobilized in respiration, and the O_2 uptake will fall, as soon as we remove part of this C_4 . The quantity of C_4 retained after washing, however, will still be sufficient to transport smaller quantities of H which are oxidised in experiments with purified enzymes or which are necessary for the reduction of the dyes, added in Thunberg experiments.

Certainly, the C_4 can be washed out to some extent. That they are retained at all, shows how firmly they are bound by their enzymes.

8. The Donator, its Activator and the Yellow Enzyme.

If we mince the breast muscle of the pigeon, quickly extract it with water, and press it out through muslin, we get a turbid fluid. In the respirometer this fluid shows a vigorous respiration, almost equal to that of the whole muscle. The turbidity of this fluid is due to microscopic granulae which can be separated on the centrifuge. These granulae contain the *Warburg-Keilin-System*, the succino- and the malico-dehydrogenases. I will call these granulae, separated on the centrifuge, just „enzyme“. The remaining clear fluid I will just call „fluid“. Neither the enzyme nor the fluid show any considerable oxygen uptake, by mixing them, however, we restore the original respiration. According to Fig. 6 and 7, the „fluid“ should contain, as necessary factors, only the donator and the co-enzymes. If, however, we boil up the fluid and add it to the enzyme in a boiled condition, it is unable to restore respiration, though donator and co-enzymes are not destroyed by boiling. The fluid thus contains a thermolabile substance which is necessary to complete the system. *Banga* (42) has shown this substance to be of protein nature and has given methodes for its preparation. I will briefly call it „activator“. A few mg. of this activator added to the enzyme plus the boiled juice, will restore respiration. If, instead of the boiled juice we add co-enzyme to this system, the only missing member will be the donator. This enabled us to isolate the donator. We had only to find out which constituent of the boiled juice is necessary to complete the system and induce respiration, which work was done by *Banga*. (42). She found that this donator is no other than hexose-phosphate, hexose-mono, and di-phosphate.

This naturally does not mean that hexose-phosphates are dehydrogenated as such. After *Laki's* (28), *Gözszy's* (22), and *G. Moruzzi's*¹¹ experiments the donator is actually a triose-phosphate, into which the hexosephosphate is split, prior to its dehydrogenation.

Enzyme, co-enzymes, hexose-phosphate and the activator

¹¹ *G. Moruzzi*. Arch. di scienze biol. 22. 1, 1936.

will give a system, which will take up fair amounts of oxygen especially if we add a trace of fumaric acid. In the presence of the „activator“ the hexose-phosphate, (split into triose), will give its H to oxaloacetic acid, which transmits it over the C₄ system to Oxygen. Without „activator“ the hexose- or triose-phosphate does not give up its H. Thus the probable function of the „activator“ is to act as dehydrogenase of the zymophosphate. *D. C. Harrison*¹² has described a hexose-diphosphate dehydrogenase, which had similar properties to our „activator“. The „activator“ requires for its function cozymase (*Warburg's* coferment 1), as shown by *F. B. Straub* (unpublished).

This system, composed of „enzyme“, co-enzymes, fumaric acid, donator and activator, takes up oxygen fairly vigorously, showing that the system is almost complete. It is not quite complete though, for the oxygen uptake does not usually reach values obtained in the normal respiration. A glance at Fig. 6 and 7 reveals where the last member of the chain is probably missing. Experience showed that dehydrogenases are unable to interact, especially if they are parts of the same solid structure. They need some electro-active substance to connect them, that is, to take away hydrogen from the one and give it to the other. This condition is fulfilled between cytochrome-oxydase and succino-dehydrogenase. Both these enzymes are part of the solid structure of the granulae. They are connected by cytochrome, which is an electroactive substance.

So *a priori* it seems improbable that succino and malico-dehydrogenase should be able to interact. *Green*¹³ has shown that they cannot. If they actually did so to any extent in our experiments, this could only be due to some electroactive substance present as impurity in our „enzyme“.

The electroactive substance, connecting succino- and malico-dehydrogenases is no other than the „yellow enzyme“ described by *Warburg* and *Christian*. The oxygen uptake of our system was due to the varying quantities of this substance retained by our „enzyme“ after washing, its last traces being very difficult to remove.

¹² *D. C. Harrison*. *Biochem. J.* 21, 1011, 1931.

¹³ s. p. 25.

Working in the purification of the lactico co-dehydrogenase, *Banga* (14, 24) and myself discovered a yellow dye with striking properties, the most fascinating of which was to give a chemically reversible redox system. It could be oxidised and reduced alternately any number of times. This clearly indicated that the substance was involved in biological oxidation. Its quantity was also found to be proportional to the intensity of respiration of different tissues. Its ubiquitous nature in animal tissues and presence in milk indicated a general importance. We were unable to tell, what its exact function was and were unable to fit it into the oxidative mechanism. So for the time being we just described it, called it „Flave“ (e. g. Cytoflave, if from tissues) and left it at that. (14, 24).

The reason for our inability to demonstrate the actual function of this substance was revealed later by *Warburg*, who showed that this substance was present in the tissues bound to a protein. This flaveprotein complex answered to the definition of an enzyme, so *Warburg* called it the „yellow enzyme“. Later *R. Kuhn*, who with his collaborators *György* and *Wagner-Jauregg*, isolated and analysed this dye, called it „flavine“. This is not a better name than „flave“ because it leads to confusion with the vegetable „flavones“.

According to its redox potential this yellow enzyme fits into our system between the malico- and succino-dehydrogenase. Its potential is, according to *Kuhn* and *Boulanger*¹⁴ in the middle, between the potential of these two systems. ($E = -0.060$ V, pH 7, 38° C). The yellow enzyme is reduced by the malico-dehydrogenase, and as *Laki* (41) showed, is oxidised by fumarate, activated on the succino-dehydrogenase. In other words this means that the yellow enzyme is capable of taking up hydrogen from the malico-dehydrogenase and is able to give this hydrogen over to the succino-dehydrogenase, connecting hereby the two enzymes.

One of the most striking properties of this flave is its ability to form a free radical, that is to say to be able to transfer hydrogen atoms one by one.

¹⁴ *Kuhn* and *Boulanger*. Ber. 69, 1557, 1936.

With the introduction of this yellow enzyme, the system is complete. It takes up oxygen vigorously, which oxygen uptake is equal to that of intensively respiring tissues.

The whole theory presented in these chapters is summed up in Fig. 8. The circle means a granulum of the muscle cell. A similar granular structure is found in liver and kidney. The figure is somewhat simplified by not writing the three cytochromes in series.¹⁵

9. Remarks on Dehydrogenases.

I have shown in a previous chapter that „succino-dehydrogenase“ and „malico-dehydrogenase“ are not „dehydrogenases“ at all. They are, according to their function „hydrogen-trans-portases“ and will act as „dehydrogenases“ only under artificial conditions. It would be better if they were called accordingly, but I do not propose to use new names, for it does not matter what we call them, as long as we know what we mean.

I am afraid that the observations presented will necessitate subjecting our ideas about dehydrogenases to a further revision.

Our knowledge of dehydrogenases is mostly founded on *Thunberg's* experiment, which I have outlined on page 14. The discolouration of the dye was always taken as an indication of the presence of a dehydrogenase, inter-acting with its substrate. The reaction was explained as follows: the H of the donator becomes activated by the dehydrogenase and, owing to its redox potential reduces the dye, so that from the redox potential of the dye we have even drawn conclusions on the redox potential of the donator.

¹⁵ Sometimes students find it hard to believe the first reaction on the left. They object that O_2 must go somewhere since it cannot dissolve into 4 electrons. It is easiest to picture this reaction as O_2 oxidising four H ions into water. The remaining charge of the ions serves to charge up the Fe^{++} of cytochrome into Fe^{+++} . The OH ions, formerly in equilibrium with oxidised H ions, serve as the additional anions of the new, third charge of Fe^{+++} . The H of succinic acid can be thought of as reducing this OH ion to water, its charge reducing the third positive charge of the Fe^{+++} .

The discovery of the mechanism of the action of co-dehydrogenases did not cause a very profound change in these views. By „dehydrogenase“ we have to mean henceforth, a complex of the enzyme and the co-enzyme, as far as a co-enzyme is involved in that particular dehydrogenation.

Straub (43) and *Banga* (44) have shown that if cytochrome is reduced on an enzyme it is always reduced directly by the succinate present, whatever the final donator may be. If we take as example one of the most important dehydrogenation processes, the reduction of cytochrome by hexose-

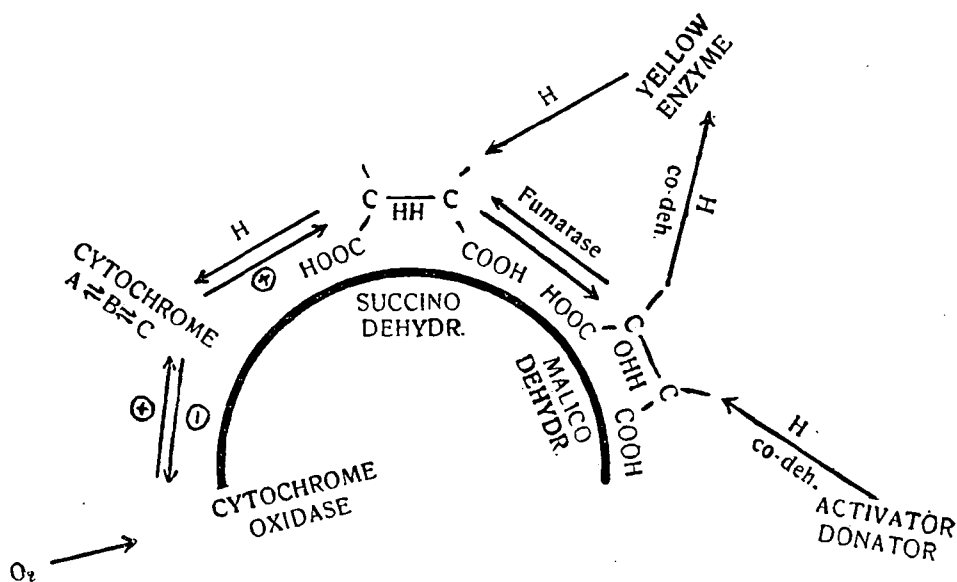


Fig. 8.

phosphate, the mechanism of the reaction will be the following: hexose-phosphate will reduce oxaloacetate, present in traces on our washed enzyme, to malate. Malate again will reduce the yellow enzyme. This on its turn will reduce the fumarate, also present in mere traces. It will be this succinate, formed by reduction of fumarate, which will reduce cytochrome. The H of hexose-phosphate will be used directly only to re-reduce the C_4 , which act as H trans-

mitters and would, by themselves, be unable to reduce the cytochrome. In this system, containing apparently but one donator, hexose-phosphate, there is a whole series of intermediary H donators: the most negative is hexose-phosphate, then comes malate and yellow enzyme. The most positive is succinate. If the cytochrome were reduced by these donators according to their potentials, they should reduce cytochrome in the order in which they have been mentioned. The fact is, however, that nature seems to neglect our knowledge of redox potentials and reduces cytochrome only by succinate.

Straub and *Banga* have shown that the same holds more or less also for dyes applied instead of cytochrome in *Thunberg's* experiment. If we add methylene blue and hexose-phosphate to our enzymic system (completed with co-enzymes), we observe a rapid decolouration. The current explanation of this would be that the zymophosphate was activated and — owing to its potential — had reduced methylene blue. But in this case also the dye is reduced by the C_4 , and not by the zymophosphate. This latter only serves to re-reduce the C_4 acids retained by the enzyme in minute quantity, and insufficient to reduce the dye by themselves. This could be shown quite simply by *Banga*, who subjected her enzymic preparation to dialysis, prior to the experiment. After the C_4 were removed from the enzyme by dialysis, the system containing hexose-diphosphate reduced methylene blue no more, or did so only at a very low rate. If traces of fumaric (0,2 mg. %) acids were added, the original conditions were restored and the dye was reduced again very fast.

The mechanism of the reduction of the dye is thus quite analogous to the reduction of cytochrome. The situation in the case of dyes is different from the reduction of cytochrome in only one respect. Cytochrome is reduced only by succinate, while the dye is reduced by malate, as well as by succinate. For if we inhibit the succino-dehydrogenase by malonate, or apply a more negative dye which cannot be reduced by succinate any more but can still be reduced by malic acid, we obtain a reduction, even if this reduction is considerably slower. Contrary to cytochrome, dyes can also be reduced by the malico-dehydrogenase. Whether this reduction is directly effected

by the malico-dehydrogenase or by the yellow enzyme or by both, has not yet been decided.¹⁶

The activator of hexose-phosphate is a real dehydrogenase, for it dehydrogenates the „donator“. All the same it does not reduce dyes, or does so only very slowly. The succino and malico-dehydrogenases are not „dehydrogenases“ but „transportases“. They reduce the dye, and the reduction given by zymophosphate is in fact only a reduction by the C_4 .

If one were to make generalisations from this experiment, one would conclude that the reduction of a dye means that the enzyme in question is not a dehydrogenase but a H transportase, and that a real dehydrogenase is characterised by its inability to reduce dyes. Generalisations are mostly wrong, but all the same it is clear that our earlier ideas are not in entire agreement with our observations.

Even if dyes are employed as acceptors, the H mobilised from foodstuffs tends to follow its physiological route over the C_4 acids, and reaches the dye easiest over succinate by the detour over the complete C_4 system.

This explains why methylene blue and dyes with a similar potential are most suited as indicators for the *Thunberg* experiment. If dyes with a more negative character are employed, which can be reduced by succinate no more, but can still be reduced by malate, the times of reduction become considerably longer. If we employ dyes which are still more negative and which, according to their potential, should be capable of being reduced by the „donator“ but not by malic acid, practically no decolouration will be obtained, this in spite of the fact that a small difference in potential is sufficient to make a reaction between two systems possible. Methylene blue is only more positive than succinate by a few millivolts, but all the same the reduction takes place at a rapid rate.

