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-synthetised 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-

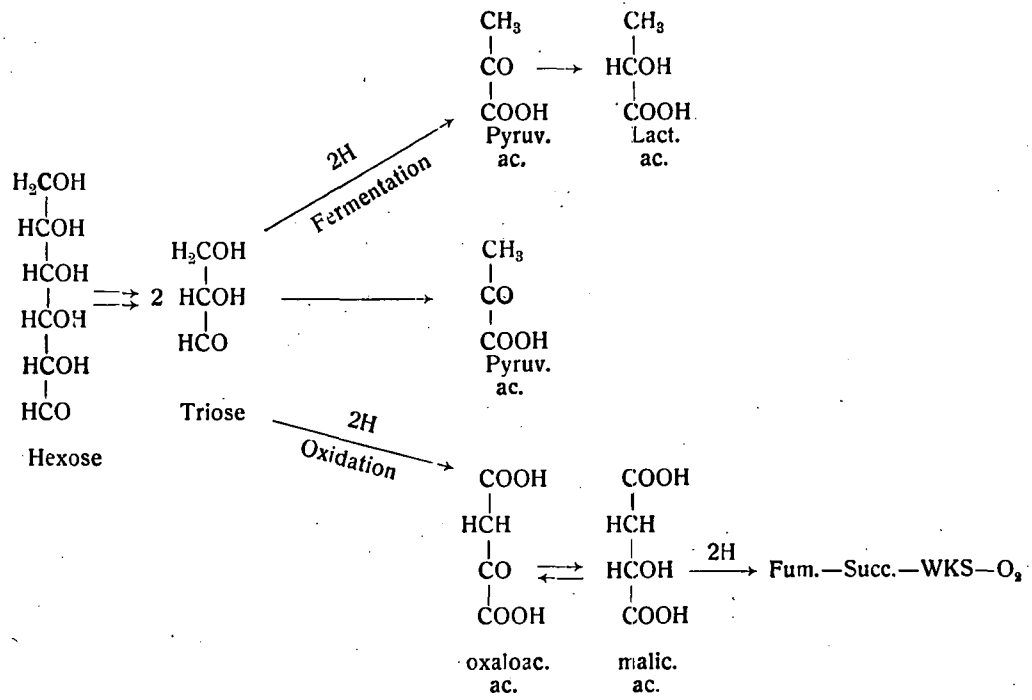
Hexose
Triose
2H
Fermentation
2H
Oxidation
Pyruv. ac.
Lact. ac.
Pyruv. ac.
oxaloac. ac.
malic. ac.
2H
Fum.—Succ.—WKS—O2

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 succesfully 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 α-β 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-acetice which is reduced by the H of the donator, 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 phosphorification 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 phosphorification 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 phosphorification 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. *Gözsy* 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 phosphorification and the rôle of adenylic acid and adenyl-pyrophosphate played therein, are of secundary 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)*.

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[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 synthetised. 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 yelow 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-furmarate. (It does not seem impossible that the yellow enzyme is also connected with phosphorification, 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 resynthetise 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 mechnism 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 synthetised 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 sufficienty, 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 $^1/_2$ O_2 was used and one molecule CO_2 was formed. This means that after loosing 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 O. 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 $C_2H_4O_2$, 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, resynthetised 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

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[25] p. 28.

resynthetise the rest into carbohydrate. The above considerations give, at least for the quotient $^1/_3$, 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 corbohydrate 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. C_4 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 C_{18} 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.

Embden 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-

[26] *F. Knoop.* Klin. Wochenschrift. 2, 60, 1923.

carboxylation of the molecule. *Embden's* discorvery 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 rout 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 hyroxybutyric 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

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[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 an other, 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 inhibted.

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-deydrogenase.

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 whithin 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 recorved 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

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[29] See. s. 20.

the point of view of his experience he justly criticises the C_4 theory[30] 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 cytocrome 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-dehyrogenase. 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-

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[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 micromethods 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 disappearence 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 excesse of oxaloacetate inhibitis succinodehydrase strongly and makes hereby the reoxidation of malate impossible.

Methods for the estimation of succinate *(Gözsy)* (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.