THE NATURE OF ENERGETIC COUPLING IN BIOLOGICAL SYNTHESES

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CONTENTS

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I. INTRODUCTION

The purpose of this review is to acquaint chemists with the great advances made recently in that branch of biological chemistry which is concerned with the chemical mechanism of cellular respiration and the nature of energetic coupling.

Biologists have for a long time been aware that, in addition to simple physicochemical processes (filtration, diffusion, osmosis, ionic reactions, hydrolysis), living organisms depend on other processes of a more complicated nature. These, the vital processes, include motion, transmission of nerve impulses, secretion, growth, etc., which take place only if furnished with energy developed from the oxidation of foodstuffs (metabolites) either by oxygen (respiration) or by double bonds occurring in organic substances (dismutations and fermentations).

When a kidney is perfused with oxygenated blood according to the method of Starling (261), normal urine is produced as the result of a simple ultrafiltration of blood plasma and a selective reabsorption of the main part of the water and all the glucose from the ultrafiltrate. If, however, the respiration of the kidney tissue is stopped by the addition of cyanide, the volume of the urine increases enormously and sugar is excreted, since urine excretion under such conditions is merely the result of a simple ultrafiltration process (16).

The secretion by glands and the transmission of impulses in the nervous system are also processes which depend on cellular respiration.

The contraction of muscles is dependent on energy which, however, does not require respiration, since a frog muscle in an oxygen-free at-

mosphere is able to carry out several hundred single contractions or a tetanus of long duration. As shown by Meyerhof (198), the energy supply in such cases is derived from an internal oxidation of sugar: this process leads to the formation of lactic acid and is called glycolysis or animal fermentation.

Lundsgaard (185), in connection with his discovery of the complete inhibition of lactic acid formation by iodoacetic acid, observed that even when glycolysis is stopped a muscle is able to contract to a limited extent. After forty to fifty single contractions a muscle poisoned with iodoacetic acid is exhausted, the contractions decrease rapidly, and simultaneously the relaxation becomes more and more incomplete, i.e., the muscle is completely exhausted in a state of rigor. Lundsgaard further demonstrated that in muscles which contract without respiration or fermentation the liberation of inorganic phosphate from certain phosphate esters (creatine phosphate, adenosine polyphosphate) takes place to a much greater extent than in normal muscles. Apparently respiration and fermentation restore these phosphate esters, whereas muscles poisoned with iodoacetic acid and without oxygen supply very soon consume the limited stores of creatine phosphate and adenylic polyphosphates which appear to be the most direct energy source for the contraction mechanism. Thus Lundsgaard observed that, simultaneously with the appearance of rigor and complete exhaustion, the supply of creatine phosphate was used up and that of adenylic polyphosphate greatly diminished. These fundamental experiments therefore indicate that the "level" of creatine phosphate + adenosine polyphosphate is maintained by oxidoreductions (respiration or fermentation), whereas the contractile system is "charged" by energy supplied by the liberation of inorganic phosphate from creatine phosphate and adenosine polyphosphates. Since muscles, contracting at the expense of creatine phosphate and adenosine phosphates alone, are exhausted in a state of rigor, the energy furnished by these two dephosphorylations most likely is used not for the contraction but for the relaxation of the contracted muscle, and the relaxation therefore is probably the charged state of the contractile system (see section X).

The restoration of creatine phosphate and adenosine polyphosphates by oxidoreductions will be discussed in detail in this review, since the mechanism of this coupling is understood to a considerable extent. This understanding is mainly due to the magnificent work of Warburg and coworkers (292, 299, 220) who, by revealing the nature of oxidoreduction enzymes, have made it possible to understand chemically how the energy of respiration and fermentations can be utilized for biological syntheses. The phosphorylations of creatine, adenylic acid, or glucose offer a good illustration of biological syntheses which are coupled to oxidation.

The term "synthesis" as applied in organic chemistry merely means the conversion of one substance into another more complex substance, regardless of thermodynamic concept. In biology the term "synthesis or assimilation", however, has for quite a long time been applied to reactions which lead to an increased free energy (positive ΔF) of some of the members of a given system.

Since an increase of free energy[2] as an end result is thermodynamically impossible, biological syntheses must be characterized by a coupling between two reactions, the one representing an increase in free energy ($+\Delta F_1$), the other a fall ($-\Delta F_2$), where ΔF_2 has to be equal to or larger than ΔF_1. Such a definition of biological syntheses includes, in addition to highly complex reactions, certain relatively simple processes, such as the dismutation of sugars into polyalcohols and sugar acids or alcoholic fermentation. Most biologists would hesitate to call alcoholic fermentation a synthesis, because of the liberation of energy ($-\Delta F_2$ much greater than $+\Delta F_1$) and the formation of substances of lower molecular weights than the original.

Nevertheless it is important to realize that if the conversion of sugars into fatty acids is called a synthesis (assimilation), and it is generally so termed among biologists, several dismutations and fermentations also belong to the group of syntheses, owing to the similarity of these last processes to the conversion of sugar into fatty acids (see section III).

Since the formation of polysaccharides from monosaccharides requires a supply of energy, ordinarily derived from biological oxidations, this process is generally considered as a typical synthesis. Frequently, however, emphasis has been placed on the phenomena of polymerization. In this connection it is important to appreciate that the enzymatic dimerization of triose phosphate into hexose diphosphate (202, 203) and the polymerization of glucose-1-phosphate into polyhexoses (45, 130) have been shown to proceed directly, resulting in a small decrease in free energy. Calling such polymerizations syntheses would imply that the concept of biological syntheses involves no consideration of thermodynamics whatsoever.[3]

Some biologists, regarding the increase of free energy as being an essential factor in syntheses, furthermore require a coupling between qualitatively different systems like the just-mentioned uptake of inorganic phosphate into organic ester linkages. Here we have a coupling between two qualitatively different kinds of processes,—oxidation–reduction and phosphorylation.

[2] Concentration differences have also to be included in thermodynamic effects.

[3] The dimerization of free radicals liberates a large amount of free energy ($-\Delta F$ is large).

Since the concept of synthesis seems to be of a very subjective nature, I think that it should be abandoned in purely thermodynamic considerations.

Thermodynamically a process can be described as having a negative or a positive change in free energy ($-\Delta F$ and $+\Delta F$). If ΔF is negative, the reaction can occur and is able to produce work; if ΔF is positive, work must be expended to cause the reaction to occur. The first kind of reaction has been termed "exothermic", the last kind "endothermic". Recently Coryell (55) has recommended that terms like "exothermic" and "endothermic" be applied only to characterize negative and positive changes in heat ($\pm\Delta H$), and that the new terms "exergonic" and "endergonic" be applied to characterize changes in free energy (ΔF). "Ergonic" is derived from the Greek *ergon*, meaning work. Coryell's terminology will be used in the present review.