One very nice point worth mentioning in *Straub's* and

¹⁶ The potential of dyes reduced by the malico-dehydrase would indicate a direct action of the dehydrogenase. This is, however, not conclusive, for *F. G. Fisher* and *Eysenbach* have shown that the yellow enzyme can reduce systems more negative than would correspond to its normal potential. (*Liebig's Ann.* 530, 99, 1937).

Banga's experiments is, that in the case of more negative dyes the succino-dehydrogenase not only does not enhance reduction, but it inhibits it. Accordingly malonate will give an acceleration instead of an inhibition. The explanation is simple. The succinate is unable to reduce dyes if they are appreciably more negative than the succinate itself. In this case however, fumarate can act as an oxidising agent and re-oxidises the dye, reduced by the malico-dehydrogenase.

This remarkable paradoxical behaviour of malonate, inhibiting the reduction of one dye and accelerating the other, is clear evidence for the correctness of our explanations. At the same time it proves that malonate is a quite specific poison of the succino-dehydrogenase and its inhibiting effect is solely due to the poisoning of this enzyme.

Straub showed that the observations made by *Banga* on purified enzymic preparations also hold for the whole muscle. Here also, the H mobilised in respiration reaches the dye most easily by way of the double C_4 system. If the succino-dehydrogenase is cut out as transmitter, the H still reaches the dye over the malico-dehydrogenase but with some difficulty. If this enzyme is also excluded as transmitter (by the choice of a dye which is too negative to be reduced by malate) the reduction stops altogether. This explains, why methylene blue and dyes with a similar potential have the strongest effect in accelerating the O_2 uptake of tissues. More positive dyes will not reoxidise, more negative ones will not be reduced fast enough, succinate being excluded as H transmitter.

10. Other Tissues and Donators.

The C_4 theory was worked out on the minced breast muscle of the pigeon. Most of the observations hitherto presented, relate to this material. The respiratory quotient of this tissue is about unity, which shows that its fuel is carbohydrate.

The problem presents itself as to how far the results obtained on muscle in the oxidation of carbohydrate apply also to other tissues and other donators.

As far as experiment goes, it indicates that the C_4 system

plays an important rôle in the respiration of other organs and in the oxidation of other donators as well.

F. J. Stare (30) has repeated our basic experiments on minced kidney and liver. The addition of fumarate and malate produced analogous effects to those obtained on muscle.

According to the experience of my laboratory, the effect of fumarate and malonate are demonstrated less readily in liver and kidney than in muscle. This does not prove, however, that the C_4 system is of less importance in these tissues than in muscle, for slight shifts in the quantitative relation of the single members of the oxidative mechanism greatly influence the results of such experiments (see page 57). That the H is transferred also in liver and kidney from the donator to dyes by the C_4 system, is demonstrated by experiments of *Straub* (43).

Boyland and *Boyland*¹⁷ demonstrated that cancer tissue responds to the addition of fumarate and malonate in the same sense as muscle though the response is very small.

*Greville*¹⁸ showed that the respiration, increased by nitrophenols, is also carried by the C_4 system.

The ubiquitous nature of succino-dehydrogenase in organs of the higher animal distinctly shows that the system, in which this enzyme is involved, is equally widely distributed.

*F. I. Stare*¹⁹ has demonstrated that kidney and liver readily reduce oxaloacetate, proving that in these organs the malico-dehydrogenase plays an analogous rôle to that played in muscle.

It seems, however, that in certain tissues only half of the C_4 system is present. *Banga* has found that the three rapidly growing malignant tumours examined reduced practically no oxaloacetate or pyruvate. They contained a succino-dehydrogenase but no malico-dehydrogenase, (*F. L. Breusch*, unpublished). The whole process of H mobilisation in these tumours seemed to be very weak, judging by the slowness of the reduction of dyes (*Breusch*, unpublished).

What lends additional interest to these observations on

¹⁷ *Boyland* and *Boyland*. *Biochem. J.* 30, 224, 1936.

¹⁸ *G. D. Greville*. *Ibid.* 30, 877, 1936.

¹⁹ *F. I. Stare*. *Ibid.* 30, 2257, 1936.

malignant growth, is the observation of *A. Blazsó* (28). According to his experiments, corroborated by *Breusch*, the embryonic tissue behaves much in the same way as the rapidly growing malignant tissue. It reduces no oxaloacetate, though its respiration is fairly intense. Embryos contain a fairly active succino-dehydrogenase. The change into the adult type with a complete C_4 system occurs during the first two weeks of extrauterine life in the rat. The same holds for the cat. (*Breusch*, unpublished).

The C_4 system is also involved in the oxidation of substances other than zymophosphate. The reduction of methylene blue by lactate is inhibited by malonate, in presence of muscle enzyme. The aerobic oxidation of alcohol by liver is accelerated by fumarat, while that of citric acid by liver enzyme is inhibited by malonate. The decolouration of methylene blue by liver enzyme and aethyl-alcohol, is inhibited by malonate. (*Banga*, unpublished). *Annau* (28) has shown that the oxidation of pyruvic acid in liver is dependent on C_4 . In the oxidation of these different substances mentioned, the C_4 system is thus involved. It is not necessary to suppose that in these oxidations the whole C_4 system is needed. It is possible that certain donators, the potential of which is not negative enough to reduce oxaloacetic acid, reduce only fumarate by way of the yellow enzyme. This must be the case with lactic acid acting as donator. Lactic acid is activated on the same enzyme and has the same redox potential as malic acid and cannot be expected to reduce oxaloacetate. (*Laki* 21, 39).

The absence of malico-dehydrogenase in tumour and embryonic tissue, indicates that these tissues use as principal donator a substance which reduces fumarate but not oxaloacetate. This would explain the absence of half of the C_4 system from these tissues which possibly use lactic acid as donator.²⁰

²⁰ Experiments of *F. Breusch*, now in progress, suggest that this is in fact the case. Lactic acid added to the tumour suspension increases respiration and reduction of methylene blue. In other tissues lactic acid and malic acid are activated by one and the same enzyme. In tumour which has no malico-dehydrogenase, the enzyme seems to activate lactic acid only.

This substance is known to be produced by embryos and tumours.

The question of whether the C_4 system is involved in all oxidations, must be answered in the negative. *N. B. Das*²¹ has shown that it is not involved in oxidative de-amination of amino acids. Dehydrogenases, capable of reducing molecular O_2 like *Shardinger's* enzyme, xanthine oxidase, do not require the C_4 system for their activity.

²¹ *N. B. Das* The metabolism of amino acids. Diss. Szeged.

PART II.

Problems, Objections, Methods.



Introduction.

By the discovery of the catalytic function of C_4 dicarboxylic acids certain problems have become accessible to experimental solution. In the first four chapters of this part I will outline some of these problems and point out certain ways of approach rather than give their solutions. Thoughts have to be one step ahead of experiment, and I hope I shall be excused, if, after having given observations, I go one step further and indulge in speculation.

One chapter of this part I will devote to objections which can be or have actually been raised against my theory. I will do this not only in my defence but also because these objections raise problems of general interest.

In the last chapter I will say a few words and make a few critical remarks about the methods by which my results have been obtained.

1. On the Pasteur Reaction.

The problem of the quantitative relation of fermentation and oxidation has occupied biochemistry since *Pasteur's* observations. If oxidation is suppressed by the lack of oxygen, fermentation is enhanced. By increased oxidation fermentation is depressed. The mechanism of this inter-relation (the *Pasteur* Reaction) is not fully understood.

Meyerhof has shown that the lactic acid accumulated in the muscle under anaerobic conditions disappears if oxygen is readmitted, part of it being oxidised, the rest re-synthesised to carbohydrate. According to *Meyerhof* the energy needed for the resynthesis is covered by the oxidised lactic.

In this explanation of *Pasteur's* reaction the interdependence of fermentation and oxidation is only an apparent one. Fermentation proceeds in any case and oxidation removes the products of fermentation. Finding no lactic acid it seems as though there had been no fermentation at all.

The C_4 theory of respiration opens the possibility of another explanation of *Pasteur's* Reaction. To make this clear, we have to sum up the latest theory of fermentation, based on the work of *Dische*, *Parnas* and *Meyerhof*, which seems to represent the „Hauptweg“, the main route of fermentation. The essence of this theory is summed up in the middle and upper line of Fig. 9. It is this: sugar is split into two trioses. The triose is oxidised, giving off two H atoms, which are „accepted“ by a molecule of pyruvic acid. This pyruvic acid by taking up these 2 H is reduced to lactic acid. The triose itself, by the loss of two H atoms, turns into pyruvic acid, which will again take up two H atoms from another triose, and become lactic, while again a new pyruvic is formed and so it goes on till the whole sugar is turned into lactic acid. The central point of the process is the transfer of two H atoms from triose to pyruvic acid.

According to the contents of the first part of this book, hexose is split prior to oxidation also into trioses. These trioses are oxidised by loosing two H atoms, which H atoms are „accepted“ by oxaloacetic acid, while the triose itself turns into pyruvic acid. This is represented in the middle and lower line of Fig. 9.

The whole difference between fermentation and oxidation is that in the first process the 2H of the trioses are taken up by pyruvic acid, while in oxidation they are taken up by oxaloacetic acid.

The close relation of fermentation and oxidation will become still more apparent when we consider the chemical structure of both acceptors, pyruvic and oxaloacetic acid. It will be found that these substances are most closely related, oxaloacetic acid being but a „carboxy-pyruvic acid“ and malic acid but a „carboxy-lactic acid“.

Both pyruvic acid and oxaloacetic acid are activated as H acceptors. *N. B. Das* (36) has shown that they are both acti-

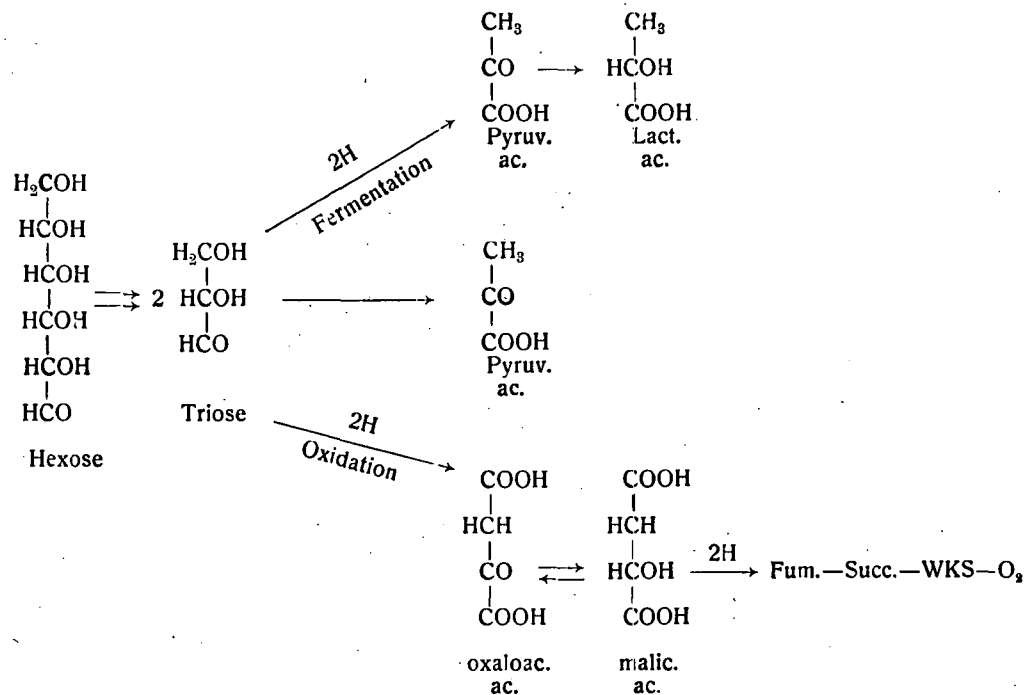


Fig. 9.

vated on one and the same enzyme. The so called „malico-dehydrase“ and „lactico-dehydrase“ are identical. Both acids are held by the enzyme, by their carboxylic group. (40). Oxaloacetic acid has two such handles, while pyruvic has only one. Accordingly, as *Das* (3) and *Laki* (40) find, the enzyme can hold oxaloacetic better, which has thus a greater affinity to the enzyme, than pyruvic, and will displace pyruvic on the enzymic surface. On the other hand pyruvic, with its CO group competes successfully with malic. So we can easily figure out what will happen if there is oxygen and what will happen if there is none. If there is oxygen, malate will be oxidised into oxaloacetic acid, which oxaloacetic acid will compete with pyruvic acid successfully and will take up the H given off by the triose. This is oxidation and in this case there will be no fermentation. If there is no oxygen, no oxaloacetic can be formed and pyruvic acid will be the sole acceptor available. Thus pyruvate will take up the H from triose. This is fermentation.

According to this picture fermentation and oxidation represent thus *alternate* ways of metabolism. And this in part is probably the explanation of *Pasteur's* effect.

This relation of fermentation and oxidation opens the way to speculation on a rather philosophical problem. Many biochemists agree that in the course of phylogenetic development the simpler process of fermentation has preceded that of oxidation, and one might wonder how Nature found its way from fermentation to oxidation. Up till now it looked as if both processes were entirely different and it was difficult to picture a discontinuous development in which, at the side of fermentation a new mechanism, that of oxidation was built.

