A Symposium on
RESPIRATORY ENZYMES

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Foreword

In 1897 Buchner published his classical study on alcoholic fermentation by cell-free yeast juice. In the same year Eijkman concluded that beri-beri among the natives of the Dutch East Indies was caused by a dietary deficiency arising from the use of polished rice. These two discoveries may be said to have initiated the modern investigations in two of the most important fields of biochemistry and medicine: the nature of the respiratory enzymes and the function of the vitamins in cellular metabolism.

For the next thirty years research workers in these two fields pursued their investigation almost independently of one another and more or less oblivious to the progress being made in the other's field. Then in the early part of the last decade it was discovered that riboflavin was the functional group in a respiratory enzyme and very soon afterward this compound was shown to be vitamin B_2 (G). Further discoveries of a similar nature soon demonstrated that the enzyme chemist and the nutritionist were to a great extent prospecting the same territory. The time seemed to be ripe, therefore, for the two groups to join in a discussion of the latest advances. Such a meeting on the "Respiratory Enzymes and the Biological Action of the Vitamins" was sponsored jointly by the Universities of Wisconsin and Chicago, institutions that have long been leaders in these fields.

This book contains the lectures and discussions given at the University of Wisconsin. It deals with the fundamental nature of those enzymes that are intimately connected with the functioning of the vitamins. Informative presentation of the latest developments, interpretation of past and present findings, and indication of some of the problems still unsolved in respiratory enzyme research are given by recognized international authorities in the field. Supplementing these explanations of the fundamental nature of respiratory enzymes are discussions applying the findings to specific problems.

The Program Committee wishes to thank the many members of the faculty for their cooperation in arranging the meetings held at the University of Wisconsin, the speakers for their papers and discussions, and the Wisconsin Alumni Research Foundation for the grant which made these sessions and the publication of the present volume possible.

Address of Welcome

C. A. DYKSTRA
President of The University of Wisconsin

THE UNIVERSITY OF WISCONSIN is a happy host today. It welcomes to its campus scientists from many laboratories who are drawn together for the discussion of common problems and common aims. It recognizes in this symposium the challenge that faces intelligent men of good will everywhere—the great need there is in the contemporary world for sitting down and reasoning together. From such a process comes progress.

We are concerned here with functions which operate in the biological and chemical world. We seek these out and discover how they work so that, knowing about them, we may cooperate with nature for the good of man. This we do by observation, experimentation, analysis, and, finally, the objective setting down of results that may yield a pattern or a principle. As we look about us and see biological specimens called men reacting to special or group interests as passion and selfishness may happen to dictate, we ask ourselves, a bit dismally perhaps, whether the statesmen and public leaders of the world can ever be persuaded to try out the scientific method as an approach to the problems of world organization. We also need desperately a healthy society and a sound international body.

Here, today, we pay tribute to the internationalism of science. As we scan our program for the week we are struck by the fact that men from different backgrounds and from many nationalities and races can come together peacefully in a symposium to present the results of long years of human effort in a field of science, check these results, and try to establish what they mean or may mean to life on this planet. Today and right here men labor together who, were they still living in their family homelands, would be enemies, legally and politically. This is the great modern paradox—that as the world of communication has made the globe a unity and as the domains of science, literature, music, art, commerce, and industry have become international, we have at the same time the phenomenon of a more bitter nationalism than ever before. Something is wrong that needs

early correction, and intelligent men must give attention to the challenge.

We meet today to talk of many things in the wonderland of science. We have the special vocabulary necessary for the accuracy of our thinking and investigation. This vocabulary is a closed book to the man in the street except for a few words, such as vitamin, for instance. This man in the street, however, does get a partial implication of your work as he hears or reads the advertiser who expounds the merits of certain food products. He may even be led to think that he can be a vigorous and whole man if only he has a box of pills or capsules in his vest pocket. He may even be duped or exploited because of this partial knowledge.