	−	+
ΔH	Exothermic	Endothermic
ΔF	Exergonic	Endergonic

In biology the concepts of assimilation (or synthesis) and dissimilation still keep their importance. A distinction between fermentations acting as purely dissimilatory systems and those acting as assimilatory as well as dissimilatory systems is very useful. The conversions of sugar into lactic acid and of glycerol into propionic acid (227) offer examples of purely dissimilatory fermentations, i.e., fermentations where the end product has lower energy content than the starting substance. The conversion of sugars into carbon dioxide and ethyl alcohol or into carbon dioxide, acetic acid, and propionic acid or butyric acid offer good illustrations of fermentations which act as assimilatory as well as dissimilatory systems. One part of the sugar molecule is sacrificed as carbon dioxide in a reaction which is "coupled" with the endergonic ($+\Delta F$) conversion of the other part into ethyl alcohol or fatty acids. The main part of this review will deal with the chemical nature of the coupling between exergonic ($-\Delta F$) and endergonic ($+\Delta F$) processes, starting with couplings between oxidoreductions and reactions of different kinds, particularly phosphate esterifications.

In an interesting review by A. J. Kluyver in 1931 (133), describing the state of understanding of biological assimilations and dissimilations at that time, he expresses the value of a coördination of experimental facts, as follows: "Personally I have no hesitation in asserting that even for an experimental science such as biochemistry the day will come when it will be wise to pause for a short time and say 'Enough matter, more art'. And

when we consider for a moment the mass of facts that has already been gathered in the biochemical field, it seems to me that this day of retrospection and synthesis should not be far off".

During the last five years a large amount of information has been collected regarding the details of biological couplings which justifies a survey of the principles which underlie biological syntheses.

II. THE PRINCIPLES OF OXIDATION–REDUCTION

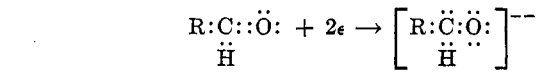
A. The characteristics of oxidoreductions

It seems advisable, before continuing the consideration of fermentations and assimilations, to give a very condensed summary of some characteristics of oxidation–reduction which are important in connection with the problems to be discussed. This section will deal only with the elementary oxidoreduction process; the specific enzymes which catalyze oxidoreduction systems will be described in a later section.

W. M. Clark (37) was the first to realize that electron transfer was the common feature of all biological oxidoreductions. When presenting a condensed summary of this subject, a quotation from a review by Michaelis and Schubert (210) is very useful:

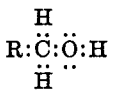
> If a substance, A, can undergo a reversible reduction by accepting an electron (ϵ) the process may be represented thus:
>
> $$A + \epsilon \rightleftharpoons A^-$$
>
> If the product, A^-, happens to be the ion of a weak acid it will tend to combine with a proton furnished either by the oxonium ion OH_3^+, if the solvent contains water, or by any other acid in Brönsted's generalized terminology:
>
> $$A^- + H^+ \rightleftharpoons AH \qquad (2)$$
>
> If these two processes occur simultaneously the net effect can be represented by the combined reaction:
>
> $$A + \epsilon + H^+ \rightleftharpoons AH \qquad (3)$$
>
> or
>
> $$A + H \rightleftharpoons AH \qquad (3a)$$
>
> Only in such a case is the term "reduction" entirely equivalent to hydrogenation.
>
> Exactly the same argument applies to the bivalent oxidation–reduction process of a substance B when the two steps occur simultaneously. The corresponding reactions are:
>
> $$B + 2\epsilon \rightleftharpoons B^{--} \qquad (4)$$
>
> $$B + 2\epsilon + 2H^+ \rightleftharpoons BH_2 \qquad (4a)$$
>
> $$B + 2H \rightleftharpoons BH_2 \qquad (4b)$$

The electronic structures corresponding to equation 4 are:



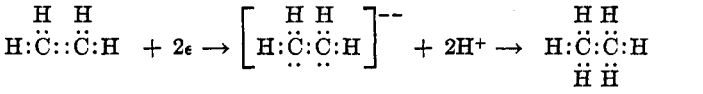
(6)

The product formed would be the divalent ion of an unmeasurably weak acid and would immediately attach protons to form the alcohol:



But this addition of protons has nothing to do with the reduction process, which is entirely contained in equation 6.

A similar case is afforded by the reduction of ethylene to ethane, in which the reduction and proton-attaching processes are:



(7)

The pairs of molecules AH and A^- or BH_2 and B^{--} of equations 2 and 4 may be said to be two states of ionization of the same acid, of which the ionized form in some cases may be practically incapable of existence.

Semiquinones

In a certain number of instances it has been demonstrated that the bivalent redox process illustrated in equation 2 actually occurs in two successive univalent steps involving a half-reduced (or half-oxidized) intermediate.

$$B + \epsilon \rightleftharpoons B^-$$

$$B^- + \epsilon \rightleftharpoons B^{--}$$

This intermediate, which contains an unpaired electron and therefore has the character of an organic free radical, is called a semiquinone (208). The investigations of Shaffer in 1933 (257) showed the importance of one-step oxidation for inorganic redox systems, and a few years later Michaelis and collaborators (208) showed the occurrence of one-step oxidation in a number of important organic redox systems. Michaelis and collaborators were able to demonstrate the occurrence of semiquinones electrometrically and by the magnetic susceptibility due to the unneutralized spin of the odd electron (210, page 441):

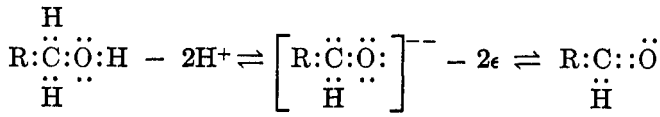
> It is an essential property of these intermediate oxidation levels that they are always in a mobile equilibrium with the compounds on the next higher and the next lower step of oxidation, whereas ordinary valence-saturated organic compounds are usually inert with respect to establishing equilibria with other valence-saturated compounds. Acetaldehyde does not dismute to ethyl alcohol and acetic acid, . . . although it would be possible, speaking purely thermodynamically. In contrast, the establishment of the equilibrium of a radical of the type mentioned with an

electron donor or acceptor is just as unhampered as that of an acid or base with a proton donor or acceptor.

In a later section in this review the one-step oxidation will be discussed again.

B. Redox potentials and chemical structure

As a consequence of the formulation of equations 1 or 6 and 7 it follows that in oxidizing a substance AH_2 a removal of protons (proton dissociation) would have to precede the removal of electrons:



The ionization of the electron donor may be a factor of importance for the emission of electrons and may account for the fact that potentials of biological oxidoreduction systems as a rule drop rapidly (i.e., increasing ability to emit ϵ) in going from systems where the electron donor is an immeasurably weak acid to systems where the electron donor has a measurable H^+ dissociation.

In table 1 the redox potentials of some typical metabolites are presented, using the system of W. M. Clark.

Positive redox potentials mean oxidizing systems; negative ones mean reducing systems. A positive ΔF means that a spontaneous reaction is impossible; ΔF negative means that a spontaneous reaction is possible.

$\Delta \tilde{F}$ represents the free-energy change (in calories) under standard conditions, i.e., 1 molar (unit activity) water solution, pH 0, temperature 25°C. ($T = 298°$). $\Delta \tilde{F} = n\mathcal{F} \cdot \tilde{E}$, where $\tilde{E}$ designates the normal potential (red./ox. = 1) under standard conditions, n is the number of electrons involved in the redox process, and $\mathcal{F}$ is the Faraday constant. E_0' represents the normal potential under conditions other than standard, i.e., when the activity of the reactants is not unity and the pH is different from 0.

Many of the redox potentials have been obtained only from thermal data. Owing to the fundamental work of Parks and Huffman (237) and of Huffman and Borsook (116) and to later work (*cf.* 24), very exact data for the free energy of formation of a number of important biological substances have been obtained.[4] This has made it possible to obtain redox potentials independently of direct potentiometric measurements.

