ENERGY RELATIONSHIPS IN GLYCOLYSIS AND PHOSPHORYLATION

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Our knowledge of the energy relationships in glycolysis and phosphorylation developed in close connection with the problem of the energy-transferring chemical reactions of muscle activity. I may be allowed, therefore, to start with that topic. Leaving aside the concepts of the so-called classical period of physiology and starting with the myothermic work of Hill and Hartree about 1920, we may broadly distinguish three historic periods in these investigations.

In the first period, which fills a little more than a decade from 1920 to 1932, the relationship of the anaerobic muscle metabolism to the oxidative metabolism, as well as to the production of heat and work, was studied in the living isolated muscle. As a result, the three most important reversible systems connected with muscular activity were brought to light: (1) Splitting of carbohydrate to lactic acid and aerobic resynthesis; (2) splitting of creatinephosphate into creatine and phosphate and anaerobic and aerobic resynthesis; (3) splitting of adenylpyrophosphate and anaerobic and aerobic resynthesis.

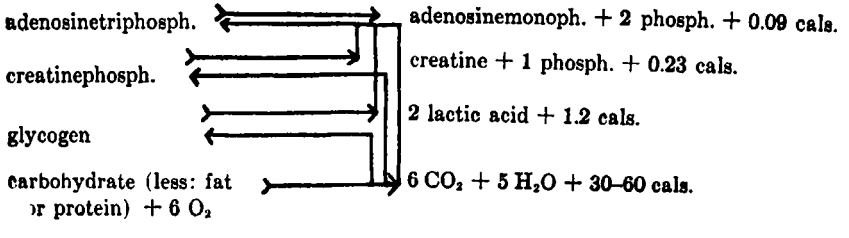
In the second period, by a close study of the enzymatic breakdown of carbohydrate in solution, the intermediates in this breakdown were isolated, transfer of phosphate and hydrogen could be ascribed to single reaction steps, and the equilibria in solution could be established, as well as the total energy of the different steps. This period again roughly fills a decade and closes with the latest discovered intermediate, 1,3 diphosphoglyceric acid, by Warburg, Christian and Negelein in 1939.

The final period, in which we now stand, thus far has shown three different trends: firstly, to develop a more general theoretical view of the energy transfer by means of energy-rich phosphate bonds, especially in the contributions of Lipmann[1] and Kalckar[2] to this subject; secondly, to generalize the relation between hydrogen transfer and phosphorylation to all oxidative steps, that is, to the respiration of carbohydrate as well as to the oxidoreduction. In addition to the work of the above investigators, one may mention several papers from Cori's laboratory[3] and also some by Ochoa.[4] The third tendency has been to apply our knowledge to a final understanding of the contraction mechanism of muscle by means of the hypothesis that the phosphate-bond energy is finally transferred to the contractile protein itself. I

here refer to the work of Engelhardt, followed by that of J. Needham and others. Although these attempts, at the moment, are highly speculative, eventually they may lead to the clue to this last energy transfer. I shall restrict my report mainly to the first two periods, in which I was able to participate more actively. I expect that Dr. Kalckar, as a representative of a younger generation, will devote more of his time to the more recent developments.

Before going into details, I may state that the heat of the different reactions can be measured by direct calorimetry with considerable accuracy,—much more accurately than by calculation from measured combustion heats, which involve small differences between large values. The latter method is subject to great errors, especially if the heats of solution and dilution are not known or if the effect of phosphorylation is disregarded. But in some cases, we must rely on the latter procedure, where, for any reason, direct calorimetry is inapplicable. All energy-rich phosphate bonds were found by direct calorimetry. On the other hand, the change of free energy in the reaction can be measured directly only in those instances where the equilibrium is not shifted too far to one side (with the proportion of the reactants not higher than 99:1), so that the equilibrium concentrations can still be accurately measured. This corresponds, for monomolecular reactions, to K values between 10^2 and 10^{-2}; for bimolecular splitting or synthesis, to K values between 10^4 and 10^{-4}. For our ranges of temperature and concentration, $\triangle F$ is then always smaller than approximately 5000 calories. Since phosphorylation potentials cannot be determined by means of electromotive force measurements like oxidation potentials, free energy changes of those types of phosphate transfer which yield great energy ($-\triangle F$ over 5000 calories), and which are more interesting, must be calculated more or less with the help of thermal data.