We therefore have the obligation in our special fields of science which promise so much to all to attempt such simplification and general statement that those things for which we can vouch will become common knowledge at the earliest possible moment. Just now there is a wide spread of interest in many areas which this symposium deals with. It is a good time, therefore, to capitalize on this popular interest, for we have a receptive public. We of the public are willing and anxious to learn from you.

We here at Wisconsin are glad you are with us. We are happy too in the cooperation of our sister institution, the University of Chicago, in the enterprise here represented. Our welcome is genuine, and we wish for the conference unusual and distinguished success.

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A SYMPOSIUM ON RESPIRATORY ENZYMES

Addresses given at an Institute
Held at the University of Wisconsin
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Intermediate Carbohydrate Metabolism

OTTO MEYERHOF
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THIS REVIEW OF THE intermediary carbohydrate metabolism must necessarily be treated broadly and generally, for the subject has many different aspects, and the detailed questions of hydrogen transport, Pasteur effect, pyridine nucleotides, cocarboxylase, metabolic cycles, phosphorylations, indeed all the items which are intrinsic elements of the present picture of carbohydrate breakdown, will be dealt with by competent investigators of these subjects. Moreover, I had the opportunity to discuss the special question of oxidoreduction and dismutation in carbohydrate metabolism at the Chicago congress some months ago.

If we take this occasion to look back fifty years and to compare our present knowledge with that which existed at the end of the last century we have reason to be very proud, for at that time this whole field appeared nearly as *tabula rasa.* But two outstanding achievements had already been accomplished: first, Claude Bernard's work on the interconversion of glucose and glycogen in the liver and on the role of blood sugar under normal and diabetic conditions; second, the work of Pasteur on the different microbic fermentations as manifestations of the anaerobic metabolism of these organisms. Nothing was known about the oxidative breakdown of sugar. Although lactic acid formation in the blood and especially in the muscles had been observed by Claude Bernard and others, it was not known whether nor how this cleavage was connected with respiration.

Since then the interconversion of glycogen and blood sugar have continued to claim the attention of medical investigators, and recently, as you know, a highly interesting development was reported: Professor Cori's discovery of glucose-1-monophosphoric acid as intermediary. The old problem of diabetes was shifted by the isolation of insulin from the study of blood sugar regulation to the biochemical task of studying tissue metabolism under the influence of added hormones. The third old problem of the connection between

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fermentation and respiration remained for a long time a subject of speculation, and even now many a question is unanswered. However, I should like to follow this latter trend of ideas a little more in detail.

Pfeffer and Pflüger, following Pasteur, held a rather simple view of this relationship: the first step of respiration was assumed to be always anaerobic. If no oxygen is present, the products of anaerobic cleavage accumulate: alcohol in yeast and higher plants, lactic acid in the tissues of higher animals and in some bacteria. But if oxygen is present, these products are oxidized to carbon dioxide and water.

That this concept required modification became apparent twenty years ago from studies of metabolism of muscle. In 1907 Fletcher and Hopkins (1) showed that under anaerobic conditions frog muscles formed lactic acid steadily during both activity and rest, and that this lactic acid disappeared when oxygen was admitted. Parnas (2), working some years later in the same Cambridge laboratory, claimed to have found that this disappearance was a complete oxidation, thus apparently confirming the views of Pfeffer and Pflüger. In 1920, because of the controversial state of this question, I repeated the experiments of Parnas, avoiding especially all kinds of irritation or injury of the muscles which would lead to extra-consumption of oxygen (3). Under these conditions much more lactic acid disappeared in oxygen than could be accounted for by oxidation, and the lactic acid unaccounted for was reconverted into carbohydrate. This was true for the lactic acid formed during activity as well as for that formed during rest. Similarly, it was shown that in equal periods of rest much more lactic acid was formed anaerobically than could be burnt aerobically by the resting respiration. Indeed, the amount of oxygen which failed to be used in a period of anaerobiosis was about the same as the excess consumed after that period. This oxygen was sufficient only to oxidize from a quarter to a sixth of the lactic acid which disappeared.