[4] Franke in 1933 pointed out in an interesting review (86) the essential importance of thermal data for the understanding of metabolic processes. Franke's calculations were, however, very rough and therefore not able to demonstrate the exact agreement between thermal data and potentiometric measurements.

There is excellent agreement between the accurate potentiometric measurements of Lehmann (161) and of Borsook and Schott (27) and the redox potentials calculated from thermal data (Parks and Huffman). Very good agreement also has been obtained for other redox systems. Owing to the new methods of specific heat determination developed by Rossini, Huffman, Parks, and others, very exact values for entropies will be possible and therefore also more accurate values for free energies and free-energy changes. Furthermore, W. M. Clark (35) and, more recently, Borsook (26) have calculated redox potentials from equilibrium constants and have thus obtained relatively accurate data for some systems where thermal data have not yet been obtained.

It is well known that the redox potential varies with pH according to the equation:

$$E_0' = \tilde{E} - N\frac{RT}{n\mathcal{F}} \times \text{pH}$$

if $n/N = 2$,

$$E_0' = \tilde{E} - 0.03 \times \text{pH}$$

If $n/N = 1$,

$$E_0' = \tilde{E} - 0.06 \times \text{pH}$$

n designates the number of electrons and N the number of protons involved in the redox process. In the oxidation of carbonyl to carboxyl, $n/N = 2/3$.

Most biological hydrogen donors are oxidized in two steps according to the reaction:

$$AH_2 = A + 2H^+ + 2\epsilon$$

and the pH curve (at 30°C.) therefore follows the 0.06 slope. If in a two-step oxidation (removal of 2ϵ) only one proton is removed from the group which is involved in the oxidation, the pH curve will follow the 0.03 slope. The cozymase (pyridine nucleotide) represents a system which has a 0.03 slope of the redox pH curve. The 0.03 pH curve shows that only one proton is removed when two electrons are removed, in agreement with the nature of the group which is oxidized or reduced (see section IV).

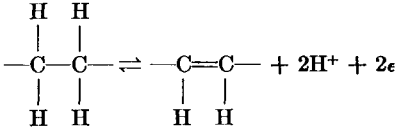
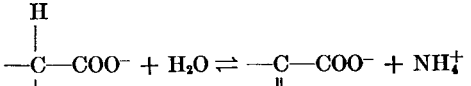
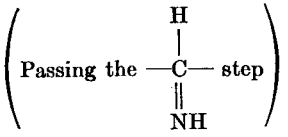
According to the equation

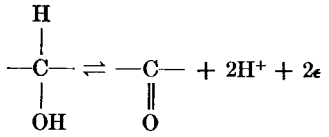
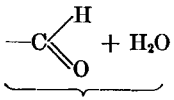
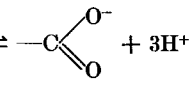
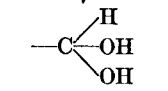
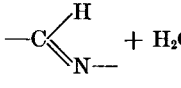
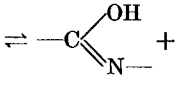
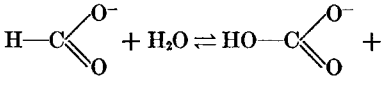
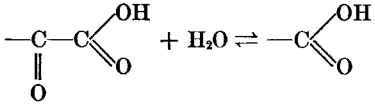
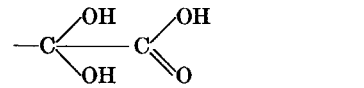
$$E_0' = \tilde{E} - 0.0615 \times \text{pH}$$

the normal potential of a redox system in which 2ϵ and $2H^+$ are involved increases 430 millivolts (= 19.096 calories) when the pH is changed from 7 to 0. A corresponding change in pH will, however, increase the redox potential of the pyridine system only 215 millivolts (*cf.* Borsook (25, 26)).

TABLE 1

Redox potentials of some typical metabolites

KIND OF REDOX SYSTEM	METABOLITES*	$\Delta\bar{F} = nF\bar{E}$ i.e., at unit activity, pH 0, $T = 298°$	E_0' (pH 7)	REMARKS
		calories	*millivolts*	
	I			
	(1) Succinate$^{--} \rightleftharpoons$ fumarate$^{--} + 2H^+ + 2\epsilon$	+20.450	±0	Full agreement between potentiometric measurements (161) and thermal data (237)
	(2) Propionate$^- \rightleftharpoons$ acrylate$^- + 2H^+ + 2\epsilon$	+20.660	+10	$\Delta\bar{F}$ calculated from ΔH; ΔS estimated from analogous cases (Huffman). E_0' calculated from $\Delta\bar{F}$ (Borsook (26))
	(3) Butyrate$^- \rightleftharpoons$ crotonate$^- + 2H^+ + 2\epsilon$	+19.066	−25	
 Paraffins to olefins	(4) Palmitate$^- \rightleftharpoons$ oleinate$^- + 2H^+ + 2\epsilon$	ΔF_0 = +21.440	+25	Since these fatty acids are water-insoluble, only ΔF_0† can be calculated
 $+ 2H^+ + 2\epsilon$  i.e., amino → iminocarbonyl	II			
	(1) Alanine$^{+-} + H_2O \rightleftharpoons$ pyruvate$^- + (NH_4)^+ + 2H^+ + 2\epsilon$	+18.380	−40	E_0' measured by Wurmser and Wurmser (310); $\Delta\bar{F}$ calculated from E_0' (26)
	(2) Glutamate$^{+--} + H_2O \rightleftharpoons \alpha$-ketoglutarate$^{--} + (NH_4)^+ + 2H^+ + 2\epsilon$	+18.820	−30	$\Delta\bar{F}$ and E_0' calculated from II(1) and from the transamination equilibrium (see Borsook (26))