The above considerations give a more satisfactory picture of this process. It has been shown that fermentation and oxidation are closely related processes, H being transferred in the former to pyruvic, in the latter to carboxy-pyruvic acid. To shift over from fermentation to oxidation nothing more was needed than to carboxylate pyruvic acid. By this carboxylation the molecule was taken out from the carbohydrate cycle and stabilised as a catalyst. At the same time the second carboxylic group changed also the affinity in the necessary way.

Older theories of fermentation, such as the theory of *Neuberg*, involve no oxido-reduction between triose and pyruvate. *Banga* (46) has given a nice demonstration of the relation between the two possible ways of fermentation. The enzyme responsible for the activation of pyruvate (and oxaloacetate) is bound to the microscopic granulation of the cell. This granulation can be separated from extracts on the centrifuge. The resulting clear liquid produce lactic acid unabated, but there will be no oxido-reduction between triose and pyruvate, this latter not being activated. Thus the activated and labilised triose re-arranges into lactate without the oxido-reduction. This way of lactic acid formation is apparently the more primitive one and is probably connected only with the formation of one molecule of creatine phosphate. This method of lactic fermentation is probably still used by rapidly growing malignant tumours and embryonic tissues which produce much lactic acid but are unable to activate pyruvate. If we try to arrange events phylogenetically, we would have to place this method of fermentation at the top of the list. This way of fermentation was probably completed later by the activation of pyruvic acid, which makes the oxido-reduction and therewith the formation of a second creatine-phosphate possible. The latest process is oxidation, in which pyruvic was replaced by carboxy-pyruvic (oxaloacetate). This process made an additional apparatus necessary which serves to free the reduced oxaloacetate again of its accepted H. This appendix, (in which probably one or two more phosphates are bound) is the succino-dehydrogenase-Warburg-Keilin-System.

This relation of fermentation and oxidation also answers the question why Nature has chosen the C_4 dicarboxylic acids as central catalyst of oxidation. But apart from this, there might be another additional reason. It is known that the α and β position to a carboxylic group, lends the C atom a special reactivity. Now there is only one group of substances which contain two unsubstituted C atoms, both α and β at the same time. This unique group is that of the C_4 dicarboxylic acids. The second COOH group thus not only stabilizes the molecule but at the same time gives it a special reactivity, increasing its affinity to the enzyme. This increased affinity enables the

traces of C_4 acids present in tissue to carry the whole respiration, the most intense function of our body.

Using different words for the same idea, we can also say that the $\alpha\beta$ double bond - as shown by *F. G. Fisher*,²² has a special reactivity and is hydrogenated by biological systems with special ease. The double bond of fumaric and oxyfumaric acids is $\alpha\beta$ to two carboxylic groups.

There is some reason to believe that if a substance is hydrogenated in the cell this hydrogenation always happens over an activated double bond. As mentioned before it is probably not oxalo-acetic which is reduced by the H of the donor, but oxyfumaric acid. Activated double bond means two free valencies.

There is one substance in the cell which shares this double $\alpha\beta$ position of a double bond: this is aconitic acid, which according to *Knoop* and *Martius*²³ is readily formed in the cell from citric acid. Most animal and vegetable cells contain, as generally known, a very powerful dehydrogenase for citric acid. Experiments of *F. L. Breusch* now in progress, suggest that citric acid with its dehydrogenase is also utilised by the cell as H transmitter analogous to malic acid. More work will be needed before definite statements can be made.

2. Energetics.

The object of the cell is not to oxidise but to liberate energy and one wonders how the energy is liberated in a form which can be used to support cellular activity. Energy turned into heat is more or less lost.

The close relation of oxidation with fermentation allows us partly to answer this question, partly to make certain guesses.

Fermentation is not quite as simple as represented in Fig. 9. Firstly it is not hexose that is fermented, but hexose-diphosphate. Accordingly it is not triose but triose-phosphate that is formed by its splitting. This triose-phosphate, after having lost two H atoms, does not turn immediately into pyruvic acid but first into 3 phospho-glyceric acid, which then turns into 2 ph-glyceric acid, this turns into phospho-

²² See *Liebigs Ann.* 530, 99, 1937.

²³ *F. Knoop. Z. physiol. Chem.* 246, I, 1937. *C. Martius. Ibid.* 247, 104, 1937.

enol-pyruvic-acid and only this last goes over into pyruvic, giving up its phosphate. Pyruvic acid, in the end, is reduced to lactic acid.

Lactic acid contains less energy than the triose-phosphate. There is thus a liberation of energy during this transformation. As we know, chiefly owing to the work of *Meyerhof* and his collaborators, the liberation of energy is concentrated round two points. The first is the oxido-reduction between triose and pyruvic acid, the second is the transformation of phospho-enol-pyruvic acid into pyruvic acid and phosphate.

The energy liberated in both of these reactions is used to synthetize one mol. of creatine-phosphate (*Meyerhof*, *Parnas*). The mechanism of the phosphorylation of creatine is different in these two reactions. When the phospho-enol-pyruvic acid turns into pyruvic acid, it is actually the phosphoric acid of the enol-pyruvic which is given over to creatine (*Parnas* reaction). In the phosphorylation connected with the oxido-reduction between triose-phosphate and pyruvic acid the phosphate must be taken from an outside source, from inorganic phosphate. In this phosphorylation cozymase has a leading rôle. As mentioned in chapter 1, the oxido-reduction is mediated by cozymase. According to *Warburg's* conception in this oxido-reduction the H of the triose is taken over and passed on to pyruvic acid by cozymase. According to the latest report of *Meyerhof*,²⁴ cozymase, while being reduced, is capable of binding one molecule of phosphoric acid. The energy liberated in the oxido-reduction is thus invested into the ester-link of phosphoric acid on cozymase which in its turn gives its phosphate over to creatine. *Gözszy* in unpublished experiments also found a few years ago that our lactic co-dehydrogenase (cozymase) is capable of binding one molecule of phosphate, when interacting with zymo-phosphate.

The actual mechanism of the phosphorylation and the rôle of adenylic acid and adenylyl-pyrophosphate played therein, are of secondary interest here and will not be touched in spite of the general importance of these substances which occupy a central position in the transference of phosphoric acid (*K. Lohmann*).

²⁴ *Meyerhof*. Naturwiss. 25, 443, 1937.

To sum up we can say that in the oxido-reduction between triose-phosphate and pyruvate one mol. creatine-phosphate is synthesised. The dehydrogenated triose-phosphate then turns into pyruvic acid and transmits its energy together with its phosphate to a second molecule of creatine.

In this form the energy invested into the ester-link of creatine-phosphate can leave the scene of fermentation and support different activities of the cell which need energy. By splitting one creatine-phosphate into creatine and phosphate 11,000 cal. are liberated. Thus by the synthesis of the two mol. creatine-phosphate 22,000 cal. are preserved, which is nearly equal to the total energy liberated in fermentation. The energy difference between triose and lactic acid is 25,000 cal.

As I have tried to show in the previous chapter, fermentation and oxidation are identical processes with the one difference, that in oxidation the oxido-reduction takes place between triose and oxaloacetate instead of triose and pyruvate. The mechanism, co-enzymes and enzymes of both oxido-reductions are the same. Thus we can expect that in the oxido-reduction between triose and oxalo-acetate one mol. creatine-phosphate is built up, just the same as in the oxido-reduction with pyruvate. Equally we can suppose that the dehydrogenated triose, while turning into pyruvate, transmits its energy and phosphate to a second molecule of creatine. This together is 22,000 cal. preserved. We can even go one step further and say that if the oxido-reduction between triose and oxaloacetate is connected with the formation of one creatine-phosphate, the oxido-reduction between malic acid and yellow enzyme, mediated by the same cozymase, is probably also connected with the synthesis of one creatine-phosphate. This gives us the probable reason why Nature slipped in one more oxido-reduction between malic acid and cytochrome, using its C_4 molecules once more in the form of succinate-fumarate. (It does not seem impossible that the yellow enzyme is also connected with phosphorylation, and that a further phosphate is bound by this enzyme during its oxidation or reduction).

In this way we can give partial answer to the question as to why Nature effects that very simple reaction $2H + O = H_2O$ piecemeal in so many steps. We can give a reason for the

existence of half of the chain, from the donator to succinate and tell with fair probability how the energy, liberated in this part of the oxidative mechanism, is preserved and transmitted to other functions.

We do not know how the energy liberated in the other half of the oxidative mechanism, in the reaction between O_2 , cytochrome and succinate, is preserved. Judging by redox-potentials, the quantity of this energy is very considerable, in fact the greater half of the total energy of oxidation must be liberated in this part of the system. In what form this energy is preserved and kept from turning into heat we do not know. It is an attractive idea to think that the granulae of the cell might work in an analogous manner to the chloroplasts and resynthesise lactic acid into sugar, using the energy of oxidation. This is, however, at the moment no more than speculation. The energetics of this part of the oxidative mechanism represent an urgent and fundamental problem.

It is difficult to believe that the energy liberated in this part of the mechanism should be thrown away as heat. All the same at the moment we cannot exclude this possibility for the energy of three molecules of creatine-phosphate is sufficient to answer for the 35 % efficiency of muscle.

The theory summed up in Fig. 8 also tells us how the oxidative energy of donators, other than hexose, is preserved and transferred to other processes. We have only to replace zymo-phosphate by other donators. If this donator reduces oxalo-acetate, two molecules of creatine-phosphate may be synthesised by the oxidative energy of dehydrogenation. If the donator only reduces fumarate (resp. the yellow enzyme), one molecule of creatine phosphate will be built up.

Before leaving this fascinating field of energetics I would like to point out that the theory summed up in Fig. 8. opens the possibility of certain experiments. If we inhibit the mobilisation of the donator but supply the system with sufficient quantity of succinic acid, we can find out how far the energy evolved between succinate and O_2 can be utilized for cellular activity. If, however, we cut out the *Wargurg-Keilin* system, let us say by cyanide but provide the system with oxaloacetic acid or fumaric acid we can find out how far the



energy liberated in the H transfer from donator to oxaloacetate or from malate to fumarate can be utilized to maintain cellular function. Advance in knowledge of such a complicated system as that of respiration often depends on the possibility of cutting the system to bits and bringing therewith certain parts of it to measurement.

3. Intermediary Metabolism.

According to Fig. 9 triose is oxidised both in fermentation and oxidation to pyruvic acid. In fermentation this pyruvic acid gets reduced to lactic acid. In oxidation no such reduction takes place and the question is: what happens to this pyruvic acid, the first oxidation product of sugar? Under normal conditions we find only very little or no pyruvic acid in the tissue, so we can safely conclude that the tissue is able to deal with pyruvic acid fairly rapidly.

E. Annau (32) occupied himself with this process. He could show that tissues contain an active dehydrogenase for pyruvic acid, which has previously not been appreciated sufficiently, since its function is dependent on a hitherto unknown coenzyme. There was thus an enzyme to oxidise pyruvic acid by splitting of two of its H atoms. *Annau* (28) also showed that this H of pyruvic acid is oxidized by the C_4 system.

He also succeeded in establishing the whole balance of the reaction and showed that for every disappearing pyruvic acid molecule $\frac{1}{2}$ O_2 was used and one molecule CO_2 was formed. This means that after losing two H atoms the molecule was decarboxylated.

We must stop at this statement to consider its implications.

At this point we meet CO_2 , one of the main products of biological oxidation, for the first time. This CO_2 was not produced under the direct oxidative influence of O_2 but by decarboxylation, more or less independently of oxidation. The first stages of oxidation, the oxidation of triose into pyruvate, proceeded without any CO_2 formation with an R. Q. of 0. The CO_2 production only followed the dehydrogenation

of pyruvate, with an R. Q. of 2. This agrees also with the statements of *G. Moruzzi*.²⁵

Another fascinating problem is suggested by the consideration of the fate of the molecule of pyruvic acid, after it has lost two atoms of H and one CO_2 . The molecule resulting in this reaction must have the composition of $\text{C}_2\text{H}_4\text{O}_2$, which answers to the definition of a carbohydrate. Since only one atom of Oxygen was taken up per mol. pyruvate, it is evident that the product was not oxidised any further. The oxidation had to be either acetic acid or glycolic aldehyde, but none of these products could be found in the system, though acetic acid added could be recovered unchanged. So it becomes most probable that the oxidised pyruvic acid is polymerised to sugar. This is also in agreement with the old teaching of physiology, that pyruvic acid is one of the strongest glycogenic substances.

If this is true, it represents a most fascinating and fundamental biological principle.

If a substance like triose is oxidised, the products of oxidation are CO_2 and H_2O and we find a corresponding decrease in the quantity of carbohydrate. This looks as if the disappearing carbohydrate had been oxidised completely to CO_2 and water. The study of pyruvic acid, however, suggests an entirely different procedure. Here the molecule has lost altogether four H atoms (forming H_2O) and one CO_2 , the rest being re-converted into carbohydrate, which can be split into triose again, dehydrogenated and decarboxylated, resynthesised and so on. The net effect will be the same as if the lost molecules had been oxidised completely to CO_2 and water.

This represents a most important principle, suggested already years ago by *J. B. Connant*. Nature seems to work with such little cycles. She does not oxidise the molecule down to its last elements but oxidises it only in two steps, then puts it back into the original form, and starts over again making the whole machinery exceedingly simple.

The main product of fermentation in muscle is lactic acid. According to *Meyerhof* one-third to one-sixth of the lactic acid is oxidised and the energy liberated is used to

²⁵ p. 28.

resynthesise the rest into carbohydrate. The above considerations give, at least for the quotient $\frac{1}{3}$, a much simpler explanation. They make it probable that what actually happens is not that every third molecule, but only one-third of every molecule is oxidised, the remaining two C atoms being polymerised into sugar. No energy transfer is needed for this and the whole chain of events can take place with free energy decreasing at every step. The chain of reactions would be: Lactate $- 2H \rightarrow$ pyruvate. Pyruvate $- 2H - CO_2 - H_2O \rightarrow$ carbohydrate.