These facts, which are independent of special interpretations, are sufficient to invalidate the original theory of Pfeffer and Pflüger in that they show that the oxidative removal of fermentation products is not necessarily identical with the oxidation of these products. But we can pose the more limited question whether the oxidation on the whole attacks the end products of anaerobic breakdown. With respect to lactic acid formed in a preceding anaerobic period, we must surely answer in the affirmative. We know that lactic acid is easily oxidized by way of pyruvic acid. For example, Barron *et al.*

(4) showed that specially treated, washed bacteria may lose the power to oxidize sugar and other substrates, but retain the power to oxidize lactic to pyruvic acid. Experiments on muscle lead to the same conclusion. After a muscle is poisoned with iodoacetic acid the formation of lactic acid is blocked; at the same time the respiratory quotient drops to 0.7, and is not changed by the addition of sugar, but is brought to 0.95 by the addition of lactic acid. Respiration is increased, and oxygen consumption is essentially equivalent to the disappearance of lactic acid (5). Similar results were obtained by Krebs with respiration of brain and testis after poisoning with iodoacetic acid (6). Since oxidation of sugar is completely checked, no interpretation is possible except that lactic acid is directly oxidized.

But this is not necessarily the pathway of sugar oxidation in the aerobic steady state. That independent ways of sugar oxidation exist may be gathered from many observations, such as the rapid oxidation of fructose in brain tissue, where, in contrast to glucose (7), it does not give rise to anaerobic lactic acid. Furthermore, Warburg and Christian showed that hexosemonophosphate can be oxidized by the triphosphopyridine nucleotide in yeast extract to phosphogluconic acid (8), and Lipmann demonstrated the complete oxidation to carbon dioxide in this manner (9).

On the other hand, the oxidation of sugar by way of pyruvic acid is also firmly established, and in this case the steps up to the formation of the acid are identical in respiration and in anaerobic glycolysis. As was discovered by Peters (10), pyruvic acid accumulates during oxidation of carbohydrate by cells and tissues in cases of vitamin B_1 deficiency, which means that lack of cocarboxylase blocks the oxidative decarboxylation of pyruvic acid. Many other findings, such as the similarity of the oxidation of pyruvic acid to that of sugar in tissue pulps and extracts, point in the same direction, namely, that sugar is oxidized via pyruvic acid (11). Thus several pathways of sugar oxidation exist, the choice of which may depend upon the special set of enzymes in different tissues and also upon hormonal and other controlling influences.

All this probably has some bearing on the relationship already mentioned between oxidation and interference with the mechanism of fermentation. I have mentioned before the two possible cases of this relationship—the actual synthesis of split products to the initial substance and the non-formation of the split products during the stationary state of respiration. Without fearing to be accused of a

biased judgment I dare say that both cases are characterized by the same numerical relationship—the oxidation quotient, which expresses the ratio of the aerobic disappearance of splitting metabolism in moles sugar to the oxidized sugar equivalents (12). Critics have objected that under extreme conditions this number may range from zero to infinite, but it is equally true, and more important, I think, that under physiological conditions living cells exhibit quotients between 3 and 6—approaching 6 more and more as the conditions of temperature, oxygen pressure, nutritional state, and milieu become optimal for the cells in question. The same preference for the quotient of 6 was demonstrated by O. Warburg for different warm-blooded tissues where the anaerobic glycolysis is high enough to allow the calculation of the quotient (13).

The original concept of a metabolic carbohydrate cycle involved the assumption that in the stationary state the quotient results from a continuous overlapping of anaerobic glycolysis and of oxidative resynthesis of the cleavage products—the endothermic resynthesis made possible by coupling with oxidation. Today it seems possible to refine this scheme and to modify it somewhat without rejecting the main argument. Indeed, in the past fifteen years a tremendous amount of material has been collected to prove that the general concept of these cycles in carbohydrate breakdown holds good, that every oxidative step is coupled with an involuntary phosphorylation, and that the several intermediate stages of the anaerobic breakdown can be reversed by means of the "energy-rich phosphate bonds" (31) created in this way.