 Hydroxy (alcohol) to carbonyl	III (1) Malate^{--} $\rightleftharpoons$ oxaloacetate^{--} + $2H^+$ + 2ϵ	+15.500	−102	E_0' measured by Lehmann and Jörgensen (162)
	(2) Lactate$^-$ $\rightleftharpoons$ pyruvate$^-$ + $2H^+$ + 2ϵ	+11.880	−180	E_0' measured by Barron and Hastings (15)
	(3) Ethyl alcohol $\rightleftharpoons$ acetaldehyde + $2H^+$ + 2ϵ	+11.730	−163	$\Delta\tilde{F}$ and E_0' calculated from Negelein and Wulff and from the potential of cozymase (Borsook (26))
	(4) β-Hydroxybutyrate$^-$ $\rightleftharpoons$ acetoacetate$^-$ + $2H^+$ + 2ϵ	+6.580	−290	E_0' measured by Jörgensen (115)
$H_2 \rightleftharpoons 2H^+ + 2\epsilon$ (hydrogen electrode)		0	−430	
 + H_2O $\rightleftharpoons$  + $3H^+$ + 2ϵ	IV			
	(1) Acetaldehyde (+H_2O) $\rightleftharpoons$ acetate$^-$ + $3H^+$ + 2ϵ	−1.790	−468	$\Delta\tilde{F}$ calculated from Parks and Huffman (237)
	(2) Sugar (+H_2O) $\rightleftharpoons$ sugar acid$^-$ + $3H^+$ + 2ϵ		−400 to −450	*Cf.* Green *et al.* (99). The biological sugar oxidations seem to involve phosphate instead of water (Negelein and Brömel (220))
 + H_2O $\rightleftharpoons$  + $2H^+$ + 2ϵ	(3) Hypoxanthine + H_2O $\rightleftharpoons$ xanthine + $2H^+$ + 2ϵ		−407	Potentiometric measurements with methyl viologen (Filitti (81))
	(4) Xanthine + H_2O $\rightleftharpoons$ uric acid + $2H^+$ + 2ϵ		−355	Potentiometric measurements (Green (95))
	V (1) Formate$^-$ $\rightleftharpoons$ CO_2 + H_2(gas)		−425	E_0' measured as an equilibrium constant by Woods (309). $\Delta\tilde{F}$ calculated from Parks and Huffman. The gas pressure replaces the hydrogen-ion concentration
	(2) Pyruvate$^-$ + $2H_2O$ $\rightleftharpoons$ acetate$^-$ + HCO_3^- + $3H^+$ + 2ϵ	−9.400	−630	$\Delta\tilde{F}$ calculated from thermal data and from II(1) and II(2) (Borsook (26)). The biological oxidation of pyruvic acid involves phosphate instead of water (Lipmann (175))
	(3) α-Ketoglutarate^{--} + $2H_2O$ $\rightleftharpoons$ succinate^{--} + HCO_3^- + $3H^+$ + 2ϵ	−8.000	−600	$\Delta\tilde{F}$ calculated from thermal data and from II(1) and II(2) (26)

* The metabolites are not ionized at standard conditions.

† ΔF_0 is the change in free energy between pure solids or liquids.

It is obvious, therefore, that the relationship between the redox potentials of various systems depends upon the pH at which they are compared. Since n/N varies from 0.67 to 2, the comparison between redox potentials of biologically important substances must be made at a pH near that of the tissue, for instance, at pH 7.

Table 1 shows that the system

$$\text{hydrocarbon} \underset{+2\epsilon}{\overset{-2\epsilon}{\rightleftharpoons}} \text{olefin}$$

is the most positive system of all metabolites. The system

$$\text{amino acid} + H_2O \rightleftharpoons \text{keto acid} + NH_4^+ + 2H^+ + 2\epsilon$$

forming the imino acid as the primary product, is considerably more positive than the system

$$\text{hydroxy acid} \underset{+2\epsilon}{\overset{-2\epsilon}{\rightleftharpoons}} \text{keto acid}$$

This fact might explain the observation of Krebs and Cohen (150) that the dismutation of α-ketoglutaric acid is increased markedly in the presence of ammonia.

The potential of the sugar–sugar acid system is not known exactly. It seems, however, quite certain that phosphorylation of aldehyde and carboxyl groups raises the potential considerably (see section V).

Borsook's calculations (26) of the free-energy change in the system

$$\text{pyruvate}^- + H_2O \underset{+2\epsilon}{\overset{-2\epsilon}{\rightleftharpoons}} \text{acetate}^- + CO_2$$

shows a very strong negativity of this system, approximately 200 millivolts more negative than the hydrogen electrode. The enzymatic system has never been measured potentiometrically. The recent discovery of Lipmann (174) that phosphate is involved in the enzymatic oxidation of pyruvic acid will be discussed later; in the same place it will also be understood why the enzymatic redox system must have a normal potential considerably more positive than that calculated from thermal data. This fact does not diminish the great biological importance of the redox potential calculated from thermal data, since the end result of the enzymatic oxidation of pyruvic acid is acetic acid and carbon dioxide and the phosphate does not enter the final balance. It is also interesting that the system

$$\alpha\text{-ketoglutaric acid}^{--} \rightleftharpoons \text{succinic acid}^{--} + CO_2 + 2H^+ + 2\epsilon$$

which is so closely related to the pyruvic acid–acetic acid system, also has a redox potential more than 100 millivolts more negative than the hydrogen electrode (26). The system

$$\text{formic acid} \rightleftharpoons CO_2 + H_2$$

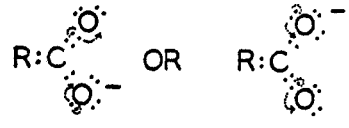
is very near the level of the hydrogen electrode (309).

If hydrogen gas (H_2) is formed, the hydrogen pressure rather than the hydrogen-ion concentration determines the redox potential. Woods' equilibrium determinations (309) were carried out at an alkaline reaction (pH about 8) but at a hydrogen pressure very near 1 atmosphere, i.e., under conditions corresponding to pH 0 for redox systems which do not form hydrogen gas.

Looking at table 1, we find as a general feature that the higher the proton dissociation (acidic properties) the better the electron donor, i.e., reducing agent. The degree of proton dissociation of the group to be oxidized is not, however, the general factor which determines the potential of redox systems.

The high potentials of systems like dienols $\rightleftharpoons$ diketones, diphenols $\rightleftharpoons$ quinones, nitrite $\rightleftharpoons$ nitrate, sulfite $\rightleftharpoons$ sulfate contradict the proton hypothesis just mentioned. One factor, however, seems to be able to account for all the facts concerning oxidoreduction potentials,—*viz.*, the so-called resonance energy. It would be out of place here to attempt even an outline of this interesting development in modern physical chemistry. This field has been described in the fundamental monograph of G. N. Lewis (166) and in the comprehensive monograph of L. Pauling (240). A study of these two monographs, supplemented by discussions with Dr. Coryell, has led the author of this review to believe that the modern concepts of structural chemistry will actually be able to account for the thermodynamic properties of biological redox systems in general and phosphoric esters in particular.

In trying to illustrate the great importance of resonance for biological oxidoreduction, let us examine, for instance, a carboxyl group, written in the electronic terminology introduced by G. N. Lewis in 1916 (*cf.* 166). A carboxyl group, which is ionized, can be illustrated by two equivalent structures:



The calculation of the stability of the carboxyl group from the sums of double-bond and single-bond energies gives a much lower value than that found experimentally. This extra stability is called resonance energy (240). Owing to the resonance energy, such structures as the carboxylate ion (with resonating structures) are characterized by a high degree of stability; this means that a large amount of energy is necessary to transform such structures into other groups not belonging to the resonating type, as is the case, for instance, with aldehydes. The fact that carboxyl groups have a very small tendency to accept electrons, i.e., are poor oxidiz-

ing agents, may be attributed to the high resonance energy they have in relation to the hydrogenated product, aldehydes. Since carbon dioxide has more resonance than the carboxylate ion, this last group of compounds in the table are very good electron donors. The ethylene group, having no resonating structure, is known to have a large tendency to accept electrons. Quinones have less resonance than hydroquinones, a fact which agrees with the strong oxidizing action of the diketo groups. Nitrates and sulfates, having high resonance energies, can be reduced to nitrites and sulfites, which also possess a high degree of resonance; the small change in stability means that nitrates and sulfates are good electron-acceptor systems. The much larger stability of water than that of oxygen, due to the ionic character of O—H bonds in water, lends the latter substance a large affinity for electrons.

In the section dealing with the coupling between oxidoreduction and phosphorylations I shall return to the field of structural chemistry which is of direct significance for this problem.