I shall now come to the first historic period of which I have spoken. How are we to picture, with our present-day knowledge, the over-all energy exchange of a working muscle under aerobic and anaerobic conditions? Here the general scheme which summarizes the results of this period still holds.[5]



The calories here refer to the available energy stores for the different reactions in one gram of unfatigued frog muscle. Every reaction below that shown in the uppermost line of the diagram is farther away from the immediate supply of energy for work, and has, at the same time, a larger store of energy. Moreover, it is coupled with the reversal of the adjacent reaction, that is, with the recombination of the split products. But it is also coupled with all others by shunts. This is very important. For instance, the oxidation of carbohydrate can bring about the synthesis of creatine-phosphate not only by way of intermediate lactic-acid formation, but directly as well. Under strict aerobic conditions, this applies not only to the alactacid muscle poisoned with iodoacetic acid[5a] but to the normal muscle as well. Such a muscle, apparently, does not form lactic acid at all, and the oxidation serves for immediate resynthesis of creatinephosphate. Lactic acid formation is already an emergency mechanism, which, however, is set in motion under normal conditions in a muscle working *in situ;* especially, until the blood supply is adapted to the sudden increase of the demand of oxygen by the start of work. Intermediate lactic acid formation, therefore, is in response to the lack of oxygen, while breakdown of creatinephosphate in the working muscle occurs alike in presence and absence of oxygen.

This view is somewhat different from the older assumption that, even under normal oxygen tension, lactic acid appears as an intermediate in the active non-poisoned muscle; and it removes a curious difficulty or paradox encountered in the relationship of phosphocreatine breakdown and lactic acid formation during anaerobic fatigue of an isolated muscle.[6] In the first contractions of such a series of twitches or tetani, the quotient of $\frac{\text{creatinephosphate split (mole)}}{\text{lactic acid formed (mole)}}$ is high,—about 3 to 4. For high degrees of fatigue it is very low, probably less than 0.2. But "creatinephosphate split" in the numerator refers to that residual amount of split creatinephosphate which is found by analysis after the contraction, and which is not immediately resynthesized during lactic acid formation. From this, it must be concluded that, at first, extremely small amounts of creatinephosphate are resynthesized by coupling with lactic acid formation and, in high degrees of fatigue, nearly all newly split creatinephosphate is synthesized in that way. This paradoxical result, that anaerobic resynthesis of creatinephosphate becomes more and more evident with increasing anaerobic fatigue, and is nearly absent in the beginning, is easy to understand when one considers that the mechanism of coupling with lactic acid is only an emer-

gency device. Usually creatinephosphate is synthesized by coupling with *oxidation*. But when a larger part is broken down and oxygen is not available, lactic acid formation sets in, which counteracts the depletion of the energy store of creatinephosphate. Theoretically, it would seem possible that muscle contraction could proceed in oxygen solely with the breakdown of adenosinetriphosphate and the subsequent resynthesis, without intermediate breakdown of creatinephosphate. The available store of .09 calories would be enough for about 30–50 single maximal contractions. But by registration with glass electrodes from the muscle surface, Dubuisson[7a] has obtained a curve of pH change, which shows an alkalinization even in the very first contractions, and this alkalinization is interpreted as a breakdown of phosphocreatine.

From these newer concepts of muscle metabolism, we can expect an approximately constant caloric quotient of lactic acid in muscle, $\frac{\text{calories produced anaerobically}}{\text{gms. lactic acid formed}}$, only with similar degrees of anaerobic fatigue. Although a quite short activity of muscle is unsuited for chemical analysis, relatively short periods of stimulation formerly gave caloric quotients of about 400 calories, while, in the highest degrees of fatigue, the quotients found were 280 to 250 calories. Since 205 calories are developed enzymatically by splitting 0.9 grams of dissolved glycogen into lactic acid [180 calories (difference of combusion heat) + 25 calories (heat of neutralisation with bicarbonate and phosphate)], the minimum of 250 calories corresponds to nearly exclusive lactic acid formation with an approximate balance of split and resynthesized creatinephosphate, and with some additional heat derived from the great neutralization heat of protein.* The higher caloric quotients correspond to additional breakdown of creatinephosphate. When the molecular heat for the enzymatic hydrolysis of creatinephosphate (11,000 calories per mole) is taken as the basis, and the muscle is analyzed for both lactic acid and creatinephosphate, it seems that the total heat is still somewhat greater than explained by this breakdown, even if the pH change and the large neutralization heat of protein is taken into account.[8a]

Finally, we may ask how the myothermic measurements of Hartree and Hill can be explained for single twitches of a muscle in oxygen when only creatinephosphate is split, as in the case of muscle poisoned by iodoacetic acid. According to Hartree and Hill, about 50 per cent

* The heat of neutralization of protein amounts to 12,500 calories per equivalent, or 140 calories per gm. of lactic acid, but probably very little is neutralized in this way.