On the other hand, the original concept of a single complete cycle passing through the stage of lactic acid cannot be exactly true for a very simple reason, which has become clear since 1933; namely, that pyruvic acid is the necessary precursor of lactic acid in glycolysis and of alcohol in yeast fermentation (14). Under anaerobic conditions the reduction of pyruvic to lactic acid is compensated for by the oxidation of phosphoglyceraldehyde to phosphoglyceric acid. The latter, in turn, is decomposed via two intermediaries to pyruvic acid (15). The hydrogen transfer proceeds in both directions by the way of cozymase, the diphosphopyridine nucleotide of Warburg.

But if oxygen is present the dihydrocozymase can transfer its two hydrogen atoms to oxygen instead of to pyruvic acid by a long chain of oxidative catalysts: the pheohemin enzyme of Warburg, the three cytochromes, and the flavinproteins; consequently the pyruvic acid

is not reduced. On the contrary, such an oxidation of dihydrocozymase shifts the equilibrium in the opposite direction, so that lactic acid, if present, would be oxidized by cozymase to pyruvic acid, whereas in the stationary state of sugar oxidation pyruvic acid would be continuously formed by way of phosphoglyceric acid, without a compensating reduction.

Therefore only pyruvic acid, and not lactic acid, is formed in the stationary state of oxidation. This interpretation at the same time gives a clue to the oxidation quotient, the numerical relationship between the oxygen consumed and the lactic acid that is prevented from being formed: if one atom of oxygen is required to oxidize the two hydrogen atoms of dihydrocozymase, then this atom prevents one molecule of pyruvic acid from being reduced to lactic acid or in yeast fermentation to alcohol. Therefore six atoms of oxygen (corresponding to the complete oxidation of one molecule of lactic acid) can prevent six molecules of lactic acid from being formed, and we obtain the normal oxidation quotient of 6. Of course this refers only to the principle. The cozymase reoxidized by oxidative catalysts must dehydrogenate other intermediary stages besides triosephosphate, because every oxidative step in the breakdown of sugar acts in the same way, preventing the formation of one molecule of lactic acid per one atom of oxygen taken up.

And this is only one side of the picture. If the breakdown of sugar in oxygen and in nitrogen proceeded with the same speed to the stage of pyruvic acid, and the only difference consisted in the fate of pyruvic acid to be reduced or further oxidized, then the oxidation would not prevent, as it actually does, by this so-called "Pasteur effect," the greater part of sugar from disappearing. But here the concept of metabolic cycles has its place. Actually every oxidative step is coupled with the phosphorylation of the adenylic system, and by this means a corresponding phase of anaerobic breakdown is reversed, so that for every oxygen atom consumed one three-carbon molecule can return to its initial stage as sugar or glycogen. This state of affairs is very neatly shown by the recent experiments of Cori, Kalckar, and co-workers (16) with dialyzed extracts of kidney and heart, and by experiments of Belitzer and Tzibakowa (17) with washed pigeon muscle. Cori and his group found that in the presence of the complete glycolytic coenzyme system the organ extracts oxidize glucose and phosphorylate an excess of it, so that for every hexose molecule burned to carbon dioxide, ten molecules of phosphate are taken up to form five molecules hexosediphosphate; and

since the oxidized molecule also had to be phosphorylated, altogether twelve molecules of phosphates are taken up for one molecule glucose or twelve oxygen atoms consumed. Therefore every step of glucose oxidation consisting in an oxidoreduction between cozymase and an oxidizable intermediary is coupled with phosphorylation. Not only is this true for the two steps where it is already known, i.e., the oxidation of phosphoglyceraldehyde and that of pyruvic acid, in which Lipmann discovered acetylphosphate as the primary product of oxidation (18), but for every such step an energy-rich phosphate bond is created in adenosinetriphosphate, which enables a synthetic step to take place.