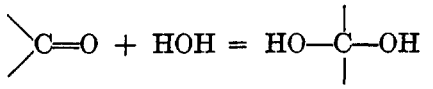
Regarding the concepts of oxidoreduction potentials and free-energy changes, it is always important to bear in mind that what determines whether an oxidation or a reduction of a substance is endergonic ($+\Delta F$) or exergonic ($-\Delta F$) is the change in the stability of the molecular group involved in such reactions. Going from more stable to less stable structures means an increase in free energy ($+\Delta F$) and *vice versa*. In order to know the stability of structures, the conditions under which the change is assumed to occur have to be stated, whether at unit activity and pH 0, usually called standard conditions, ($\tilde{F}$, $\tilde{E}$), or at pH 7, etc. I emphasize this, because in a recent review Kollath and Stadler (142) define a reduction as "Energiebindung" and an oxidation as "Energieabgabe."[5] Such a definition is not only misleading but wrong. Quite apart from the fact that the authors in their definition do not include the concentration of the substances involved in oxidoreductions, particularly the hydrogen ions, one of the consequences of their statement is that the reduction of oxygen, known to be an extremely exergonic reaction (high negative ΔF), is an "Energiebindung." The transfer of electrons from one iron porphyrin system to another having a more "positive" redox potential (i.e., a stronger oxidizing system) is known to be a spontaneous reaction, which therefore represents an exergonic process. Since the E_0' values (pH 7) of iron porphyrins are positive (i.e., the removal of electrons from such compounds represents an endergonic process), the release of energy in transferring electrons from one "positive" system to another more "positive" must be due

[5] "Unter Reduktion versteht man chemische Vorgänge in der Materie, die mit Energie*bindung*, unter Oxydation solche, die mit Energie*abgabe* einhergehen." (142).

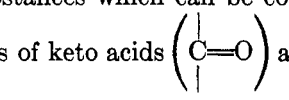
to the reduction of the most "positive" system. These examples are sufficient to illustrate that the definition proposed by Kollath and Stadler does not make any sense whatsoever.

C. Hydrogen donors and acceptors

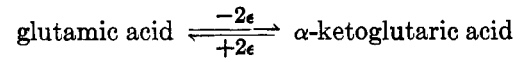
Since protons as well as electrons are involved in most biological oxidoreductions, it has been customary to identify oxidation with dehydrogenation and reduction with hydrogenation. On several occasions it is therefore convenient to use the Wieland–Thunberg terminology: "hydrogen donors" for the substances which emit protons and electrons, and "hydrogen acceptors" for substances which take up protons and electrons. Wieland (302) was the first to advance the idea that carbonyl groups in order to be oxidized have to form hydrates:



This idea was partly based on model experiments which showed that a number of aldehydes in order to be oxidized require water. Aldehydes which form stable hydrates (e.g., mesoxalic acid) are oxidized in media free from water.

Hydrogen (or electron) acceptors have structures which possess double bonds. In several cases such double bonds are the result of anhydride formation as, for instance, in unsaturated fatty acids (—C=C—) which are the anhydrides of hydroxy acids, or in substances which can be considered as anhydrides; for instance, the ketones of keto acids  are the anhydrides of carbonyl hydrates.

As a rule, the hydrogen acceptor of an oxidoreduction system has to belong to the same or a more positive redox system than the hydrogen donor. Hydrogen-transfer systems which transfer hydrogen from the hydrogen donor to the acceptor usually have normal potentials between those of the hydrogen donor and of the hydrogen acceptor. Exceptions, however, are known. The normal potential of the transfer system triphosphopyridine nucleotide is considerably lower than that of the system



nevertheless, this transfer system can accept the hydrogen of glutamic acid and transfer it to a more positive system. The reason for this surprising fact is that in the presence of oxygen practically all of the pyridine nucleo-

tide is kept in the oxidized form; in the absence of oxygen the dehydrogenation of glutamic acid by pyridine stops immediately.

This example illustrates that it is essential that the potential of the final hydrogen-acceptor system be higher than that of the hydrogen-donor system, or the oxidation of the hydrogen donor will stop very soon.

D. Internal and external oxidoreductions

The distinction between internal and external oxidoreductions depends on the manner in which the hydrogen acceptor is supplied, that is, whether it is formed from the hydrogen donor or whether it is supplied from an external source.

Internal oxidoreductions form the hydrogen acceptor from the hydrogen donor. Two main types exist: (*1*) Dismutations, where the hydrogen acceptor is formed by removal of water from the hydrogen donor or by the formation of a ketone from an aldehyde. The double bond thus created is the hydrogen acceptor proper. (*2*) Fermentations, where the hydrogen acceptor is formed by the removal of water from the first or second oxidation level of the hydrogen donor. The double bond thus created is the hydrogen acceptor proper.

External oxidoreductions do not use hydrogen acceptors formed from the hydrogen donor but take up hydrogen acceptors from the environment. If oxygen (O_2) is used, the process is called respiration. If nitrate (HNO_3) or nitrite (HNO_2) is used, the process is called denitrification. Analogous to this reduction is the reduction of sulfates and the reduction of carbonates (chemosynthesis). The uptake of nitrogen is, however, a different process because of the high stability of the N≡N bond (166) (see section VII).

This review will deal mainly with the internal oxidoreductions, because of the close relation of these processes to biological synthesis. The only group of external oxidoreductions which will be examined here is respiration, since it is so closely connected with fermentations and synthesis.

E. Dismutations

A dismutation is an oxidoreduction involving a molecular group and the anhydride of this group, the first acting as an electron and proton donor, the latter as an electron and proton acceptor.[6] The classical example is the dismutation of the malic acid–fumaric acid system into 50 per cent succinic acid and 50 per cent oxaloacetic acid. In the presence of an enzyme, fumarase, which occurs in all biological systems, malic acid is partly transformed into its anhydride, fumaric acid, and *vice versa.*

[6] The conversion of methylglyoxal into lactic acid (226) represents an oxidation–reduction in the same molecule; the hydrated aldehyde group is the electron donor and the keto group is the electron acceptor.

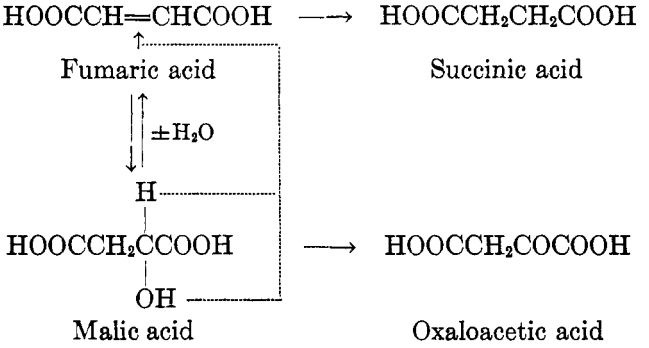
In potentiometric experiments with the succinic acid–fumaric acid system in the presence of the specific enzyme (but in the absence of fumarase), Lehmann (161) established that fumaric acid is reduced to succinic acid. The fine experiments of Lehmann furthermore illustrate the complete reversibility of the system

$$\text{succinic acid} \rightleftharpoons \text{fumaric acid} + 2H^+ + 2\epsilon$$

Moreover, experiments by F. G. Fischer (82) show the presence of an enzyme different from Lehmann's, capable of reducing fumaric acid to succinic acid, using a reduced dye (leuco-janus red) as hydrogen donor. The enzyme described by Fischer was called fumarate hydrase.