of the total heat of contraction is given up anaerobically in the initial phase during contraction, while 50 per cent is delayed heat in oxygen. For each mole of oxygen, burning carbohydrate, 113,000 calories are developed; and for each mole of creatinephosphate hydrolyzed, 11,000 calories are liberated. Therefore, 5 moles of creatinephosphate must break down and be resynthesized by one mole of oxygen. This would give 55,000 calories for the initial heat, and 113,000 — 55,000 = 58,000 calories for the oxidative heat. Actually, Nachmansohn and I, in 1930,[9] found between 3.6 and 5.4 moles of creatinephosphate (averaging 4.7 moles) resynthesized for each mole of oxygen consumed, during the first part of the recovery period after short stimulation, when about 5 times as much creatinephosphate synthesized is about 5 times the lactic acid which disappears. In the minced muscle, Belitzer and Tzibakowa[10] found, that when three- or four-carbon acids were oxidized, 4 moles of creatinephosphate were resynthesized for each mole of oxygen consumed. Theoretically, on the basis of known mechanisms of phosphate transfer, the relation of 4 moles of phosphate to one mole of oxygen can be understood, while, at the present time, higher proportions cannot be interpreted by means of known coupled reactions. But, nevertheless, they seem to occur, since recently Ochoa[4] has reported the transfer of phosphate to sugar in heart extracts in the proportion of 6 moles of phosphate to each mole of oxygen. From a thermodynamic viewpoint, such high yields of esterified phosphate per mole of oxygen are possible, even if the phosphate should be stored in an energy-rich linkage as in creatinephosphate, and would not form the usual phosphoric acid ester. Oxidation during the recovery period in the isolated amphibian muscle has no higher efficiency than 50 per cent, equivalent to the creation of 4 or 5 energy-rich phosphate bonds per mole of oxygen. But, since we know that anaerobic glycolysis has an efficiency of 100 per cent in creating such phosphate bonds, special means may exist in respiration, to bring this about, such as, for instance, the large gap of oxidation reduction potential between the cytochromes and the pyridine nucleotide.

The second part of my report is concerned with the results of the second historic period of our problem. In this period, the whole pathway of breakdown leading from glycogen to lactic acid was cleared up by studying the intermediate reactions in muscle extract, yeast extract, in other cell extracts and more or less isolated enzymatic systems. Two general reaction types became known. The first includes those reactions easily reversible in the absence of other additional chemical systems,

which therefore display a small change of free energy. These can be called, in a special sense, "equilibria reactions." The second type includes those, which can be reversed only by coupling with a second system, where every single reaction shows a relatively great change of free energy. But, by the combination of inducing and induced reaction, a reversible system can be built up, which is nearly "ergo-neutral." In general, the first type of reaction goes on in dialyzed extracts, where the coenzyme systems are removed, while the latter type needs coenzymes, which form a part of the coupled systems. In the stationary state of lactic acid formation from glycogen, about twelve consecutive steps are passed through, of which eight belong to the first-named group, while the rest are so combined that the change of free energy therein is equal to the change of free energy in the total chain of reactions from glycogen to lactic acid. By coupling with the adenylic system as the phosphorylating coenzyme, this free energy is transferred to two energy-rich phosphate bonds per mole of lactic acid produced.

I shall not describe here the single reactions, because I have done this previously[11] and they are well-known. However, some of the equilibrium reactions, undoubtedly, are very interesting from chemical and thermodynamic viewpoints. I mention, for instance, the zymohexase reaction between hexosediphosphate and triosephosphate, which has a strong negative heat in the direction of splitting and obeys closely the Van't Hoff law of isochores. I shall restrict myself to the reactions of the second type, with appreciable yield of free energy, and shall only discuss those points where either some progress has been made in recent times or the situation is still somewhat obscure. The concept of the energy-rich phosphate bond, developed mainly by Lipmann,[1] has undoubtedly helped to clarify and systematize our knowledge in this field. At present, it will be generally agreed by all experts in this matter, that the significance of the phosphorylation of the carbohydrate intermediates is based on its thermodynamic implications. In this way, the energy of the oxidative step can be easily collected in energy-rich phosphate bonds, which, at first, form a part of the intermediates themselves. By transphosphorylations, these bonds with their high energy are transferred to the adenylic system and are stored as the labile phosphate groups in the adenosinepolyphosphoric acids or are transferred still further to creatine and are stored as creatinephosphate. They can also be transferred to other systems with or without loss of their high bond-energy or possibly they can be released, when attached to protein in doing mechanical work.

By direct measurement, we know only the heat change connected with the release of such an energy-rich bond. The molar heat for the splitting of one labile group of adenosinetriphosphate is about 12,000 calories; for creatinephosphate, it is 11,000 calories; for phosphopyruvic acid, it is 8000 calories; and for argininephosphate, also 8000 calories. Lipmann has found an indirect method for calculating the free energy change of the dephosphorylation of phosphopyruvic acid and has obtained a $\triangle F^\circ$ of $-11{,}000$ calories. Assuming that the free energy is nearly the same in all energy-rich bonds, because of their mutual equilibria, this would be the true value for the other compounds too. Since a part of the calculations of Dr. Lipmann is based on somewhat doubtful numerical values, it may be of interest to submit a new computation of this $\triangle F^\circ$ value of the phosphate bond of adenosinetriphosphate itself, from equilibria measurements made in my former Institute in Heidelberg in 1938. The equilibria measurements were published, but, so far, neither I, nor anybody else, has attempted to use them for this calculation. All figures (except one for the combustion heat of glyceric acid) are completely independent of Dr. Lipmann's numerical data. Indeed, the thermal values used by me contain uncertainties similar to those in Dr. Lipmann's computation. Nevertheless, since this calculation is based on quite different reactions and leads to about the same result, it may add some more weight to the soundness of our assumptions.