So far I have avoided the word „fat“ and the reader might object to my disregarding this great group of substances, which represent, next to carbohydrate, the most important form of energy reserve. The quantity of energy, stored in the form of fat, certainly exceeds the quantity of energy stored as carbohydrate in the animal body very much.

My reason for disregarding the fats is our ignorance of their intermediary metabolism. We cannot answer the simplest questions about fats. We do not know whether fats, as such, are oxidised along ways of their own or are burned only *via* carbohydrate. As far as cellular functions have been analysed, research always revealed carbohydrate as the immediate source of energy. The carbohydrate molecule fits into the energy-producing mechanism like a cogwheel. We also know that without carbohydrate there is no animal life, and the fall of blood sugar to less than half its normal value is incompatible with life. Furthermore we know that carbohydrate is transformed into fat, and that in biochemistry roads are mostly passable both ways.

I am inclined to think that fats, as such, are not oxidised at all. *F. Knoop* emphasised some 15 years ago that the transformation of carbohydrate into fat (and vice versa) must go over low-molecular splitting products. The same holds probably for the oxidation of fat into carbohydrate and it is only a low-molecular splitting product of fat that is oxidised and polymerised into sugar afterwards.

Much confusion is due to the fact that organic chemistry calls both butyric and stearic, „fatty acids“. So many conclusions have been drawn on fat oxidation from experience

obtained on low fatty acids. Physiologically there is a profound difference between low and high fatty acids and most of our experience obtained on butyric acid fails as soon as we lengthen the chain even by one or two carbons.

It is very difficult to picture the reduction of carbohydrate into fat. Decarboxylation might give a clue to this problem. If a molecule is decarboxylated without simultaneous oxidation, this is a reduction. To give a purely theoretical example, if lactic acid, the isomer of carbohydrate, could be decarboxylated, we should get ethyl alcohol, which on paper, can be polymerized into fat resp. paraffin.

4. C₁ Acids and Acetone.

Pyruvic acid occupies a central position in oxidation as well as in fermentation. It is the primary oxidation product of triose and the mother substance of lactic acid.

The properties of pyruvic acid are quite remarkable. It is reduced and oxidised with equal ease, and is prone to decarboxylation and has an extraordinary tendency to polymerise. All these many-sided properties give a special importance to pyruvic acid and make it probable that it plays a primary rôle in the condensation and transformation of foodstuff molecules.

It is extraordinarily easy to condense three hexoses into a C₁₈ compound on paper and reduce the same to fatty acid. In reality, however, this is no more than a childish example of paper chemistry. As emphasised by *F. Knoop* fifteen years ago, the transformation of carbohydrate into fat is probably effected by the condensation of the low-molecular splitting products of the carbohydrate molecule and, the properties of pyruvic acid make this molecule specially fit for such transformations.

Embsen discovered some thirty years ago that pyruvic acid can be transformed in the liver into acetone. This transformation is a proof of the remarkable properties of pyruvic acid, for it involves a condensation, an oxidation and a de-

²⁰ *F. Knoop*, *Klin. Wochenschrift*, 2, 60, 1923.

carboxylation of the molecule. *Embden's* discovery has not found the appreciation it deserves, and in spite of it, fat was looked upon as the sole source of acetone and acetone formation as unequivocal evidence of defective fat oxidation.

As mentioned before, pyruvic acid disappears from tissues oxidatively, whereby the C_4 system acts as the H acceptor. This disappearance of pyruvate is dependent on the good working order of the C_4 system. As shown in the previous chapter, the oxidised pyruvic acid is decarboxylated and condensed probably into sugar. It is still more likely that the pyruvic acid condenses first, and oxidises and decarboxylates into sugar afterwards.

If, however, as shown by *Annau* (27), the C_4 system is inhibited in its function, the condensed pyruvate takes a different route and yields acetone. This acetone formation can, according to *Annau* (25), be greatly enhanced by traces of ammonia.

H. A. Krebs made the interesting observation that liver transforms pyruvate anaerobically into β -hydroxybutyric acid. This anaerobic condition corresponds to the aerobic experiment, in which the way to oxygen is cut by the inactivation of the C_4 catalysis. Since hydroxybutyric acid can be formed from pyruvic acid only by oxidation, it is evident that in this case pyruvic acid uses some form of chemically bound oxygen as an oxidising agent. Probably a second molecule of pyruvic acid serves as H acceptor (*H. A. Krebs* and *W. A. Johnson*).²⁷

If oxygen is present, the β -hydroxybutyric acid is oxidised to ketobutyric acid, which oxidation requires no C_4 system.

The single steps, by which pyruvic acid is transformed into hydroxybutyric acid, are unknown. *Krebs* and *Johnson* have published some very interesting observations along this line but their theory of hydroxybutyric acid formation is not entirely in agreement with *Annau's* observations and the problem cannot be regarded as definitely solved.

Whatever the changes may be by which pyruvic acid is transformed into acetone, the fact remains that pyruvic

²⁷ *Biochem. J.* 31, 645, 722, 1937.

acid can apparently be transformed into acetone, and whether this transformation will take place or not depends on the functioning of the C system. If the normal way to oxygen is cut, β -hydroxybutyric acid, acetoacetic acid and acetone appear.

It is not even necessary to add pyruvic acid to suspensions of tissues, muscle, kidney or liver in order to get acetone but it is sufficient simply to inhibit the C_4 system (e. g. by malonate) and acetone will be formed in considerable quantity. The mother substance of this acetone is unknown. What we do know is that by inhibiting the C_4 system we can induce acetone formation and by the addition of small quantities of C_4 we can prevent it.

St. Huszák (27) succeeded in showing that these reactions are not limited to tissue suspensions but can equally be demonstrated in the whole animal. Ingestion of malonate was responded to with acetone formation, which acetone formation could be suppressed by the simultaneous ingestion of fumarate. These observations suggested the problem, how far analogous events could be made responsible for acidosis and acetone formation in diabetes or other pathological conditions. One might think that the failure of the C_4 system (together with increased ammonia formation) was somehow responsible for acetone. Since the normal concentration of C_4 dicarboxylic acids in the body is very low, it might be assumed that somehow this small quantity of catalyst might be damaged in one way or another, either by its defective formation or increased destruction. This suggested a very simple experiment: to try what C_4 substances would do in cases of acidosis.

A. Korányi (50) tried this in diabetic acidosis, giving succinic acid to patients *per os*. The effect obtained was not constant. In several cases, however, acetone disappeared without the use of insulin. Not only did acetone and acidosis disappear, but the quantity of succinic acid needed was in several cases so small (1 g daily) that it allowed the conclusion that succinic acid did not act like other anti-ketogenic substances as a source of energy, but its action was catalytic.²⁸

²⁸ On hyperglycaemia, succinic acid had no direct effect. All the same, this substance seemed also to increase the carbohydrate tolerance, possibly by excluding the harmful effect of acidosis.

The great individual differences in the effects obtained indicate that the mechanism of acidosis is not the same in all cases and the effect of C_4 is limited to certain forms of it. Our clinical material was too small to allow definite conclusions and we could do no more than to draw the attention of clinicians and experimental workers to the activity of C_4 , hoping that the action of succinic acid might open new ways for the research and understanding of acidosis.

These observations suggest that — at least in certain cases of diabetes — part of the acetone might be derived from pyruvic acid and thus from carbohydrate. Acetone formation is no unequivocal proof of fat oxidation.

But before closing this chapter I must mention that *Embden's* fundamental experiment, and part of the later work based on *Embden*, is capable of a different explanation. *Embden* added pyruvate to his perfusion fluid, observed the formation of acetone and concluded that pyruvate was transformed into the latter. But if instead of pyruvate he had added malonic acid to his perfusion fluid, he had also obtained acetone, formed from an unknown source. This acetone formation by malonate is due, as shown before, to the poisoning of the C_4 system. Now pyruvic acid has an effect analogous to malonate, acting on the malico-dehydrogenase. I have mentioned before that pyruvic acid is adsorbed by the malico-dehydrogenase and displaces malic acid on this enzyme inhibiting herewith the function of this part of the C_4 system. By doing so it might induce acetone formation the presence of which, however, will not prove that it is pyruvate itself which has been transformed.

It is known that pyruvate strongly depresses the respiration of muscle. That this effect is due to the inactivation of the C_4 system was shown by *E. Annau* and *F. B. Straub* (33), who could restore respiration by the addition of small quantities of fumaric acid. By this addition of C_4 acetone formation also is inhibited.

5. Objections.

1. One objection that could be raised against the C_4 theory is the following: malonic acid strongly inhibits succino-dehydrogenase, so if this H transportase is involved in respiration, malonic acid should always inhibit O_2 uptake, as it always inhibits the succino-dehydrogenase.

I have touched this question repeatedly in the previous chapters. The O_2 uptake is the final result of the function of the whole long chain of reactions. The working of such a chain is limited by the slowest reaction, so that malonic acid will inhibit only if the H transport by the succino-dehydrogenase is or can be made into the limiting factor by malonate.

In muscle, suspended in Ringer-Phosphate, the C_4 system will limit respiration and added malonate or fumarate will produce a strong effect. In pure Ringer the reduced H mobilisation becomes the limiting factor, and fumarate or malate will have mostly no effect. Freshly suspended muscle contains enough C_4 acid to carry respiration. Accordingly the quantity of C_4 will not be a limiting factor and the respiration will not be increased immediately by the addition of fumarate but will be increased a few minutes later, after the tissue has lost some C_4 acid by diffusion, provided we do not wait long enough to allow the damage done by defective respiration to become irreversible. These examples will suffice to show that the limiting factor might be a different one under different conditions and might change even within one and the same experiment.

Malonate can be expected to inhibit only when the activation of succinate is the limiting factor. Added fumarate will increase respiration only when this latter is limited by the quantity of C_4 .

In *Thunberg's* experiments the diffusion or adsorption of the added dye or cytochrome might limit the reaction even if the quantity of C_4 is strongly reduced by the washing of the enzymic preparation.

So before drawing conclusions from the negative result of an experiment with added malonate or fumarate, quantitative relations must be carefully considered.

In the resting whole animal, respiration seems to be limited by the available quantity of O_2 or of the donator and so malonate has no influence.

In the minced and aerated breast muscle of the pigeon practically the whole C_4 is present as fumarate-malate. The oxidation of succinate and the reduction of oxaloacetate are much faster than the reduction of fumarate and the oxidation of malate. Here the oxido-reduction between these two substances seems to limit respiration.

2. It has actually been objected that the C_4 dicarboxylic acids are burned and used as fuel by the tissue, and that a substance oxidised irreversibly is not a catalyst.

This raises the general problem, as to how far a substance actually burned can act as a catalyst and how far the increased oxygen uptake, obtained on addition of such a substance can be taken as evidence of a catalytic function.

Thunberg found some thirty years ago that fumarate increased oxygen uptake in minced tissues. *Grönwall* showed this to be connected with an R. Q. above unity, which proves that fumarate is oxidised irreversibly. These experiments have since been repeated and corroborated many times (*Elliot* and *Schröder*, *Stare* and *Baumann*). Accordingly *J. M. Innes* finds a fairly rapid disappearance of fumarate from muscle and thinks that our increased oxygen uptake after addition of fumarate can be explained by the irreversible oxidation of this substance, without supposing a catalytic activity. Succinic acid given to animals is not excreted (*Flaschenträger*) and is thus probably burned.

I can corroborate all these data. I have even made my collaborator *Balassa* (48) swallow quantities of succinate, which he burned almost completely. *Banga* (28), *Annau* and *Straub* (33) have found respiratory quotients above unity. *Szegedy* (34, 35) has worked out a special very accurate, half-micro method to estimate fumarate. *Annau* and *Straub* using this method found that in an hour's time one-third of the 10 mg. of fumarate, added per g. of muscle was made to disappear. The R. Q. above unity showed that the fumarate added was burned. This rate of disappearance is not nearly fast enough to give support to the idea that fumarate could lay

on the path of the intermediary metabolism of some foodstuff of first rate importance, like fat or carbohydrate. It is evidence for the contrary. But even this slow rate of combustion would be sufficient to explain the increased oxygen uptake observed on addition of fumarate. The quantities of fumarate, normally found in muscle (0,1 mg. per g.) are very small compared with the 3 mg. which the same quantity of muscle combusted within an hour's time. This makes it seem improbable that C_4 could act catalytically.

The catalytic activity of C_4 is explained by the fact that there is a great difference in the fate of small and big quantities of added fumarate. If we add only a very small quantity, 0,03—1 mg. fumarate to our suspension corresponding to the normal concentration of fumarate in the muscle, the result will be a different one. The excess of oxygen taken up on account of fumarate will exceed the quantity of O_2 necessary for the combustion of the whole C_4 added by many 100 %. Moreover as *Stare* and *Baumann*²⁹ found, the added fumarate can be recovered at the end of the experiment. The R. Q. will not exceed unity either, but will only be raised from its abnormally low value (0,8) to its physiological level of unity. All this shows that the muscle is able to retain and protect the small quantity of C_4 needed for the catalysis for long periods, while at the same time it decomposes bigger quantities.

It is easy to give a picture of this mechanism. Small quantities necessary for the catalysis will be absorbed and protected by the enzyme, while big quantities will not. The excess of C_4 acid might also help to displace the oxaloacetic acid from the enzymic surface. Free oxaloacetic acid is very labile and undergoes, in presence of muscle, fairly rapid decarboxylation. (*Banga, Straub* (28), *Banga & Sz.* (31). This also explains the constancy of the quantity of C_4 in tissues.