That malic acid, the hydrate of fumaric acid, is oxidized to oxaloacetic acid appears from experiments by Green (96) who, by preparing muscle extracts poor in fumarase, showed that the oxygen consumption starts immediately when malic acid is added, but only after an induction period when fumaric acid is added. This induction period is due to the slow conversion of fumaric acid into malic acid in such extracts.

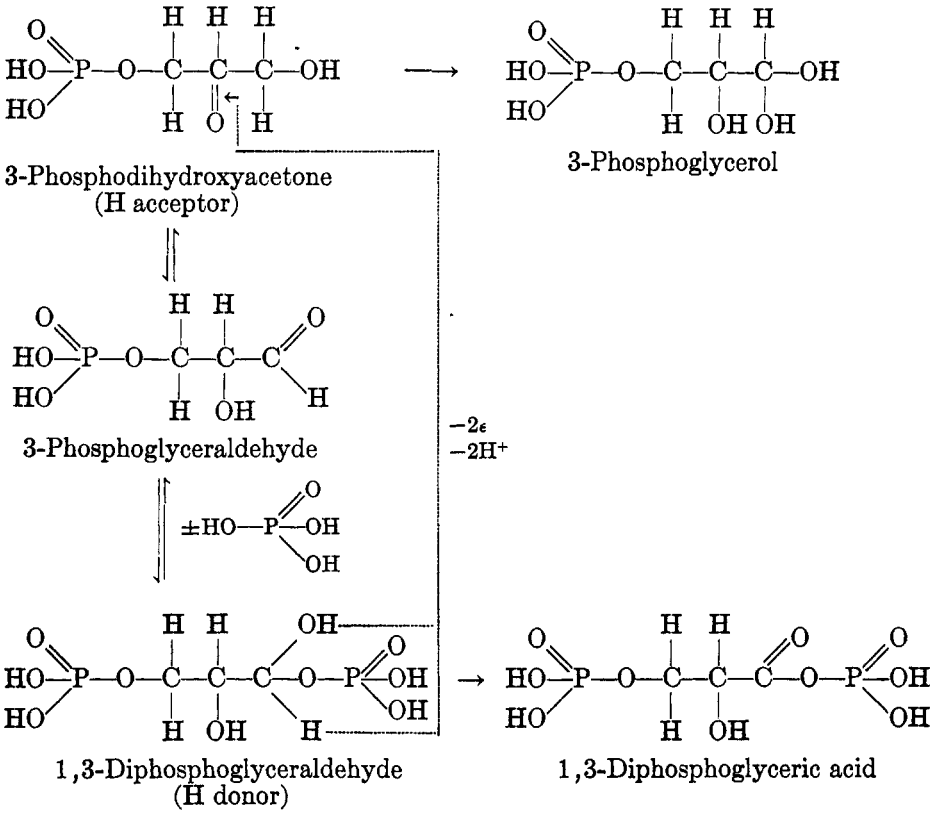
The dismutation of malic (fumaric) acid can be illustrated by the following scheme:



The dismutation of triose into glycerol and glyceric acid is a somewhat more complicated kind of dismutation, which requires phosphate (see page 88).

In the dismutation of triose into glycerol and glyceric acid, the ketotriose (dihydroxyacetone phosphate) acts as hydrogen acceptor, and the phosphorylated aldotriose (phosphate replacing water) as hydrogen donor. This has been established by important experiments of H. O. L. Fischer and Baer (84), of Kiessling and Schuster (131), and of Negelein and Brömel (220).

The dismutation of the fumaric acid–malic acid system is a confirmation of Wieland's assumption that hydrate formation creates hydrogen donors. The dismutation of triose phosphate and of pyruvic acid, however, shows, as pointed out by Lipmann (175), that phosphate in some cases replaces



water. Perhaps the significance of phosphate for biological dehydrogenations is greater than that of hydrate formation (*cf.* section V).

III. FERMENTATIONS

A. The characteristics of fermentations

The principles of fermentation are best illustrated in the simple case of lactic acid fermentation ("glycolysis"). The sugar is oxidized one level[7] by an α-keto acid (pyruvic acid) which is thereby reduced to the hydroxy acid; the first oxidation product of the sugar (a sugar acid) is transformed into a keto acid by formation of the anhydride (*cf.* malic acid $\rightleftharpoons$ fumaric acid). The hydrogen acceptor thus regenerated is able to oxidize a new molecule of the "active" sugar. Whereas a dismutation is oxidation of a compound by its anhydride (or keto-form), a fermentation in general can be characterized as an oxidation of a substance by the

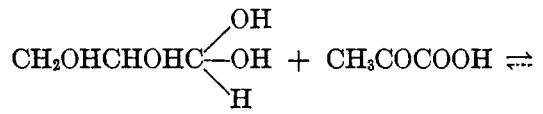
[7] The expression "one-level" oxidation will be used to designate the oxidation of one valence-saturated state to the next, i.e., in most cases the removal of two electrons.

anhydride (or keto-form) of its own oxidation product, oxidized one or two levels higher.

Glycolysis takes place on a large scale in animal tissue unable to use oxygen because of lack of oxygen or of oxygen-"activating" enzymes. In addition, the so-called lactic acid bacteria and some colorless algae (*Prototheca*) show this sort of fermentation in the absence of oxygen.

Disregarding the essential rôle of phosphate in oxidations at this point, the main features of the oxidoreduction of glycolysis can be illustrated as follows:

Oxidoreduction



Glyceraldehyde hydrate (H donor) + Pyruvic acid (H acceptor) $\rightleftharpoons$ $CH_2OHCHOHCOOH$ + $CH_3CHOHCOOH$

Glyceric acid (oxidation product; sugar acid) — Lactic acid (end product)

Regeneration of the hydrogen acceptor

$$CH_2OHCHOHCOOH - H_2O \rightleftharpoons CH_2{=}C(OH)COOH \rightarrow CH_3COCOOH$$

Glyceric acid — Water — Pyruvic acid (enol) — Pyruvic acid (keto)

It is worth while to emphasize two characteristic features of glycolysis: (*1*) Sugar is converted quantitatively into one single substance, lactic acid, which has a lower energy content than the original. (*2*) No carbon dioxide is formed.

All the other sugar fermentations can be described as very simple variations of glycolysis, the variations occurring in most cases after the formation of pyruvic acid.

The pyruvic acid may undergo cleavage as follows: (*a*) Decarboxylation to acetaldehyde and carbon dioxide (alcoholic fermentation); (*b*) hydrolysis to formic and acetic acids (*coli* fermentation).

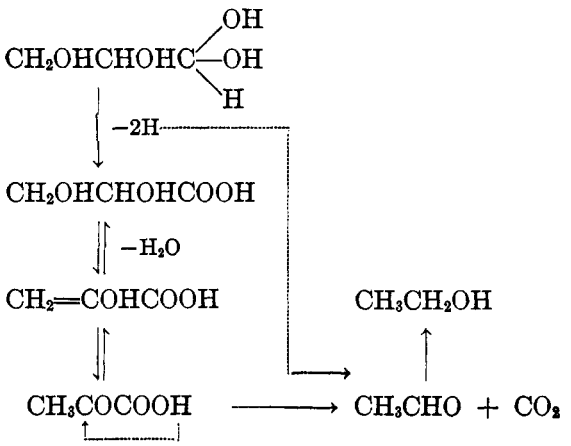
The pyruvic acid may be oxidized to acetic acid and carbon dioxide (fermentation of fatty acids).