The coupling reaction, which was described in 1938,[12] shows a stoichiometric balance, in which one mole of glyceraldehydephosphate is oxidized by one mole of cozymase to 3-phosphoglyceric acid, while one mole of phosphate combines with one mole of adenosinediphosphate to form adenosine triphosphate.

3-glyceraldehydephosphate + cozymase $(\overset{+}{DPN})$ + adenosinediphosphate + H_3PO_4

$\Downarrow\Uparrow$

3-phosphoglyceric acid + dihydrocozymase (DPNH) + adenosinetriphosphate

The equilibrium of this reason was studied in a large series of experiments with different concentrations of the reactants. Although it was demonstrated by Warburg and Christian[13] and also by Negelein and Brömel[14] that this reaction is really composed of several steps leading over 1, 3-diphosphoglyceric acid, as a connecting link between oxidation and phosphorylation, this does not matter for the calculation of the

change of free energy, which is determined solely by the concentration of the initial and final products in the over-all equilibrium.

I have reproduced here the three most exact experiments, with their equilibrium concentrations. They were made under identical conditions of volume, temperature, etc., and with the same pure preparations of the different reactants. Experiment I, where all participants were used in about equivalent amounts, was made from both sides of the equation and reached nearly the same equilibrium point in both ways. It may, therefore, be taken as the most reliable. Experiment II was made only from the left side of the equation with the same initial concentrations, but with 10 millimole excess of 3-phosphoglyceric acid (PGA). Experiment III was made from the right side with 10 millimole excess of inorganic phosphate. Dihydrocozymse was determined spectrographically. In experiment I, the other components were all determined separately; in experiments II and III, they were partly derived from the value of dihydrocozymase. Glyceraldehydephosphate (GAP) is calculated from the zymohexase equilibrium.[15] The agreement of the K value is as good as can be expected.

EQUILIBRIUM OF THE "COUPLING REACTION"*

pH, 7.8; temperature, 20° C.

(I) $K_c =$

$$\frac{1.27 \cdot 10^{-3}\ \text{DPNH} \times 1.05 \cdot 10^{-3}\ \text{PGA} \times 1.16 \cdot 10^{-3}\ \text{ATP}}{2.13 \cdot 10^{-3}\ \text{DPN}^{+} \times 0.04 \cdot 10^{-3}\ \text{GAP} \times 2.6 \cdot 10^{-3}\ \text{H}_3\text{PO}_4 \times 2.45 \cdot 10^{-3}\ \text{ADP}} = 2.8 \cdot 10^{3}$$

(II) $K_c =$

$$\frac{0.62 \cdot 10^{-3}\ \text{DPNH} \times 10.7 \cdot 10^{-3}\ \text{PGA} \times 0.60 \cdot 10^{-3}\ \text{ATP}}{2.79 \cdot 10^{-3}\ \text{DPN}^{+} \times 0.045 \cdot 10^{-3}\ \text{GAP} \times 3.2 \cdot 10^{-3}\ \text{H}_3\text{PO}_4 \times 3.0 \cdot 10^{-3}\ \text{ADP}} = 3.2 \cdot 10^{-3}$$

(III) $K_c =$

$$\frac{1.74 \cdot 10^{-3}\ \text{DPNH} \times 1.74 \cdot 10^{-3}\ \text{PGA} \times 1.74 \cdot 10^{-3}\ \text{ATP}}{1.92 \cdot 10^{-3}\ \text{DPN}^{+} \times 0.035 \cdot 10^{-3}\ \text{GAP} \times 11.9 \cdot 10^{-3}\ \text{H}_3\text{PO}_4 \times 1.9 \cdot 10^{-3}\ \text{ADP}} = 3.5 \cdot 10^{-3}$$

For $K = 3 \cdot 10^{3}$, $\triangle F'^{\circ}_{c} = -RT\ln K = -1342 \times \log. K = -4700$ calories. In calculating from this equilibrium the $\triangle F^{\circ}$ for ATP →

* The data are taken from, (12) table V, pages 125 and 126. For experiment I, the intermediate values for the equilibrium concentrations attained from both sides are used.