3. An objection of another kind has been raised against the C_4 theory by *F. Knoop* to whom we owe much of our knowledge of intermediary metabolism. He is struck by the great number of substances, imino acid, aldehydes and ketones, double bonds, which can be reduced in animal tissues. So from

²⁹ See. s. 20.

the point of view of his experience he justly criticises the C_4 theory³⁰ in giving expression to his belief that H, split off from the primary donator, can take many ways and can be transported towards oxygen by a great number of substances. The C_4 acid is just one of the many possible H acceptors. *A. H. Krebs* is more or less inclined to a similar conception, regarding the reduction of oxaloacetate just as one example of the reduction of CO groups.

This problem can naturally be decided only by experiment. As far as our experience goes, it shows that the main process of respiration follows strictly prescribed routes. According to thermodynamics, cytochrome-oxidase and cytochrome should oxidize with preference the most negative substances, like the donator. But in spite of thermodynamics cytochrome-oxidase oxidises only cytochrome and cytochrome only succinate. They just refuse to oxidise any other substance, for they are made and fitted together that way. This is what we call organisation, and any H, to be oxidised by cytochrome, has to pass succinate.

The oxido-reductions representing the H transport from donator to oxygen are the core of oxidation, the final source of animal energy. It is difficult to believe that such a process should not have its fixed apparatus and H should be transported by acceptors more or less accidentally formed.

Substances taking part in this system of H transport must also answer to very strict thermodynamic exigencies. Maleinic acid is a good example of this. It is known from *Thunberg's* work that the isomer of fumaric acid, maleinic acid, instead of being a catalyst, is a strong poison of respiration. It can take the place of fumarate on the enzyme, but it cannot take its function, though it is activated by the succino-dehydrogenase. The sole reason that could be found for this is its more negative potential. (*Laki* 27). So a substance has to answer to strict specifications if it is to be a member of the oxidative mechanism.

There is no doubt that the H atoms mobilised can also reduce substances other than oxaloacetate or fumarate. The re-

³⁰ *F. Knoop*. Münchener med. W. p. 633. 1936.

duction of imino acids (*Knoop*) or the lactic fermentation (reduction of pyruvate) clearly show this. Accidentally a H atom might even find its way to oxygen independently of the *Warburg-Keilin-System*, as shown by the small cyanide insensitive fraction of respiration. The main bulk of H, however, will be combusted along fixed routes by an apparatus of which the C_4 system forms an integral part. To prove this is the main object of this book.

6. Methods.

When undertaking a research one has first to decide upon ones material. In the last decades muscle has been used very extensively in biochemical research, for muscle, by the nature of its function is capable of powerful energetic changes which lend themselves well to measurement. Corresponding to these violent energetic changes we find rapid chemical reactions which can be measured within short periods. This is a very important point, for the biochemist, studying vital functions, finds himself mostly confronted with the difficulty of being unable to measure changes in the living, undamaged system. But as soon as he damages his system, the labile, subtle edifice of the cell collapses, and his results will give him no more information about the normal life of the cell than a city could give about society, if we studied it immediately after a violent earthquake.

Damaged tissues are known to become disorganized rather rapidly. So it is often very important to work with fresh material and to apply methodes which would allow us to make measurments in short intervals. The value of experiments in which chemical changes are studied after prolonged incubation of the damaged tissue, is often very restricted. But even if working under optimum conditions, with fresh tissue, within short periods, we have to be very cautious in drawing conclusions from our results on normal cellular functions. It is often one of the most difficult points of research to correlate findings in damaged cells with normal life.

One of the points frequently neglected is that of quantitative relations. Supposing we find a certain change, such

as the reduction of a carbonyl or an imino group, and we feel inclined to ascribe an important rôle to this reaction in the H transfer in respiration. If we want to find out whether this reaction can be an important link in the respiratory system, we must correlate the rate of this reaction with the rate of respiration. Respiration is a very active process in which great quantities of O_2 are consumed. So before we can suppose that our reaction might act as a link in respiration we must show that it can proceed at a rate at least equal to the rate of total respiration. It is not enough to state that a reaction can, or has taken place, and I have no confidence whatever in experiments in which the research worker comes back after lunch to see what has happened in his system, and to tell us how a cell is living. In such an involved system as a disintegrating cell any change admitted by thermodynamics might occur.

But even if we have made up our mind to use muscle, we must specify our further choice for there are considerable differences between muscle and muscle according to their function and the living habits of the animal. The intensity of metabolic changes in the muscle will also depend on whether the animal leads a sedentary or active life. Rabbits and rats for instance do not move far from their holes. There is therefore a great difference between the metabolism of the muscles of the limb which in the rat and rabbit have usually little work to do, and the diaphragm which works day and night, and the heart which works the hardest. So for years I worked with the diaphragm of the pig which has much advantage over other muscles. Later I turned my attention to hearts. The drawback of this is that our experimental animals have but small hearts and those of bigger animals cannot be obtained without much delay. Most of the work reported in this book was done with the breast-muscle of the pigeon which has a very active metabolism according to its very strenuous function, extended frequently over long periods.

Naturally this muscle cannot be studied undamaged, and this damage greatly distorts normal conditions. This distortion, however, might serve to our advantage. Progress in the study of the mechanism of such internally balanced cyclic functions as respiration, often depends on the possibility of dis-

connecting or distorting the system, and by doing so, of bringing certain members of it to observation. The mincing of the muscle probably entails the release of the enzymes responsible for the mobilisation of the donator. So we get a much more active respiration than corresponds to the normal, resting muscle. At the same time C_4 acids are lost by diffusion, making the activation of the latter the limiting factor. This brings the C_4 system under observation, so that the damage and distortion of the system can serve to our advantage. Our success in analysing will often depend less on the question of how far we can preserve, but on the question of how far we can distort normal conditions.

The choice of muscle as material has its dangers. Contraction, the specific function of the muscle, involves rapid production of energy, and correspondingly rapid chemical changes. These changes, however, might be connected solely with the specific function and give little information about the „private life“ of the muscle cell. So it is important to try to show that changes observed in the muscle are also found in other tissues.

In our experiments a modified *Latapie* Mincer was used (27). The most important difference between *Latapie*'s and other common mincers is that the material to be minced is not pressed against the perforated disc by a worm, but by a piston, which reduces the damage inflicted on the tissue. This mincer cuts the tissue into distinct particles of the necessary average size. These particles can be looked upon as slices which have been sliced again in two planes, perpendicular to each other and to the original plane. As *Annau* has shown (27), there is no considerable difference in the behaviour of sliced and minced tissues obtained by the *Latapie*. The mince was suspended first in Phosphate (27), later in Ringer-Phosphate.

Thunberg experiments were made in a modified *Thunberg* apparatus (Sz.) (27), which enables us to measure a whole series under identical gas pressure. *Laki* (39—41) has shown that the same apparatus can also be used for measuring the velocity of hydrogenation, if certain circumstances are taken into account.

The solution of several problems was dependent on micro-methods which would allow the rapid and accurate estimation

of very small quantities of pyruvic and oxaloacetic acid. The great difficulty of this was that both methods had to be specific and had to allow the estimation of both substances in the same material. Owing to the close relation of these substances this task seemed to be unsolvable, but eventually we were successful. Pyruvic acid was estimated (*Straub*) (28) by condensation with salicylaldehyde which method is an adaptation of *Csonka's* estimation of acetone. This reaction is not given by oxaloacetic acid. Oxaloacetic acid was estimated as 4-nitroso-pyrazolon-3-carbonic acid (*Straub, Bruckner*) (28), which estimation is highly specific. It is given only β ketones, but even under these oxaloacetic acid behaves in a special way, having a very high extinction quotient. This method will answer to the highest demands and I expect it will be useful in future research, for as I have shown, the reduction of oxaloacetic acid admits the measurement of a new magnitude, the quantity of H mobilized. In *Banga's* (31) paper the conditions are given under which the disappearance of oxaloacetate can be taken as measure of its reduction. It is a fortunate circumstance that these experiments in which the reduction of oxaloacetate is studied, need not be made anaerobically, for the excess of oxaloacetate inhibits succinate dehydrogenase strongly and makes hereby the reoxidation of malate impossible.

Methods for the estimation of succinate (*Gözszy*) (27), malic acid (*Straub*) (28), fumaric acid (*Straub*) (27), *Szegedy* (34, 35), *Balassa* (48) and the RQ. (*Straub*) (28) are also given. They are less satisfactory and still accessible to improvement.

PART III.

Vegetable Systems.

Introduction.

From the very beginning my research work in Biochemistry was led by the conviction that there can be no real difference in the fundamental chemical mechanisms of plants and animals. We are all but young buds of the same old tree of life, expression of the same fundamental principles appearing under different disguise.

Results of the last few decades have greatly corroborated me in this conviction. The existence of Vitamins is convincing evidence on this line, as it shows that plants and animals work with the same cogwheels. The substance, Vitamin B₁, instrumental in producing thoughts in our brains, is equally indispensable for the silver membrane of rice. Insulin, the highly specified product of a highly specified organ of our body is found also in the yeast cell. There can be no fundamental difference in two mechanisms, the parts of which are interchangeable.

So if we want to elucidate some fundamental biological principle, it does not matter whether we study a high or low animal, a plant, or the yeast cell. There is no fundamental difference between kings and cabbages.

Vegetable material has many advantages over the animal. Its protein structure is less labile, and often tolerates treatment with anhydric solvents that would denaturate animal protein.

Vegetable tissue might have still other advantages. Animal tissues all live under the identical condition of their „internal environment“. Different plants or different parts of the same plant live under very varied conditions, e. g. under the ground or high up in the air. So we may expect that from

the different oxidative mechanisms the one or the other will predominate, according to the special conditions. Moreover in the plant the picture will not be complicated by specific functions, like muscular contraction etc.

Naturally the vegetable material has also its disadvantages. The chemical changes are on the whole less rapid in the plant than in the animal, and therefore more difficult to measure. Owing to the rigity of the cell wall, the damage done to the cells by our methods is often graver than in the animal. Accordingly it will be still more difficult to correlate the results obtained under artificial conditions with normal cell life.

The Polyphenoloxidase System gives a good example of this. As I will show presently, we can easily demonstrate the presence of this most powerful oxidative mechanism in plants. After having isolated and measured all the single members of this mechanism we are left with the question: has this anything to do with respiration? And we are unable to answer this question. The same applies to the Peroxidase System.

These two oxidative mechanisms just mentioned, the Polyphenoloxidase and Peroxidase System, often dominate the whole appearance of the plant. But in the shadow of these powerful systems we also find cytochrome, which as I have shown, dominates the picture in the animal and carries most, if not the whole of respiration. Thus we are confronted with the question as to whether cytochrome carries the respiration in plants the same way as it does in animals, while the other two systems have some different function. On the other hand, if the Polyphenoloxidase and Peroxidase Systems are involved in respiration, are the same systems not found in the animal cell also?

It is a depressing fact that we are unable to answer these simple and fundamental questions. I am rather inclined to think that all the three systems are involved in respiration of all cells but it will depend on conditions which one will predominate. The conditions of the „internal environment“ of the higher animal favour the system of which cytochrome is a member. There are also reasons to believe that the other two, the Polyphenoloxidase and Peroxydase System, are also involved in

animal respiration even if they carry only a small fraction of total oxygen uptake and are perhaps only serving some specific function of the cell. What makes me think that these systems are involved in animal respiration is that two of the most important members of the „Peroxidase System“ have been shown to be of Vitamin nature (Vitamin C and P) and thus to be essential for the functioning of the animal tissue also. The enzyme which is the most characteristic member of the chain, the „Perioxidase“, has been shown to exist also in the animal tissue. Polyphenols are found in the animal too and are very closely related to polyphenols acting in the vegetable polyphenoloxidase system (Adrenalin, Dopa). The existence of Phenoloxidases has been demonstrated also in animals.

The problem, however, is undecided and my object will be to present briefly the underlying experimental facts. Even if the two great vegetable systems, the Polyphenoloxidase and Peroxidase System have nothing to do with respiration and have no bearing on the higher animal, their study has led to results which are not without importance for animal biochemistry: the isolation of Vitamin C and the recognition of the Vitamin nature of phenyl-benzo,pyrone dyes. In this case my research will be a further example of the fact that even a wrong theory may lead to correct results and any theory is good as long as it suggests experiments.

1. The Polyphenoloxidase System.

It is an age-old observation that certain plants turn brown if damaged. The potato, the apple, the pear, the banana are every-day examples of this. It was *Palladin*, the great Russian botanist, who gave his attention to this phenomenon. He has shown that this discolouration is connected with the function of certain enzymes oxidizing a polyphenol, (a phenol with more than one OH group). In the intact plant, or under anaerobic conditions, the oxidised polyphenol becomes reduced again to the uncoloured compound. If, however, the plant is damaged, this reduction does not take place and the oxidised phenol appears as a „respiratory pigment“.

Looking at these observations to-day it becomes evident

at once that these „respiratory pigments“ are links in a chain of a respiratory system, at one end of which stands oxygen, oxidising „the respiratory pigment“ by the interaction of special enzymes. At the other end stands the dehydrogenase releasing H from the foodstuff, reducing the chromogen to its leuco compound. We are faced here with the same fundamental principles as in the animal respiration: the splitting off of H from the „donator“, and its piecemeal oxidation.

The enzymes responsible for the oxidation of phenols have been the object of numerous studies (*Bertrand, Bach, Chodat, Wolf, Robinson, Raper* etc.).