The pyruvic acid may undergo condensations as follows: (*a*) with carbon dioxide to oxaloacetic acid (?) (*cf.* Wood and Werkmann (306) and Elsden (64)); (*b*) with amino acids as an acetylation (du Vigneaud (284)).

Other variations are due to secondary reactions of acetaldehyde, e.g.,

carbinol formation (*aerogenes* fermentation), or to secondary reactions of acetic acid, e.g., condensation to acetoacetic acid (*Clostridium butyricum*). These secondary products, replacing pyruvic acid as hydrogen acceptor, give rise to new end products.

The characteristics of *alcoholic fermentation* may be illustrated in the following way:



The scheme illustrates that acetaldehyde, not pyruvic acid, is the hydrogen acceptor in alcoholic fermentation. One of the end products, alcohol, has a higher energy content (per carbon atom) than sugar; the other end product, carbon dioxide, has a lower energy content. Whereas glycolysis is only a dissimilation, alcoholic fermentation is a mixture of dissimilation and assimilation.

In *coli* fermentation pyruvic acid is split in another manner:

$$\underset{\text{Pyruvic acid}}{CH_3COCOOH} + H_2O\ [\rightarrow CH_3C(OH)_2COOH] \rightarrow \underset{\text{Acetic acid}}{CH_3COOH} + \underset{\text{Formic acid}}{HCOOH}$$

The formic acid is degraded (decarboxylated or dehydrogenated) by a special enzyme into carbon dioxide and hydrogen:

$$HCOOH \rightleftharpoons H_2 + CO_2$$

Woods (309) has demonstrated the reversibility of the last reaction. Carbon dioxide can be reduced to formic acid not only by molecular hydrogen but also by organic hydrogen donors, a reaction described by Winogradsky (305) as early as 1890 (so-called chemosynthesis). It is of interest to investigate whether the reaction pyruvic acid = acetic acid + formic acid is reversible. A demonstration of the formation of pyruvic acid by the condensation of formic acid with acetic acid would make pos-

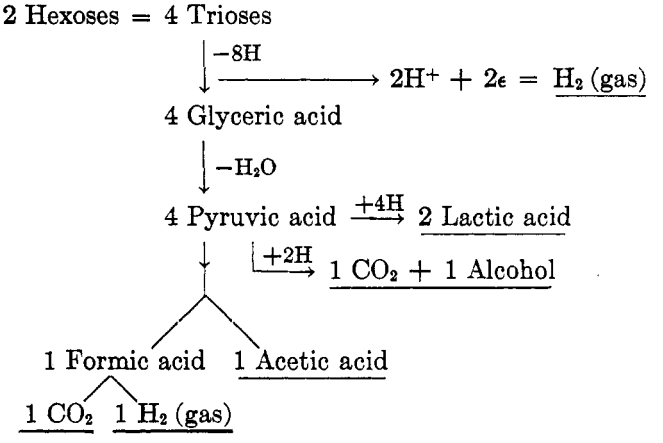
sible a complete chemical explanation of sugar formation from carbon dioxide.

We can summarize the facts known about *coli* fermentation as follows:

The equation of the ordinary *coli* fermentation (108) is

$$2C_6H_{12}O_6 + H_2O = 2CH_3CHOHCOOH + CH_3COOH + C_2H_5OH + 2CO_2 + 2H_2$$

This equation is most readily explained by the following scheme:



Of the eight hydrogen atoms liberated from sugar, only six hydrogen atoms are used in the reduction of pyruvic acid and of acetaldehyde. The two hydrogen atoms left form 1 molecule of hydrogen gas.

Adding the end products from the different processes we get: 2 lactic acid + 1 acetic acid + 1 alcohol + $2CO_2$ + $2H_2$.

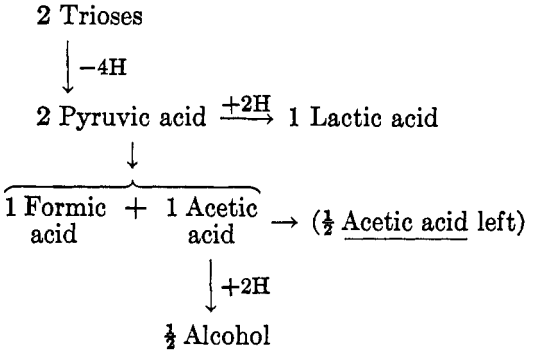
Since 1 mole of alcohol is formed, 1 mole of carbon dioxide is derived from a simple decarboxylation of pyruvic acid which means, the ratio CO_2:H_2 being 1, that 1 mole of hydrogen is formed from the dehydrogenation of triose; this oxidation will just furnish the two hydrogen atoms left in the hydrogen balance. A consequence of these considerations is therefore that the electrode potential of the system triose–glyceric acid is not too far from the level of the hydrogen electrode.[8] Stephenson and Stickland (263) have already pointed out that hydrogen must be formed from other sources than formate and have given evidence for this claim.

One objection against the scheme just presented is the fact that B. typhosum accumulates formic acid and does not form hydrogen (254). This might indicate that the only source of hydrogen in *coli* fermentation

[8] This assumption is made on the basis of the old Wieland scheme of aldehyde oxidation. If phosphate is taken up in the aldehyde group (Negelein–Lipmann reaction) the oxidation can hardly yield hydrogen gas.

is formic acid. Since such an assumption, however, seems to be in disagreement with Harden's equation, it is rather worth while to try to explain the lack of hydrogen in B. typhosum fermentation as due to a reduction of acetic acid to ethyl alcohol by the hydrogen of the triose.[9] Such a reduction of free acetic acid is, however, a strong endergonic reaction.[10]

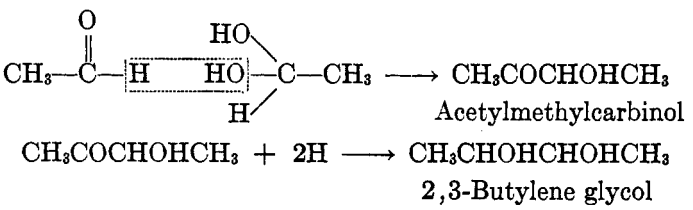
Scheme of B. typhosum fermentation



The end products formed are: 1 mole of lactic acid; 1 mole of formic acid; 0.5 mole of acetic acid; and 0.5 mole of ethyl alcohol. Both qualitative and quantitative figures agree with the experimental data.

B. Different kinds of fermentations

Some soil bacteria, *Aerobacter aerogenes*, contain an enzyme which catalyzes the condensation of acetaldehyde to a carbinol which, replacing aldehyde as hydrogen acceptor, is reduced to a glycol (107).



Thus the glycol replaces the ethyl alcohol as end product in the *aerogenes* fermentation.

Some strains of *aerogenes* are also able to reduce glycerol to trimethylene glycol, a reduction which very few bacteria are able to carry out. Braak (29) in Kluyver's laboratory discovered this interesting reduction from the observation that strains of *aerogenes* were able to grow very well on glycerol as the only carbon source in the absence of oxygen. Braak demonstrated a dismutation of glycerol by isolating the reduction product, trimethylene glycol, $CH_2OHCH_2CH_2OH$, in a high yield.