$ADP + H_3PO_4$, I had the kind help of Dr. H. Kalckar, who especially assisted me to transform the thermal data of the reaction (phospho) glyceraldehyde $+ H_2O \rightarrow$ (phospho) glyceric acid $+2H^+$ into values of free energy.

The total equilibrium of the coupling reaction may be divided into the three partial equilibria K_1, K_2, K_3, and their corresponding $\triangle F'^\circ$ values $\triangle F'^\circ_1$, $\triangle F'^\circ_2$, $\triangle F'^\circ_3$, respectively.

$$K_1 = \frac{DPNH}{DPN^+ \times 2H^+} \qquad K_2 = \frac{3\text{-Phosphoglycerate} \times 2H^+}{3\text{-Glyceraldehydephosphate } (+ H_2O)}$$

$$K_3 = \frac{ATP}{ADP \times H_3PO_4}$$

and $\triangle F'^\circ_c = \triangle F'^\circ_1 + \triangle F'^\circ_2 + \triangle F'^\circ_3$.
thus: $\triangle F'^\circ_3 = \triangle F'^\circ_c - \triangle F'^\circ_1 - \triangle F'^\circ_2$

$\triangle F'^\circ_1$ can be taken from the value of Borsook[16] for E'° (-0.28 volt at pH 7) of this equilibrium. When corrected for pH 7.8, this gives, in the endergonic direction, + 13,500 calories. $\triangle F'^\circ_2$ can be calculated only tentatively with the help of not too accurate thermal data according to the equation: $\triangle F_2 = \triangle H_2 - T\triangle S_2$. In general, $\triangle H$ refers to the difference of the heat content of the pure substances. The subtrahend should include, besides the difference of the entropies (S) of the pure substances, the difference of the entropy changes in dissolving the substances at standard conditions (molar concentration, pH = 0). The terms for the ionization of the acid formed by oxidation and for shifting the pH from 0 to the pH of the equilibrium (7.8) finally must be added. In our cases, however, $\triangle H_2$ corresponds to the difference of the heat content of the dissolved diluted substances, since only the heats of reaction of the dissolved phosphorylated compounds are known and not their heats of combustion. It would be entirely misleading to use instead of this the combustion heats of the unphosphorylated compounds without a thorough and critical evaluation. The combustion heat of the dimeric racemic glyceraldehyde[17] cannot be reconciled with other heat measurements on trioses, as was pointed out earlier.[18] The combustion heat of dioxyacetone[18] with the addition of the heat of solution is in better agreement with the value calculated from dissolved fructose and the endothermic reaction of the zymohexase : fructose-1, 6 diphosphate $\rightleftharpoons$ 2 dioxyacetonephosphate. Since the isomerisation of the triosephosphates is thermoneutral ($\triangle H > 1000$ calories), the value of H for dissolved glyceraldehyde may then be compared with the calculated value of dissolved glyceric acid.[1] Other possible calculations are based on the measured heat of the dismutations : triosephosphate + acetaldehyde = phosphoglycerate + ethyl alcohol; and triosephos-

phate + pyruvate = phosphoglycerate + lactate.[12, 20] In these cases the difference of the heat content of dissolved acetaldehyde and ethyl alcohol, and likewise of pyruvic and lactic acid must be subtracted. These values can be taken from known combustion heats and heats of solution and dilution. Moreover, one must take into account the concomitant phosphorylation of hexose in the first mentioned enzymatic dismutation, and of creatine in the second one, as well as the heat of neutralization. In the mean, $\triangle H$ for the reaction glyceraldehydephosphate + $H_2O \rightarrow$ phosphoglycerate + $2H^+$ turns out to be + 4000 calories ($\pm$ 1500 calories).* $T\triangle S$ for glyceraldehyde (phosph)/glyceric acid (phosph) in their pure state probably is small, judged from analogies in the table of Parks and Huffman[21] (table 40, p. 210). The possible differences of the change of entropy by solution must be disregarded owing to lack of suitable data.† Thus the $T\triangle S$ value, which has to be taken into account, is that of the molecule of water which disappears: $T\triangle S_{(H_2O)} = \triangle H_{(H_2O)} - \triangle F_{(H_2O)} = -68{,}300 + 56{,}600$ calories $= -11{,}700$ calories. The ionization of glyceric acid to glycerate$^-$ at pH 0 is endergonic. The pK of the glyceric acid group in phosphoglyceric acid = 3.42.[22] Since $-RT\ln K = -1342 \cdot (-3.42) = +4600$ calories at 20° C, $\triangle F^\circ{}_2$ for

$$\text{glyceraldehydephosphate} + H_2O \xrightarrow[-3H^+]{-2e} \text{phosphoglycerate}^- =$$

$+ 4000 - 11{,}700 + 4600$ calories $= -3100$ calories.