In the study of these enzymes the classical guaiac-reaction played an important rôle. If the cut surface of these plants is wetted with a solution of guaiac resin, a brilliant green colour very soon develops due to the oxidation of the resin. Since the guaiac reaction was known as a specific reagent of peroxides and peroxidases, complicated theories were elaborated about the mechanism of the action of these polyphenol-oxidases. *M. W. Onslow* has shown that the plants giving the guaiac reaction contain catechol or its derivatives. It was thought that these catechols activated oxygen somehow, forming peroxides. I could show, however, that what happened was much simpler: the oxygen, interacting with the polyphenoloxidase, oxidised off two H atoms from the phenolic OH group. The catechol thus became dehydrogenated into an o-quinol. o-Quinols are very powerful oxidising agents and oxidise the guaiac into its coloured compound. (51, 52, 53).

If the phenol is oxidised by the oxidase in the intact plant the quinol is reduced to phenol again. If, however, by damaging the plant we damage dehydrogenases, this reduction does not take place, and the quinol combines with nitrogenous substances present, into highly coloured substances (*Raper and Wormall*).³¹ The o-quinols themselves are mostly not very highly coloured.

Whether the quinol in the plant is reduced immediately by the dehydrogenase, and whether there are other substances acting as links between quinol and dehydrogenase, we do not

³¹ *Biochem. J.* 21, 26, 1927.

know. The polyphenoloxidase plants do not contain ascorbic acid mostly in a higher concentration. All the same they contain some and we can expect that in presence of ascorbic acid the quinol will oxidise the ascorbic acid present, and the dehydrogenase will reduce only the dehydro-ascorbic acid. Quinols oxidise ascorbic acid reversibly at a very high rate. (57, 72).

Oxidising enzymes acting on monophenols, like cresol or tyrosin seem to be members of an analogous system.

The phenol-oxidases present a clear case of a vegetable respiratory system. The only trouble is that the system works too well. It calls to mind the detective stories in which suspicion is roused by the fact that the evidence is too plain.

Vietorisz and myself (59) have measured the activity of the polyphenoloxidase in the potato. The inner layers of the undamaged tissue of this plant show no colouration. Thus, if the phenol is oxidised at all, it must be reduced at an equal rate. Now if we squash the potato and damage the tissue, discolouration appears at once. Thus after damaging the cell the phenol is either oxidised faster or is reduced more slowly.

Without any trick it is not possible to measure the rate of the oxidation of the phenol, for its quantity is too small and it is oxidised before the measurement could start. So if we want to measure the rate of the oxidation of the phenol, we have to reduce the quinol all the time. This is easily done by the addition of ascorbic acid. The experiment shows that the squashed tissue takes up at least fifteen times as much oxygen for the oxidation of the phenol as it uses in its normal respiration. Thus even if the oxidase has acted on the phenol in the undamaged tissue, its activity was at least fifteen times smaller than in the damaged tissue, and the potato contains at least fifteen times more oxidase than it needs for its respiration. This seems to be senseless. If no more than one fifteenth of the enzyme could be active in the plant, it could be just as well for the enzyme not to be active at all and have some quite different function than to support respiration. Thus the intact tissue is colourless, not because the quinol is reduced, but because the oxidase does not act.

The fact that damage of the cell releases the phenoloxidase,

suggests that this enzyme might have something to do with the protection of the plant against damage, with natural immunity. Quinols are known to have a strong bactericidal activity and to have a tanning effect on protein. One would think that the polyphenoloxidase together with catechol are stored by the plant to be released only if the cell is damaged. By killing the bacteria and tanning the damaged surface the system will close the gates to further progress of the damaging influence.

Other oxidising enzymes might have analogous functions. Milk contains a most powerful oxidising enzyme, although has no respiration. This enzyme described by *Shandinger* oxidises aldehydes, producing an equivalent quantity of peroxide. Peroxides are known to kill bacteria and bacteria are known to produce aldehydes.

2. „The Peroxidase System.“

M. W. Onslow has termed plants such as cabbages, lemons etc. which do not discolour on injury and correspondingly give no guaiac reaction and contain no catechol, „peroxidase plants“. This name was given because the most conspicuous constituent of these plants is a very active peroxidase, the enzyme activating peroxides. In the presence of peroxidase, peroxides will be able to oxidise substances which without peroxidase are not acted upon. It is easy to demonstrate the existence of a peroxidase in a plant juice by adding peroxide and a substance like guaiac resin or benzidine. In presence of peroxidase the rapidly developing colour will indicate the oxidation of our reagents and hence the presence of peroxidase. If there is none present the system will remain uncoloured.

In order to find out something about the respiratory system in which the peroxidase might be involved, I performed many years ago these guaiac and benzidin reactions with the juice of „peroxidase plants“. I observed that the reaction occurred only with delay of a second or so, while a purified peroxidase gives the reaction at once. This insignificant delay of the reaction has given me work for many years, for I found it to be due to the presence of a most fascinating substance,

(54) which had strong reducing properties and was capable of reversible oxidation and reduction. This substance, first called O_{x11} by its protocol number, was found to be related to carbohydrates, so I called it ignose, not knowing which carbohydrate it was. This name was turned down by my editor. „God-nose“ was not more successful, so in the end „hexuronic acid“ was agreed upon. To-day the substance is called „ascorbic acid“ (75) and I will use this name.

The delay of the peroxidase reaction in the plant juice was simply due to the fact that the ascorbic acid present, reduced the oxidised guaiac or benzidine again. This went on till the ascorbic acid was used up, which took about a second.

The discovery of ascorbic acid is a classic example of the fact that a wrong theory may yield good results. My studies of oxidation originated from my desire to understand what the adrenal cortex was doing. I thought this gland was involved, by its product, in biological oxidation and so I studied oxidation in order to understand the adrenal gland. It is known that human patients, in whom the gland does not work (*Addison's disease*) turn brown before they die, in the same way as potatoes, apples or bananas. So my excitement was fully justified when I found that the adrenal cortex contained relatively big quantities of ascorbic acid, the same substances which, if added to polyphenoloxidase plant tissues, prevented pigment formation and which seemed to carry respiration in the cabbage. The adrenal gland contains so much ascorbic acid, that for a long time this gland was found to be the only suitable material for the big scale preparation of this substance. My excitement was increased still more by the fact that Addison patients could be bleached out by the injection of ascorbic acid.³² (57).

To-day we know that the theory was wrong and the specific product of the adrenal gland is „cortine“ and several other tissues like the corpora lutea and the adrenal medulla (*Huszák*)

³² Ascorbic acid prevents only the formation of melanoid pigments. As showed by *P. Koller* (unpublished) embryonic iris tissue growing in a solution of ascorbic acid forms pigment unhampered. Ascorbic acid has thus no effect on normal pigment. This explains why the hopes of solving the colour problem by means of ascorbic acid were disappointed.

(64, 66) contain ascorbic acid in equal concentration. Possibly it serves to protect the autooxidisable hormones.

I could also show later (58) that, along with ascorbic acid, peroxidase plants contain an enzyme capable of oxidising off two H atoms from ascorbic acid which reaction is reversible. The oxidised ascorbic acid will take up H again, if such is offered. I could also show that this „ascorbic acid oxidase“ did not act on ascorbic acid just by good luck. The oxidase is made to oxidise ascorbic acid only, and made to act upon it with a maximum velocity, even if only very small quantities are present.

Since peroxidase plants are mostly relatively rich in ascorbic acid, it is natural to suppose that all the three substances, peroxidase, ascorbic acid, and its oxidase are all members of the same oxidising mechanism.

Peroxidase and peroxide do not oxidise ascorbic acid, or do so only at a very slow rate. If, however, peroxide is added to the plant juice containing peroxidase, it will oxidise ascorbic acid very quickly. It is evident that the plant juice contains some other substance also, necessary for the reaction.

The experiments showed that this substance was of aromatic nature. Its function was to connect peroxidase and ascorbic acid. In its presence peroxidase oxidised ascorbic acid immediately. The mechanism of the reaction was this: peroxide plus peroxidase oxidised this aromatic substance into the corresponding quinol, which in its turn oxidised ascorbic acid.

I tried to isolate this aromatic substance from lemon juice years ago (unpublished). The successive purification of this juice left me in the end with a substance apparently belonging to the great group of yellow water-soluble vegetable dyes.

We owe our knowledge of the chemistry of these substances chiefly to the pioneer work of *v. Kostanecky* and *A. G. Perkins*.

The nucleus of the molecule of these substances is a benzoyl-pyrone. In the most widely distributed representatives of these dyes, this nucleus is substituted at the position 2 by a phenol. Such substances we call flavanones. (Fig. 10.) If there is a

double bond at 2—3 we call the substance a flavone. If in addition to this there is an OH group in position 3, call it a flavonol.

The most common representative of this group is the flavonole quercetine. (Fig. 11.) Like most other members of this group, quercetine has two OH groups on the benzo-pyrone moiety in position 5 and 7. These two OH groups (5 and 7) serve to bind sugar, for most members of this group of dyes are found in the living cell as glucosides. The two OH groups at 3', 4', are free and seem to be connected with the function of the substance, as I will show presently.

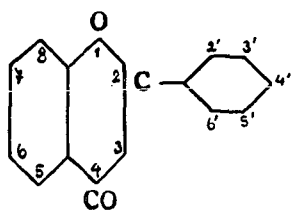


Fig. 10.

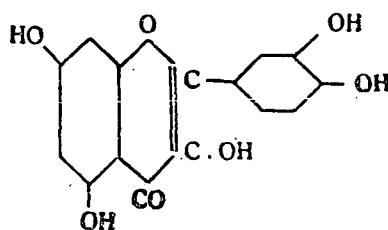


Fig. 11. Quercetine.

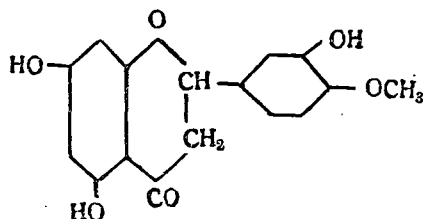


Fig. 12. Hesperetine.

Lemons are known to contain the flavanone hesperetine (Fig. 12.) in the form of a glucoside, called „hesperidine“. This glucoside has the same structure as quercitrine, the glucoside of quercetine. Naturally in hesperidine there is no double link at 2—3 and no OH at C 3. Moreover the OH group at 4' is bound by a methyl. The lemon builds up its store of hesperidine for life at a very early stage, so the unripe lime contains amazing quantities of hesperidine. If one looks at the quantity of hesperidine obtained from one small unripe lime, one wonders where the fruit has been.

The crystalline flavanone fraction obtained from ripe

lemon showed an amazing reactivity, not shared by pure hesperidine which is a very stable substance. The analysis by *Bruckner* (83) and myself has led to the interesting observation that on ripening, the plant demethylates hesperidine, freeing the OH group at 4'. The demethylated hesperetidine is called eriodictyol, which dye was not known before as glucoside. By the liberation of the second OH group the dye gets a very high reactivity. The unripe plant seems to store its reserve dye in an inactive, methylated form and demethylates the glucoside in the course of its life according to need. The ripe lemon contains mostly eriodictyol glucoside and little hesperidine, while the reverse is true for the unripe fruit. Hesperidine is insoluble, the eriodictyol glucoside is soluble in water.

St. Huszák (67) has studied these dyes from the point of view of the peroxidase systems. He has shown them to catalyse the reaction between peroxide, peroxidase, and ascorbic acid. They are in this respect active only as far as they have a free o dihydroxy group at 3, 4. Without this grouping they can give no quinols. Accordingly hesperidine is inactive, quercetine and eriodictyol or their glycosides are active. They catalyse the reaction by getting oxidised themselves by the peroxidase plus peroxide and oxidising in their turn ascorbic acid.

This catalytic activity of these compounds was found to be more or less specific. Eriodictyol, quercetine and their glucosides are about 100 times more active than the equivalent catechol.

Naturally, peroxidase cannot work if there is no peroxide. The question was therefore, as to whether there is any peroxide in plants and if so, where does it come from. This question was answered by *Huszák* (67), who hereby completed the whole system. The peroxide was formed in the oxidation of ascorbic acid by the ascorbic acid oxidase.

If we observe thus a rapid disappearance of ascorbic acid in the cabbage juice, or the juice of other peroxidase plants, this apparently simple reaction has the following mechanism: molecular oxygen interacts with ascorbic acid oxidase and oxidises off two H atoms from ascorbic acid. The oxygen itself

is hereby reduced by these two H atoms to hydrogen peroxide, while ascorbic acid itself becomes, by the loss of its two H atoms, dehydro-ascorbic acid. The H_2O_2 formed reacts with peroxidase and oxidises a flavone (or flavonole or flavanone). The oxidised flavone in its turn oxidises a second molecule of ascorbic acid. In this way all the four valencies of the O_2 molecule are utilised for oxidation of ascorbic acid. Dehydro-ascorbic acid is reduced by tissues again. It is true that dehydrogenases do not reduce ascorbic acid, but dehydrogenases, acting on hexosephosphate (*Meldrum*) or citric acid (*Banga*, unpublished) reduce glutathion and glutathion reduces dehydro-ascorbic acid. *F. G. Hopkins* and *Morgan*³³ have shown that this reduction is greatly accelerated by the ascorbic acid oxidase. Herewith the system is complete, and represents again a chain of reactions linking up the „Donator“ with the O_2 by the piecemeal oxidation of the H.

3. On Vitamin C.

My first observations on ascorbic acid (54) were made in a cellar room of the physiological laboratory at Groningen. The isolation of this substance was made possible by the hospitality of Sir *F. G. Hopkins* at Cambridge. (55). It could be prepared from cabbages, oranges and adrenal glands in small quantities sufficient for the first chemical analysis. (56). I could show that the substance answered to the general formula of $C_6H_8O_6$, demonstrate its reversible oxidisability and show that it was one of the fundamental, widely distributed, reducing agents of the cell, animal or vegetable. Unfortunately the plants mentioned allowed preparation on a small scale only and were unsuitable for big-scale preparation. My raids on greengrocers stores had no result. I could find no suitable material for big-scale preparations. Further progress in the study of the function and chemical constitution of the substance depended on the preparation of larger quantities. The only suitable material for work on a large scale was the adrenal gland. This however was not available

³³ Biochem. J. 30, 1446, 1936.

at Cambridge and so Professor *A. Krogh* kindly tried to help me by sending big quantities of glands by air from Denmark, but the material on its arrival had deteriorated.

