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 *Warburgs* 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 loose 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

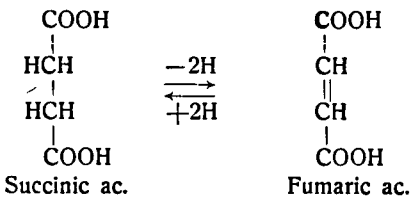

Fig. 1.

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 studing 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), *Gözsi* (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 adenyl 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-*

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[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 phosporic 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. l. Ogston* and *D. E. Green*. Biochem. J. 29. 1983, 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

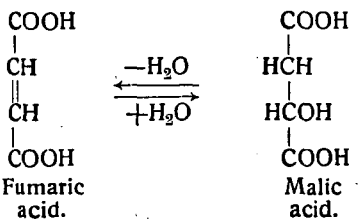

Fig. 2.

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. (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

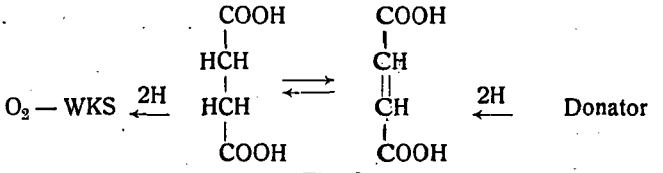

Fig. 3.

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. *Gözsy* (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.

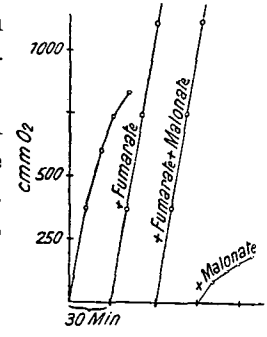

Fig. 4.

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 succesfully 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

[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 enzyme. Thus malonate is unable to compete with the added fumarate, the quantity of which is mostly very great compered 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 succesfully 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, oxalocetate 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.

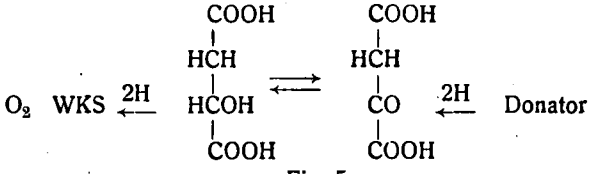

Fig. 5.

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 ist 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;

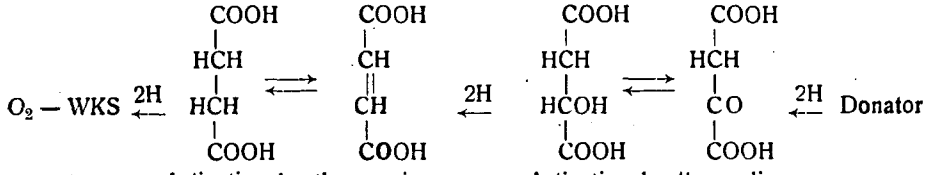

Fig. 6.

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. Probdably 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

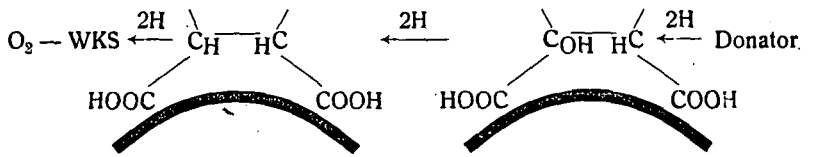

Fig. 7.

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), *Gözsy*'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 C_4 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.

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[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. ($E = -0{\cdot}060$ 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.

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[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-transportases" 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-

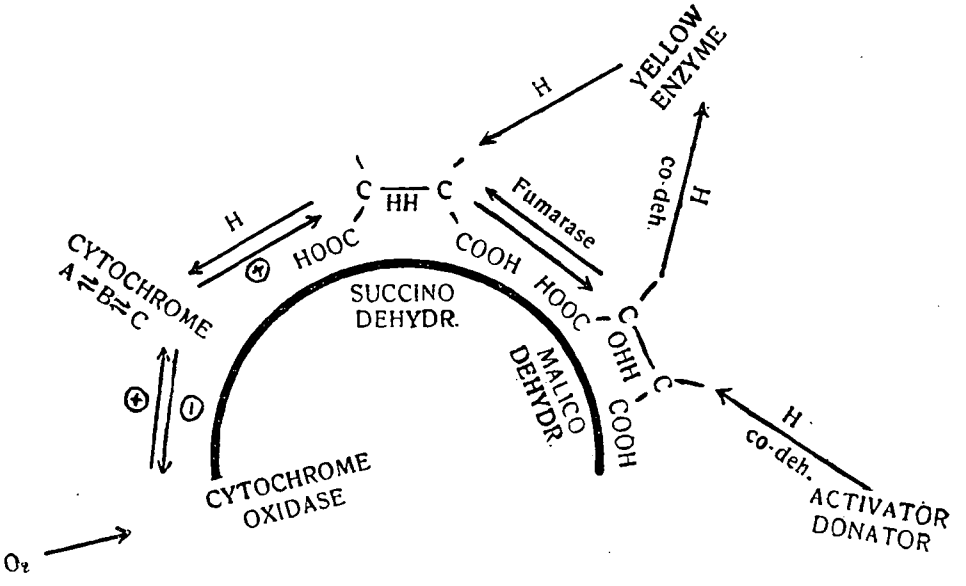

Fig. 8.

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. (Liebigs Ann. *530*, 99, 1937).

Banga's experiments is, that in the case of more negative dyes the succino-dehydrogenase not only does not enhances 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*, unspublished). 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

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[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 methylenne 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. Dehyrogenases, 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.