The shift of the system of pH 0 to pH 7.8 follows at 20° C, a 58 millivolt slope, as far as pH 3.42 and an 87 millivolt slope from pH 3.42 to 7.8. This corresponds to $-27{,}000$ calories. Thus, $\triangle F'^\circ{}_{2\ (pH\ 7.8)} = -3100 - 27{,}000$ calories $= -30{,}100$ calories. No correction is applied for the increase in strength of the second phosphate group of phosphoglycerate compared with glyceraldehydephosphate (pK 5.98 instead of pK 6.75),[22] which possibly would add -1000 calories.

Inserting the $\triangle F$ values in the complete equation we get: $\triangle F'^\circ{}_3 = -4700 - 13{,}500 + 30{,}100$ calories $= +12{,}000$ calories. In the exergonic direction (ATP $\rightarrow$ ADP + H_3PO_4), $\triangle F'^\circ{}_3 = -12{,}000$ calories.

* For this calculation, the heat of formation of the glyceraldehyde group (dissolved) is taken as $-140{,}700$ calories, of water as $-68{,}300$ calories and of the glyceric acid group (diss.) as $-205{,}000$ calories. The values of $\triangle H_2$ obtained by the four different methods of calculation are:
From dioxyactone (diss.), glyceric acid (diss.), and thermoneutrality of the isomerisation,[19a] + 3500 calories.
From fructose (diss.) → hexosediphosphate → 2 triosephosphate, glyceric acid (diss.),[19a] + 5300 calories.
From the dismutation with acetaldehyde/alcohol,[13] + 5000 calories.
From the dismutation with pyruvate/lactate,[20] between + 2500 and 4500 calories.

† From $K_{isomerase} = 22$ for glyceraldehydephosphate → dioxyacetone phosphate it follows that $\triangle F$ of this isomerisation equals -1800 calories (in dilute solution). But this equilibrium does not enter the computation in the text.

This value is in good agreement with the value for $\triangle F'^{\circ}$ obtained by Dr. Lipmann for the dephosphorylation of phosphopyruvic acid, namely, $-11{,}250$ calories.

It is worth while to compare this total equilibrium of the coupling reaction with the intermediate equilibrium found by Warburg and Christian in the presence of cozymase and pure oxidizing enzyme:

$$K_{\text{oxidation}} = \frac{\text{DPNH} \times \text{1, 3-diphosphoglyceric acid}}{\overset{+}{\text{DPN}} \times \text{3-glyceraldehydephosphate} \times H_3PO_4}$$

This equilibrium constant is strongly dependent on the pH, and at 7.8 and 20° C probably is about 3, only 1/1000 of that of the total equilibrium. Therefore, $\triangle F'^{\circ}$ for glyceraldehydephosphate + $H_3PO_4 \rightarrow$ 1, 3 diphosphoglyceric acid is about $-14{,}000$ calories, since $\triangle F'^{\circ}$ for the oxidoreduction with DPN is only some 100 calories. We can deduce from this result that the consecutive reaction: 1, 3-diphosphoglyceric + ADP$\rightarrow$ 3-phosphoglyceric + ATP, must have a $\triangle F'^{\circ}$ of about -4000 calories in order to obtain the $\triangle F'^{\circ}$ of the coupling reaction. Such a value, corresponding to a log K of 3 for the transphosphorylation would mean, that, with equal concentrations of the reactants, 3–4 per cent of diphosphoglyceric acid and ADP are in equilibrium with 96–97 per cent of 3-phosphoglyceric acid and ATP. Although it was announced at one time from Warburg's laboratory that the enzyme in question was isolated, nothing is known so far to verify this computation. I had, however, concluded from experiments on dephosphorylation, where this reaction was involved, that the equilibrium would be far to the right.[29]

Let us now consider briefly the implications of the high phosphate-bond energy liberated by splitting off of phosphate, or absorbed by uptake of phosphate. In the oxidoreduction step, nearly as much of the free energy of the oxidation of glyceraldehyde to the glyceric acid level is taken up by phosphorylation of ADP to ATP, as is taken up for the reduction of $\overset{+}{\text{DPN}}$ to DPNH. The former is a net gain in free energy, while the dihydropyridine is reoxidized in the reduction of one mole of pyruvic to lactic acid. The free energy change of this reduction (pyruvate to lactate) at pH 7 is about + 8300 calories. With a small loss in free energy, the reoxidation of dihydrocozymase reconverts the product of oxidation to lactic acid, that is, to the oxidative level of glucose.