Further progress was made possible by the generous invitation of the *Mayo-Foundation* and the hospitality of Professor *E. C. Kendall*. I was able to use the material of Americas huge slaughterhouses and prepared about 25 gr. ascorbic acid from adrenal glands, working up this expensive material by the hundredweight.

This material enabled me to study the substance from the point of view of vegetable respiration (58), and to find the ascorbic acid oxidase. I could also study its effect on Addison disease (57). My chief concern, however, was the elucidation of the chemical configuration of the molecule. For this reason I shared my material with Professor *W. N. Haworth*, who from the beginning showed a vivid interest in this substance. We both arrived at the same conclusion: this quantity of material was insufficient to make any progress. So the material went and there was no chance of any further preparation. Plants failed as material and repeated preparation from adrenal glands was impossible because the expense was prohibitive. The research was given up in despair and nothing more was left than a small quantity of ascorbic acid in the bottom of one of my tubes.

From the very beginning I suspected ascorbic acid to be identical with Vitamin C but my roaming life was unsuited for Vitamin tests and moreover, somehow or other, Vitamins were my pet aversion. Vitamins owe their great popularity to their paradoxical behaviour, making us ill if we do not eat them, while all other substances make us ill only when we do eat them. What food must contain to be wholesome is a question of primary interest to the cook rather than to the scientist. Accordingly the appreciation of results of vitaminology are often out of all proportion to their scientific importance. Moreover the vitamin nature of ascorbic acid could not contribute much to its real scientific interest, since its importance and presence in plant and animal tissues has been demonstrated already.

Two years later I was condemned to be Professor and sent to the chair (of biochemistry) in *Szeged*. At the same time

fate has sent me a clever collaborator *J. L. Svirbely* who had had some experience in vitamin C test and brought with him the conviction that my ascorbic acid could not be Vitamin C. All the same I made him test my old suspicion to see whether the small quantity of powder left in the bottom of one of my tubes was not the vitamin C. In November 1931 he had definite evidence that it was. At this time also Tillmans directed attention towards the possible identity. We did not publish until we could repeat our experiment on a large number animals. (69–72). Simultaneously *King* and *Waugh* reported the isolation of crystals from lemon juice which had antiscorbutic properties and seemed to be identical with ascorbic acid, that time „hexuronic acid“.

By its identity with vitamin C general attention turned towards ascorbic acid. But there is little use in knowing that a substance is interesting if there is none of it. My stores were completely exhausted and there was no chance of further preparation. Big quantities of ascorbic acid were urgently needed to find out the exact configuration, and to perform the chemical work necessary to demonstrate definitely that ascorbic acid really was the vitamin C and the vitamin activity of our crystals was not due only to some enclosed impurity.

Szeged happens to be the centre of the „Paprika“ — Hungarian red Pepper industry. This fruit (*capsicum annuum*) was about the only one I had never tested. For some unknown reason nature has supplied Hungarian red Pepper with a most wonderful store of ascorbic acid. In two successive seasons I was able to prepare 3 1/2 kg of crystalline ascorbic acid from this fruit.

From this substance *L. Vargha* prepared monoacetone ascorbic acid, which in itself is quite inactive, but crystallizes beautifully. After repeated recrystallisations the ascorbic acid could be split off again and was found to be fully active still. This was the first definite evidence that ascorbic acid really was vitamin C. (74).

Most of my ascorbic acid was distributed among all workers who were interested in it. So this material greatly contributed to the rapid elucidation of the structure of the molecule and opened the way to synthesis. Thus it is greatly due to the

Hungarian red Pepper that vitamin C was brought from its mysterious sphere into the domain of cheap synthetic products within the remarkably short period of two years. Now it is produced synthetically at a low price by the hundredweight.

Finding that one of his active substances is a vitamin means a little tragedy to the scientist. Quiet work on basic problems has to be given up for working out big-scale methods and their dull application. Years have to be spent in hard labour merely to make synthesis possible, which makes all the previous work valueless. Herewith one of the little cycle of hope and disappointment is closed, of which a scientist's life is composed.

All the same I am grateful to Pepper for one of the greatest impressions of my life. I am still filled with sincere gratitude for the generous international support, collaboration and comradeship I found in those days. It is encouraging to know that this spirit exists in science. If it prevailed in international politics also, we should all be approaching a more cheerful future.

4. On Vitamin P.

In the previous chapter I have deprecated vitamins and have told why I ran about for five years with crystalline ascorbic acid in my pocket without testing its vitamin activity. I have also given an example of a bad theory leading to good results. Now I want to say a few nice things about vitamins and tell why I was so anxious to find out whether flavones were not vitamins and will give an example of an incident and an error leading to discovery.

The vitamin nature of a substance can also be of great scientific importance. By vitamin we mean a vegetable substance, needed, but not made by the animal, which by its absence will cause disease. This disease often helps us to reveal the existence of such a substance. By demonstrating the vitamin nature of a substance, we prove that it plays an important rôle also in the animal, giving a new evidence of the unity of living nature.

Benzopyrone dyes are of great importance to the plant. These dyes are elaborated in the vegetable kingdom every year

afresh by the ton. It is difficult to believe that these substances should have no importance for the animal, although chemical methods fail to demonstrate their presence in the animal cell. Thus I could only hope to demonstrate their importance if they happened to be vitamins and caused disease by their absence.

The possibility of their vitamin nature was suggested by the fact that the other substance, ascorbic acid, working hand in hand with flavones in the peroxidase system, is also a vitamin. The chances of demonstrating the vitamin activity however, were very small. The most suitable animal for this demonstration seemed to be the guinea pig and the most suitable diet the scurvy diet, although animals on scurvy diet thrive well if supplied with ascorbic acid, which seems to prove that the diet either contains these flavones or else the animals do not need them.

The first indication that flavones might be vitamins was given by an incident. In the early days of ascorbic acid I had a letter from a doctor who was suffering from severe haemorrhagic diathesis. He asked for ascorbic acid to test the effect of this substance on his condition. Not having enough ascorbic acid yet, I sent him pepper in its conserved form („vitapric“). The man was cured. Later the treatment was repeated with pure ascorbic acid: it had no effect. This suggested that some other principle was responsible for the activity. To isolate this principle would have been a hopeless job, had not the idea of the vitamin nature of flavones been in the back of my mind. I set out to isolate the flavanone fraction from lemon juice, which we called citrine and my friend *St. Rusznyák* (80, 81), and his collaborators, *Armentano* and *Bentsáth* tested it on patients. The substance was active, it cured (vascular) haemorrhagic purpura and brought back the fragile and permeable capillaries to their normal state in different pathological conditions. Very fortunately the fragility and permeability of capillaries can be measured fairly accurately by the methods of *Borbély* and *Landis*. There was a sharp difference between diseases in which capillaries answered to citrine (Haemorrhagic purpura, nephritis, sepsis,

nephrosis, polyarthritis) and those in which they did not (diabetes, tuberculosis).

Such an activity suggest vitamin nature but does not prove it. Evidence of the vitamin nature could be obtained only in the animal experiment, and here it was that the error came to help us.

Encouraged by clinical observations we tried what citrine would do to guinea pigs on a scurvy diet, in spite of our bad chances. (82). The result was quite unexpected. Citrine, given in scurvy, not only prolonged life, but prevented rapid fall in weight and reduced haemorrhages. The difference with controls was quite striking and pleaded for the vitamin nature of citrine. So we called the substance vitamin P.³⁵ The difference between our control animals and the animals receiving citrine was sharp enough to use it as a test, by which the basic facts about vitamin P could be established. *Bentsáth* (86) showed that the intactness of the whole glucoside molecule is essential for the activity, the aglucons (the dyes without sugar) are inactive, but not all members of the phenylbenzo-pyrone series are active. Both constituents of citrine, the hesperidine and the eriodictyol glucoside were equally active. (*Bentsáth*, *Rusznayák* and *Sz.*) (84). But quercitrine, the most widely distributed flavonol, though otherwise not devoid of pharmacological activity (*Fukuda*, *Jeney* and *Zimmer*), was found to be inactive in our experiments. Quercitrine dif-

³⁵ It was called P, because of its effect on *Permeability* and because of *Paprika* (pepper). I had also another reason to choose P, which was not the first free letter of the alphabet. I realised that vitamin work was full of pitfalls and hoped that in case the vitamin nature of citrine would be disproved, this would happen before vitaminology reached the letter P and thus my work would cause no trouble. Moreover I knew orthodox vitaminologists would be teased by my jump in the ABC.

Naturally it would be a grave mistake to call P a „permeability vitamin“. This would be much the same as if *Burr* and *Burr* called their substances „anti-tail-drop-off“ substance. The *Burrs* have shown rats to loose their tails, if certain fatty acids are missing from their food. The function of these acids is certainly not to keep tails in their place, just as it is not the function of P to keep capillaries in the right condition. That capillaries get ill in absence of this substance is an other question.

fers from the eriodoetyl glucoside only in having a double bond at 2—3 and an OH at 3. This slight variation within the molecule is sufficient to abolish the activity. The position seemed to be analogous to that found in lipochromes, where only a few members of the big group are active as provitamins. *A. Bentsáth* (86) could also establish approximately the necessary daily dose of citrine. This was about 0,2—0,4 mg, for 1 mg had a full, 0,2 a submaximal effect. Together with ascorbic acid vitamin P is not active in quantities of γ -s, like some other vitamins. The daily dose corresponds to the daily dose of ascorbic acid. Lemons contain about five times more ascorbic acid than citrine. The daily dose of ascorbic acid is about 1,5 mg. roughly five times as much as that of citrine.

Thus our test has given valuable results and cleared the basic questions. There was only one great trouble with it. Within one series the experiments gave conclusive results but the activity was not equally reproducible in different series of experiments. The test was a subtle one and there seemed to be unknown factors at play. One factor revealed by *Bentsáth* and *Das* (85) was the importance of the food given prior to the experiment. But even this, if put right, did not assure the constancy of results. The difference between controls and animals receiving citrine became less marked as our experiments advanced. In later experiments citrine failed to prevent haemorrhages, then it failed to prevent loss of weight and in the end, lengthened the life of our animals by only a week only instead of a fortnight. A laboratory which, at our request, repeated our work with the greatest care on a large number of animals failed to find any difference. All this was very disconcerting. So it was no surprise when *Zilva* published his negative results, obtained with Hesperidine.

A great number of experiments were performed to find out the cause of this discrepancy. Eventually it was found by *Bentsáth* (86) that it was probably caused by an experimental error, without which our results could never have been obtained. This error did not take away the value of our previous experiments, but it explained the discrepancy. *Bentsáth* showed that the difference in results might have been due to the fact that our scurvy diet

still contained traces of ascorbic acid, which were too small to have any effect on the development of scurvy. The flavanones seem to be utilized by the animal only in presence of at least traces of ascorbic acid, or if we want to put it the other way round, traces of ascorbic acid can be utilized only in the presence of vitamin P.

Our peroxidase system could give a simple explanation of this. *Huszák* (67) has shown ascorbic acid and flavanones to act in the same chain of reaction. If this holds for animals, one can expect that the one substance cannot act in the entire absence of the other. So the flavanone cannot act entirely without ascorbic acid. Possibly ascorbic acid cannot act without traces of flavanones.³⁶

I am conscious of the difficulties and incertitudes. The first steps in a new field are often somewhat shaky and there is only one way to avoid error with certainty, and that is not to work or at least to avoid new fields.

Whatever the explanation may be, the fact remains that under certain conditions vitamin P has a striking effect. It prolongs life, prevents loss of weight and the development of part of the scurvy symptoms, in the first place haemorrhages. It is hoped that a better test for vitamin P will be found soon, though the factors, influencing results, are not yet fully known.

One other fact remains. This work has brought to light the activity of a group of substances and seems to have increased the inventory of the doctor by one useful tool to fight disease.³⁷

The author has prepared big quantities of citrine for distribution among clinicians, anxious to test it. Naturally these experiments could not clear the problem of therapeutic application. Such problems want very extended experience, and

³⁶ Ascorbic acid is much more readily destroyed than hesperidine and the scurvy diet might contain traces of Vitamin P.

³⁷ The substance was mostly applied intravenously. One component of citrine, hesperidine, by its insolubility, is unfit for injection, which threatens with dangerous complications. Its other constituent, however, the eriodictyol glucoside, is soluble and can be injected without any harmful effect. According to the nature of the substance it must not be applied in fractions of milligrams but in doses of 25—200 mg. a day.

these first trials were sufficient only to indicate that this substance is a useful one. The effect on resistance and permeability of capillaries is quite definite. Its curative effect in haemorrhagic diathesis (vascular type) is definite too. (81, 82). It acts also on different internal haemorrhages (intestine, kidney, gums) of unknown origin, but its effect seems not to be limited to haemorrhage. The effects obtained in the few cases of acute nephritis were striking (*A. Lajos*) and might lead in the end to the application of vitamin P in any condition threatened with nephritis. Sepsis and polyarthritis are very difficult to judge, but the observations were by no means discouraging.

I am quoting these latter observations only in the hope that they might induce clinicians to try vitamin P in a wider field, for only wide experience will help to establish definite indications.