The experiments of Belitzer and Tzibakowa are a little different, because they added creatine to cut muscle and obtained under these conditions a synthesis of creatinephosphate when lactate, pyruvate, or the four-carbon acids of the Szent-György cycle were oxidized. At the most two molecules of creatinephosphate were formed for every oxygen atom taken up. Although the presence of creatine diverts the pathway of synthesis from carbohydrate, the experiments are important in that they demonstrate the uptake of two molecules of phosphate by way of adenosinetriphosphate for one atom of oxygen consumed; this relationship is comparable to the synthesis of creatinephosphate in muscle extract, where two steps of glycolysis are involved in the transfer of phosphate, namely, the oxidoreduction and the dephosphorylation of phosphopyruvic acid (19).

Moreover, the reaction studied by Belitzer is closely analogous to the recovery period of the living muscle, especially a muscle which is only slightly fatigued. Here, during oxidative recovery, the oxidation serves mostly for the resynthesis of creatinephosphate, and to a small extent for that of glycogen. If two molecules of creatinephosphate are synthesized for every atom of oxygen taken up, then about 40 per cent of the combustion heat of sugar or lactate is consumed for the endothermic synthesis, a result which comes very close to the efficiency of the oxidative recovery in the living muscle.*

But to return from this digression to the significance, already mentioned, of the experiments for the theory of carbohydrate cycles. One objection may be raised against this interpretation of the Pasteur effect. Many cases are known where the respiration remains quantitatively the same, while the effect of the respiration on the

* Actually the same ratio of two molecules of creatinephosphate synthesized for one atom of oxygen taken up was found by O. Meyerhof and D. Nachmansohn (Biochem. Z., 222, 1, 1930) during recovery of a partially fatigued muscle.

glycolysis is suppressed. In the picture outlined above the oxygen used would automatically eliminate an equivalent lactic acid formation, in so far as the oxygen serves to reoxidize dihydrocozymase. But we must have in mind that the oxygen intervenes only indirectly by way of the oxidizing catalysts. Here the so-called "Pasteur enzyme" assumed by Warburg (20) and demonstrated by Stern and Melnick (21) plays its role in steering the oxidation. All oxidation not going by the way of cozymase would be without "Pasteur effect"; it may be oxidation of non-carbohydrate, which replaces sugar oxidation, or it may be oxidation of sugar by way of triphosphopyridine nucleotide.

Now we come to the second half of the problem, the actual conversion of lactic acid to glycogen in the oxidative recovery of the

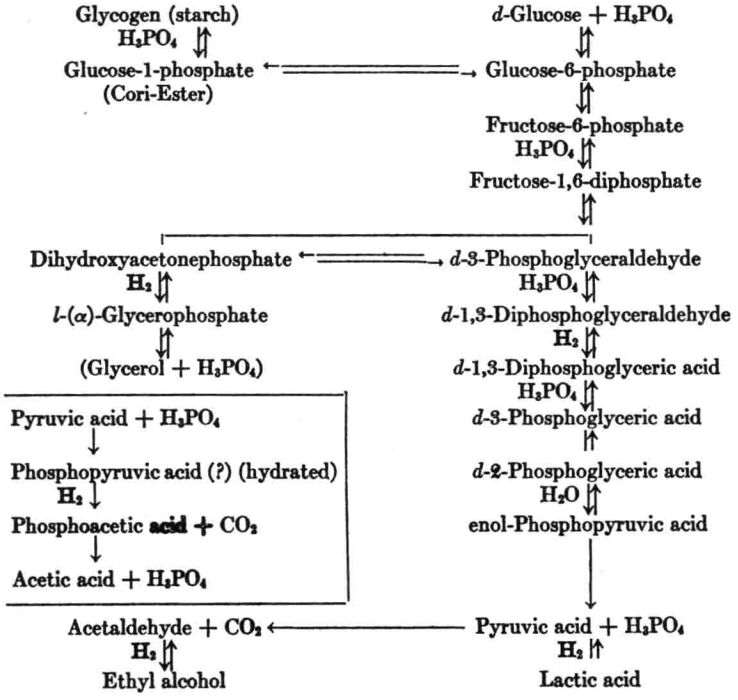

Figure 1.—Complete sequence of intermediaries in anaerobic breakdown of carbohydrate

Insertion on the left: oxidative decomposition in lactic acid bacteria, according to Lipmann (18).

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