Nature is still more skillful in accumulating the potential energy of glycolysis in phosphate-bond energy. The oxidation to glyceric acid makes possible the formation of the second energy-rich phosphate bond,

which is created by the dehydration of the intermediate 2-phosphoglyceric acid, to phosphopyruvic acid. This was already discussed some years ago by Lipmann.[1] The total heat change from glycogen to lactic acid amounts to 16,500 calories per mole of lactic acid without neutralization, or to 18,000 calories, with neutralization by ordinary buffer substances (like bicarbonate or phosphate). The creation of two energy-rich phosphate bonds would mean a gain in standard free energy of about 24,000 calories. This amount is about 30 per cent more than the total heat change—a very remarkable, but not miraculous result. Dean Burk had calculated, many years ago, that lactic acid formation in muscle under the conditions prevailing in the living organ could yield 50 to 80 per cent more free energy than total heat.[23]

In the living muscle, two moles of creatinephosphate can be synthesized for one mole of lactic acid formed, which corresponds to two energy-rich phosphate bonds per mole. We identify the steps involved as the transfer of the carboxyl phosphate of 1, 3-diphosphoglyceric acid and of the phosphate group of phosphopyruvic acid. Indeed, starting with hexosediphosphate, one easily obtains in enzyme extracts the synthesis of two moles of creatinephosphate per mole of lactic acid, where the over-all reaction is slightly endothermic and the energy available in the glycolytic splitting (18,000 calories per mole of lactic acid) is accumulated in the phosphate bonds of the two phosphocreatine molecules.[24] Nevertheless, the difficulty remains to be explained as to how, in the living muscle, hexosediphosphate has gotten its two phosphates. One phosphate group surely arises by means of the Cori reaction in the sequence: Glycogen + phosphate → glucose-1-phosphate → glucose-6-phosphate → fructose-6-phosphate. But in the muscle extract, fructose-6-phosphate is phosphorylated to fructose-1, 6-diphosphate only by means of the adenylic system, by consuming or wasting an energy-rich phosphate bond. Therefore, for one mole of hexose split, out of four energy-rich phosphate bonds, only three are completely comprehensible. If a reaction of the type, 2 fructose-6-phosphate → 1 hexosediphosphate + 1 hexose, should occur, the difficulty in explaining the creation of the fourth phosphate bond would be overcome. But such a reaction is, so far, not known.

Without doubt, the formation of the carboxyl phosphate in 1,3-diphosphoglyceric acid is responsible for the autocatalytic formation of hexosediphosphate during the so-called phosphate period of cell-free alcoholic fermentation. Every molecule of hexosediphosphate which ferments not only regenerates a new one by means of its original content of two phosphate groups, but it also regenerates two by means of

this extra phosphate taken up during oxidation. The result corresponds to the snow-ball collection practice, formerly used in promoting charitable enterprises, where every contributor not only has to pay for himself, but, at the same time, to get a second man to pay the same amount and to agree to do likewise. How this "autocatalysis" is brought about in an enzyme extract is completely obvious now, but it is still a matter of controversy as to what, in the living yeast, controls the synchronization of phosphorylation and dephosphorylation, at least after a short initial period, in which, after addition of sugar, most of the inorganic phosphate in the cell is esterified. This controlling factor, apparently, is damaged or destroyed by killing the yeast.

In discussing this problem, I shall at first briefly mention the concepts of Nilsson, in Stockholm,[25] who still in 1942,[26] in a lecture which he gave during the present war in my former Institute in Heidelberg, deemed it especially suitable to attack my scheme of fermentation and to cling to his own old idea:—that sugar is not at all fermented by way of hexosediphosphate, but exclusively by way of hexosemonophosphate. The latter then should break down into one phosphorylated and one unphosphorylated triose molecule. In the living yeast, both parts would ferment; in extracts, only the unphosphorylated halves, and the phosphorylated portions would return to hexosediphosphate through the action of aldolase. A lipoid structure in the living cell would be responsible for the fermentation of the phosphorylated triose and this structure would be damaged or destroyed by killing the yeast. The author inferred these assumptions from fermentation curves which he obtained from several types of dried yeast:—either the fermentation of sugar goes to completion with smoothly decreasing speed, or a break occurs after half of the sugar is fermented. This latter situation is the expression for the known equation of Harden and Young. In this case, provided there is less sugar present than its equivalent of inorganic phosphate, after fermentation of half of the added sugar, the other half is completely esterified to hexosedi- and monophosphate.

Some time ago, Warburg and Christian[27] backed this scheme of Nilsson's, but later, albeit tacitly, adopted my scheme. I think that it is not necessary to consider Nilsson's concept seriously in view of the bulk of the evidence that hexosediphosphate is the indispensable thoroughfare in the course of sugar breakdown. For those who are still intrigued by the slow fermentation of hexosediphosphate in yeast extracts in comparison with that of sugar, I mention only two sets of experiments which give a clue to this behavior. If one adds creatine to the maceration juice of yeast together with the phosphorylating en-

zyme from muscle, hexosediphosphate ferments to alcohol and carbon dioxide, at the same speed at which sugar ferments in the same extract. Creatine, which is foreign to yeast, serves now as the phosphate acceptor.[24, 28] In a second type of experiment, arsenate may be added in about millimolar concentration. Under otherwise favorable conditions, hexosediphosphate here again ferments as rapidly as does sugar. In this case we assume, as do Warburg and Christian, that the coupling with phosphate uptake is interrupted by the formation of 1-arsenyl-3-phosphoglyceric acid, which splits off its arsenyl group without enzymes. I have shown recently how this explains, not only the rapid formation of phosphoglyceric acid, but also its rapid dephosphorylation.[29]