The fate of Vitamin P in the body will be elucidated only by extensive studies. The first experiments of *Huszák* (68) indicate that this vitamin is not appreciably accumulated or destroyed in the animal. All the same, as *Armentano* is finding in *Rusznayák's* department, Vitamin P has to be given to man for several days before excretion starts and reaches maximal values. This indicates that a deficit in Vitamin P is a wide occurrence. Patients with fever (polyarthritis) who are benefited by Vitamin P excrete this substance at once and seem to be unable to retain it.

5. On Health, Disease and Vitamins.

In the previous chapter I made the statement that Vitamin P cured certain haemorrhagic conditions. The implications of this statement are amazing, if not bewildering. We learn at school that Vitamins have a beneficial effect only in case of a shortage. The food of these haemorrhagic patients was in no way different from average human food, thus there was no reason to suppose that their pathological condition was an avitaminosis. But if this is true, then Vitamin P must be looked upon as a therapeutic agent with pharmacological

activity, which again is in contradiction to our generally accepted ideas.

The case of Vitamin P could be dismissed as a curiosity, had not clinical research accumulated a number of analogous observations recently. The isolation and synthesis of the single vitamins enables the doctor to administer these substances parenterally in relatively big quantities to his patients. This application of Vitamins has led to astounding observations. We read reports on the beneficial effect of Vitamin C in pneumonia and myasthenia, of Vitamin B₁ in neuralgia, neuritis and even alcoholic neuritis. I mentioned that Vitamin P has an analogous effect in acute nephritis and restores capillaries to normal resistance and permeability in sepsis. Should all these conditions be avitaminoses? This is impossible. Or should Vitamins act as specific therapeutic agents? This seems equally impossible. It is evident that there must something fundamentally wrong in our ideas.

I believe the trouble is that we were badly misled by the animal experiment. The animal experiment has helped to lay the foundation of our knowledge of vitamins but has misled us in the more subtle question of health and disease. We made a mistake in calling it „health“ if the animals had no scurvy, beri-beri etc. We called it health when a dozen animals sitting in a protected cage grew well. In short, I believe that scurvy and beri-beri are not the first signs of unhealthiness, but are premortal syndromes. Cessation of growth is equally a grave disturbance of health. There is a long way to go from „full health“ to the first disturbance of growth, or the first appearance of scurvy symptoms.³⁸ Accordingly there is a wide margin between the quantity of vitamin sufficient to prevent scurvy and that required to keep us at optimum condition. This partial avitaminosis is a very wide occurrence and I believe that the greater part of humanity is living within this zone. I call the condition „full health“, in which health can be improved no more, in which we offer the greatest resistance to noxious influence and in which we can bear strain the best and show the highest ability. A dozen guinea pigs sitting in

³⁸ This might apply also to the Rumpel-Leede phenomenon.

a protected cage can tell us little about full health. Man living under civilized conditions can be compared to these sheltered animals. He might do quite well and have no indication of his deficiency, but will fail, if suddenly called upon to fight an infection, face some injury, or if by any chance he has some debility like that of the capillary system. The doctor will call the result of this failure „pneumonia“ or „nephritis“ or the like, and will bless the therapeutic effect of vitamins. But what he has done, in applying vitamins, is only to pay the old debt to nature and give to the body what is due to the body and what it needs for its smooth running.

Accordingly the necessary daily dose of a Vitamin is not the quantity which is needed to prevent scurvy. Applying Sir *John B. Orr's* words to Vitamins: we must increase the quantity of vitamins, till a further increase does not improve health. This is the correct daily dose, meaning by health full health, as defined above.

As an experimental worker I will try to demonstrate the inadequacy of the animal experiment by animal experiment. A few years ago *Jeney* and *Gagyi* reported on the beneficial effect of ascorbic acid in experimental diphtheria. These experiments have since been greatly extended and it is generally accepted to-day that healthy (not scorbutic) guinea pigs can be protected against diphtheria toxin by massive doses of Vitamin C. This observation is quite analogous to the clinical observations quoted above. The correct explanation of this result is not that ascorbic acid is an antitoxin, but that we made the mistake of calling animals which have no scurvy, normal, healthy animals. If ascorbic acid increases the resistance against diphtheria, this means that it increases the health of the animal. Health, which can be increased is not „full health“. This experiment simply shows that massive doses of ascorbic acid are needed to keep the guinea pig in „full health“.

I can support this statement with figures. *S. S. Zilva*³⁰ finds that he needs 2 mg of ascorbic acid daily to keep his animals in perfect condition. At the same time he is amazed to find

³⁰ *S. S. Zilva. Biochem. J. 30, 1419, 1936.*

that he needs ten times as much to keep his animals at maximum saturation and asks whether this maximum saturation is needed for health? He turns it down. 20 mg of ascorbic acid a day seems to be quite extravagant for a guinea pig, when the dose for man is 25–50 mg. His animals grow beautifully with 2 mg a day, so he thinks this maximum saturation is a luxury. But if he had given diphtheria toxin to his animals, he would probably have found that his saturated animals fared better than the ones kept at 2 mg and would have come to the conclusion, that a health which can be improved cannot be maximal.

But let us consider for a minute, whether 20 mg ascorbic acid a day is such an unreasonable amount for a guinea pig. Let us answer an other question first: What is a vitamin and why is ascorbic acid a vitamin for the guinea pig but not for the rabbit? Guinea pigs in their original, tropical ever-green surrounding had plenty of ascorbic acid all the year round. With every leaf consumed, ascorbic acid flowed into their bodies. Nature knows no luxury. There was no need to make ascorbic acid, so the guinea pig forgot how to make it. This the rabbit could not afford, because he would have died of scurvy in our climate, during the winter when there is no green food and no ascorbic acid is available. Thus the inability of the guinea pig to make its own ascorbic acid is an expression of its adaptation to its surroundings. All animals are perfectly adapted to their surroundings, and this is no more than natural. From the point of view of food „surroundings“ means green food for the guinea pig, all the year round. A smallish guinea pig of 300 g. consumes about 120 g of green a day. This contains about 20–60 mg ascorbic acid which is about the quantity *Zilva* finds necessary to keep his animal saturated. This is also the quantity of ascorbic acid necessary to protect a guinea pig against diphtheria, the quantity which will keep it in full health, at which a further increase of ascorbic acid does not increase resistance or raise the ascorbic acid concentration of the body. One cannot do more than saturate an animal.

I expect that the correct daily dose of any vitamin will be found in the end to be that quantity of vitamin which the animal consumed in its original surrounding.

One problem that always puzzles me when I read about „therapeutic effects“ of vitamins, is this: What would have happened to these patients, if they had had sufficient vitamin prior to their disease. It is very much easier to prevent than to cure a disease, and had these patients had a sufficient quantity of vitamin, they would probably never have become ill.

This brings me to the highest and most involved problem that ever occupied me as a scientist.

I started my studies with medicine and spent five years studying all those thousands of ailments, from which we suffer. The next twenty years I spent as a biochemist in silent admiration of the wonderful complexity, precision, harmony and adaptability of living Nature. I cannot help wondering where the contradiction lies. Is man the sole imperfect creation which is kept alive only by the artificial means which his own ingenuity has created? Or is our body not less perfect than that of other living creatures, only put to a use for which it was not made? I think that answering this problem is of more than sentimental or philosophical interest. The answer may influence our attempts to improve health, reduce suffering and increase happiness.

I am convinced that Nature never creates such an imperfect system as our body appears to be. By the rules of life no such system could survive.

I believe that any living object has to be perfect and is strictly adapted to its surroundings (or else it would die). In fact it is not only adapted to but it is part of its surroundings. The limits of life are determined by the quantity of nitrogenous material. This frame is always filled out almost completely and is present in living form. We are badly misled by the apparent feeling of our individuality. Any living system is part of its surrounding, a temporary form of matter pushing to life. The animal in the jungle is only a cell, a cogwheel of this higher organism, the jungle, into which it fits perfectly. Any imperfection in this fitting would entail its ruthless extermination.

I am convinced that man's body is just as perfect as that of his fellow animals, and his apparent imperfection arises from the disharmony between his structure and his surround-

dings. Man was not born on the pavements of cities. Man was born during innumerable years in some jungle, to the life of which he was strictly adapted. Our civilisation is of recent date and has left no marks on our body. If we want to be healthy, we must put our body back into the surroundings for which it was made.

Naturally we cannot be expected to go back to the jungle. But there is science to help us to find out which factors of our surroundings are essential, and to bring those factors into our homes. These factors are manifold: the quantity of radiation, the purity of air, amount of noise, the amount of muscular work and the reduced chance of infection, etc. etc.

One of the most important links between our body and its surroundings is certainly food. In the form of food the surroundings actually get into our body, flow through it. And certainly vitamins are one of the most important factors of that coordination.

I am convinced that if our body is put back into the surroundings for which it was made, it will work as perfectly as that of its fellow creatures. Disease is the expression of the disharmony between our make-up and our surroundings. There is no such thing as healthy or unhealthy. The fish feels uneasy on land, the rabbit under water. It all depends what we are made for.

The story of the island *Tristão da Cunha* is a full corroboration of all this.

I have a strong faith in the perfection of the human body, and I also think that vitamins are an important factor in its co-ordination with its surroundings. Vitamins, if properly understood and applied, will help us to reduce human suffering at an extent which the most fantastic mind would fail to imagine.

Post Scripta.

While this book was printed *H. A. Krebs* and *W. A. Johnson* (*Enzymologia*, 4, 148, 1937) published a paper bearing closely on the C_4 theory of respiration. According to these authors the C_4 are but members of a more complicated cycle in which oxaloacetate combines with „triose“ to form citrate. Citrate on its turn is oxidised, step by step, into succinate, succinate into oxaloacetate which again combines with triose. The carbohydrate is thus oxidised *via* citrate.

Undoubtedly, much can be said in favour of this theory, which, if found correct, would necessitate a thorough revision of this book.

Citric acid has occupied also my laboratory (*F. L. Breusch*) for some time, for its close chemical relation to C_4 , and the analogous behaviour of the citrico- and malico-dehydrogenase, both suggest a catalytic function. We can corroborate most of the observations of *Krebs* and *Johnson*. We think, however, that the observations of these authors are capable of a different interpretation. Our experiments rather suggest, that the cycle, even if it exists, has but a minor part in the total oxygen up-take of muscle.

This is not the place to enter discussion and I am quoting our observations merely as an apology for publishing this book unchanged.

In another paper *H. A. Krebs* showed (*Perspectives in Biochemistry*, Cambridge University Press, 1937) that in the oxidative processes of *Bacterium coli* fumaric acid can replace oxygen. He arrives at the conclusion that the system of succinic-fumaric acid, together with its dehydrogenase plays, as catalytic H transmitter, an important rôle also in bacterial metabolism.

I want to mention here a few further observations, made since the foregoing pages were written.

Parnas and Szankowski (*Enzymologia* 3, 220, 1937) have found oxaloacetate equivalent to pyruvate as H acceptor of triose and have given herewith strong support to the theory of the *Pasteur Reaction*, presented in this book.

H. v. Euler and *M. Malmberg* (*Z. f. physiol. Chem.* 242, 85, 1937) have found vitamin P active in promoting the formation of reticulocytes in scurvy, giving thus a new evidence for the biological activity of this substance. The authors find a certain synergism between C and P.

I have been informed that a laboratory, with which I had the pleasure of collaborating a few years ago, has also tried the effect of hesperidine combined with minute doses of vitamin C in scorbutic guinea pigs. These experiments, performed simultaneously and independently, have led to results identical with those of *Bentsáth*. Unfortunately, however, *Bentsáth* himself was unable to corroborate his previous findings in a later series of experiments. The results of a third laboratory were equally negative. It is evident, that there is a further unknown factor still, responsible for this variation. May be, a new and labile accessory food factor is hiding behind these discrepancies and I am afraid, much work will be needed, till their final cause will be revealed.

During the last weeks, at the discussions of the *Solvay* conference at Bruxelles, it became clear to me that the „ascorbic acid oxidase“ is not merely an oxidase. As mentioned on p. 77, *Hopkins* and *Morgan* showed that „ascorbic acid oxidase“ activated ascorbic acid also in its reaction with glutathion, speeding up the reduction of dehydro-ascorbic acid by SH. The function of the enzyme is thus primarily not to oxidise, but to *activate* ascorbic acid. In presence of O₂ the activation will reveal itself in an increased oxidation of ascorbic acid and the activator will act as an oxidase. The „ascorbic acid oxidase“ is thus a new example of a protein, activating a smaller molecule and ascorbic acid is an example of a vitamin activated within the cell. *v. Euler* expressed similar views.

It has been reported by *Lawrence, McCance* and *Archer* (*Brit. med. J.* No. 3995, 1937) that succinic acid, applied in two

diabetic patients, had no effect on acidosis. This suggests, that the cases, observed by *Korányi* responded more favourably, than many diabetic patients will do. Undoubtedly, acidosis represents a very complicated problem and more experience will be needed to arrive at definite conclusions and to find out whether succinic acid is useful at all fighting acidosis. There can be little doubt about the effect of succinate on acetone formation is certain experimental conditions and in tissue slices, which effect can readily be demonstrated. The system, composed of a human patient and a clinician is too involved and many things might happen in such a complex system.

I want to mention a paper at last, published by *L. F. Leloir* and *M. Dixon*, which reached me but lately (*Enzymologia*, 2, 81, 1937). This paper deals in part with the action of pyrophosphate, which substance strongly inhibits respiration. According to these authors, to the one of whom we owe much of our knowledge of respiration, pyrophosphate has an action analogous to malonate, poisoning the succino-dehydrogenase in a specific way. Its inhibitory effect on respiration is explained by its action on the C_4 system. The action of pyrophosphate is thus a further evidence for the correctness of views presented in this book.

Oct. 18, 1937.

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