There can be no doubt that sugar ferments completely by way of hexosediphosphate in the living yeast, as well as in the non-living yeast. Long ago, I had proposed tentatively as an explanation for the different kinetics in both cases, that the adenylpyrophosphatase is the most sensitive enzyme of the fifteen to twenty different partial enzymes of fermentation which form the zymase complex. By extracting or drying the yeast, it would be damaged more extensively than the others.[30] If this is true, one can assume that the adenylpyrophosphatase in the living yeast is sufficiently active for the regeneration of adenylic acid from adenosinetriphosphate, at the same speed at which hexosediphosphate arises anew from the oxidative coupling reaction with phosphate transfer to glucose. In this way, formation and dephosphorylation of hexosediphosphate would be synchronized. I admit that, so far, this idea is not definitely proved.* We shall, therefore, consider two other possibilities. Through the regeneration of hexosediphosphate, the energy-rich phosphate bond of adenosinetriphosphate is wasted. In the metabolism of muscle, this situation is avoided, because creatine takes up the energy-rich bond and stores it in the form of creatinephosphate. A continuous supply of energy-rich phosphate bonds is probably needed for many other synthetic purposes in the cell metabolism, as, for instance, for the formation of thiamindiphosphate from thiamin. The yeast cell and all other cells may contain very many such phosphate acceptors which, in a stationary state, would finally release the phosphate again by means of phosphatases, while, during growth, part of it would be preserved. I mention here briefly the related case of the autotrophic sulfur bacteria,

* I will mention, however, recent experiments which I performed to prove this point. We destroyed the yeast by freezing in liquid air or by ultrasonic vibration. The fermentation of hexosediphosphate by the extract of the cell fragments is increased many times by the addition of purified adenylpyrophosphatase from potatoes (**O. Meyerhof.** Jour. Biol. Chem. In press).

where, according to the highly interesting discovery of Umbreit, Vogler, le Page,[31, 31a] the energy of the sulfur oxidation is stored in adenosinetriphosphate and can be used anaerobically in the dark for the assimilation of carbon dioxide. Generally, synthesis may be mediated in this manner. This extra supply of energy-rich phosphate bonds in the stationary state of fermentation may, therefore, be of the greatest importance in nature. Probably such a mechanism is combined with the first-mentioned, and the adenylpyrophosphatase releases only the excess of that high energy phosphate not otherwise needed. Finally, the third possibility remains, that the living yeast makes use of a special enzyme for dephosphorylation of the carboxyl phosphate. This enzyme then would be destroyed or damaged by the extraction procedures. Such an enzyme is not known so far, but, if it exists, it would waste the energy-rich bond completely. Probably it would function similarly to the adenylpyrophosphatase in the last mentioned case. That this latter enzyme is preferentially and primarily injured by killing the yeast, is, after all, the most probable of these explanations.

When we look over the whole picture of the biological phosphorylation in carbohydrate breakdown, we arrive at some generalizations. I may cite some of these: Energy-rich phosphate bonds are only created, directly or indirectly, by the oxidative reaction steps in phosphorylated intermediates. On the other hand, all true ester phosphates, where the phosphate esterifies alcoholic groups, arise exclusively from transphosphorylation with the adenylic system or by intramolecular phosphate shift. Inorganic phosphate is never taken directly into alcoholic groups, but only into carbonyl or carboxyl groups. Probably the following generalization is also true: the function of the adenylic system as a dissociable coenzyme consists only in transphosphorylations, not in uptake or direct release of inorganic phosphate. This statement seems to contradict the important recent discoveries of Cori,[32, 32a] that adenylic acid is a prosthetic group in the phosphorylase. The mechanism of this reaction, in spite of the abundance of discoveries made by Cori and his group, is, so far, not completely understood. Since adenosinedi- and triphosphate cannot replace the adenylic acid in the phosphorylase reaction, its function here must be different; moreover, it seems from the latest statements of Dr. Cori[33] that it may form a compound with another group, perhaps a dinucleotide, so that, in principle, the last generalization may also hold.

I have purposely dealt here only with what I called the first and second historic period, leaving to Dr. Kalckar to discuss the present-

day problems. These, indeed, are of more acute interest. On the other hand, much experimental material of the past years is still available for profitable interpretation and evaluation. A conference such as ours can promote such critical examination and coordination of thermal, electrical and chemical measurements, as may be necessary for drawing thermodynamic conclusions.

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