[9] *Cf.* the reduction of butyric acid to butanol.

[10] Perhaps a phosphorylation of the carboxyl group must always precede the reduction of the carboxyl group (299, 220, 175).

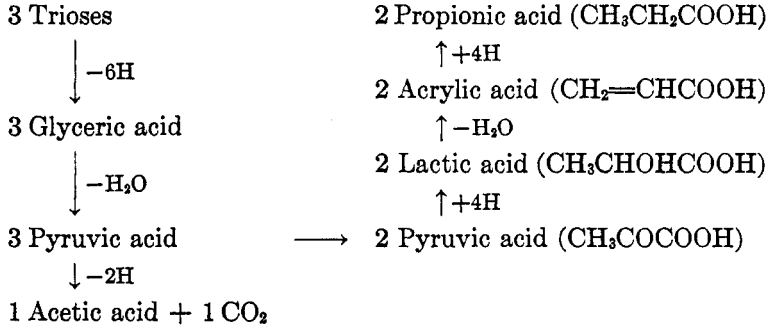
Presumably an anhydride of glycerol is formed and acts as hydrogen acceptor for glycerol. Thus acrolein is formed, together with trimethylene glycol, from glycerol by some microörganisms (287, 215).

In *propionic acid fermentation* no secondary cleavage of pyruvic acid takes place. The difference from the lactic acid fermentation is that in the propionic acid fermentation the substrate (sugar or glycerol) is oxidized two steps instead of one and, correspondingly, the acceptor (pyruvic acid) is reduced two steps, yielding propionic acid.

Fitz found the following equation for the conversion of carbohydrates into propionic acid:

$$3 \text{ trioses} = 2 \text{ propionic acid} + 1 \text{ acetic acid} + 1\ CO_2$$

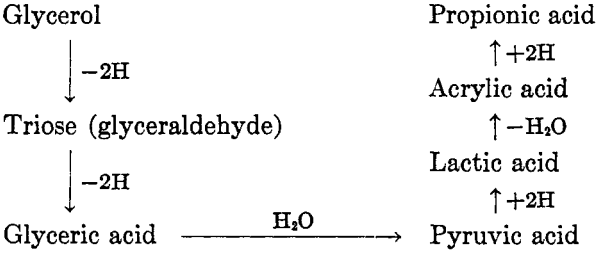
The following scheme is supported experimentally by the investigations of van Niel (227), Virtanen (285), and others:



Eight hydrogen atoms (6 + 2) are removed in dehydrogenations and eight hydrogen atoms (4 + 4) are added in reductions. Two anhydrides, pyruvic acid (the anhydride of glyceric acid) and acrylic acid (the anhydride of lactic acid), act as hydrogen acceptors.

van Niel* made the important observation that glycerol is converted quantitatively into propionic acid by propionic acid bacteria.

Applying the same principles as in the other fermentations, this fact can be interpreted by the following scheme:



The fermentation of glycerol to propionic acid is very closely related to the fermentation of sugar to lactic acid; the main difference is that in the first fermentation a two-level oxidation and a two-level reduction (± 4H) is involved, in the last only a one-level oxidation and reduction (± 2H). Both fermentations are pure dissimilations.

Wood and Werkmann (306, 307), investigating the fermentation of glycerol to propionic acid, made the fundamental observation that the addition of carbonate caused a considerable formation of succinic acid and a corresponding decrease both in the amount of propionic acid formed and in the carbonate. This might be explained by assuming that carbon dioxide is taken up by the pyruvic acid to form oxaloacetic acid[11] (the reverse reaction being well known), which is then reduced instead of pyruvic acid:

Oxaloacetic acid	$HOOCCH_2COCOOH$
↓	↓ +2H
Malic acid	$HOOCCH_2CHOHCOOH$
↓	↓ $-H_2O$
Fumaric acid	$HOOCCH{=}CHCOOH$
↓	↓ +2H
Succinic acid	$HOOCCH_2CH_2COOH$

Recent experiments by Carson and Ruben (34) with radioactive carbon dioxide (C^xO_2) show interesting new phenomena in the propionic acid fermentation. These investigators found that if radioactive carbonate is added to propionic acid bacteria in the presence of glycerol, radioactive carbon enters into not only the succinic acid formed but also the propionic acid.

This observation indicates a reversible uptake and liberation of carbon dioxide between a C_3-monocarboxylic acid and a symmetrical C_4-dicarboxylic acid, for instance:

$$\begin{array}{l} C^xOOH \\ | \\ CHOH \\ | \\ CHOH \\ | \\ COOH \end{array} \rightleftharpoons \begin{array}{l} C^xO_2 \\ + \\ CH_2OH \\ | \\ CHOH \\ | \\ COOH \end{array} \qquad \text{or} \qquad \begin{array}{l} C^xOOH \\ | \\ CHOH \\ | \\ CHOH \\ | \\ COOH \end{array} \rightleftharpoons \begin{array}{l} C^xOOH \\ | \\ CHOH \\ | \\ CH_2OH \\ + \\ CO_2 \end{array}$$

Carson and Ruben did not find an interchange between succinic acid and propionic acid; however, the reversible reaction

$$\text{fumaric acid} \rightleftharpoons \text{acrylic acid} + CO_2$$

[11] *Cf.* also Wood *et al.* (308).

or

$$\text{tartaric acid} \rightleftharpoons \text{glyceric acid} + CO_2$$

represents other possibilities which deserve attention.

Carson and Ruben find the ratio of the radioactivity of volatile acids to that of non-volatile acids equals approximately 3. Wood and Werkmann (307) find that the ratio propionic acid/succinic acid is 5 in the first three days of the fermentation of glycerol in the presence of carbonates; after the fifth day this radio decreases to 4. In Carson and Ruben's experiments the incubation period was only about 30 min. and the ratio propionic acid/succinic acid formed is very likely 5 or larger. Since the ratio of the radioactivity of volatile acids/non-volatile acids is 3, the radioactivity in propionic acid is only about 50 per cent of that of succinic acid; this is in fair agreement with the hypothesis of the reversible interchange of carbon dioxide between a C_3-monocarboxylic acid and a symmetrical C_4-dicarboxylic acid. Wood *et al.* (308) have recently shown that the carbon isotope goes only into the carboxyl group of the C_4-dicarboxylic acid. The recent experiments of Carson and Ruben show also that C^x enters only the carboxyl carbon of propionic acid.

There is reason to believe that Wood and Werkmann's carboxylation process also takes place in the fermentation of sugar to propionic acid, but since carbon dioxide is formed in this process an uptake of carbon dioxide is not easy to demonstrate. Wood and Werkmann's discovery is, furthermore, in agreement with the fact that the normal potential (calculated from thermal data) of the system

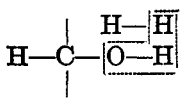
$$\text{propionic acid} \rightleftharpoons \text{acrylic acid} + 2H^+ + 2\epsilon$$

is approximately the same as that of the system

$$\text{succinic acid} \rightleftharpoons \text{fumaric acid} + 2H^+ + 2\epsilon$$

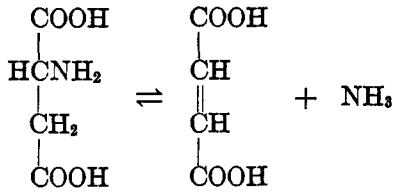
i.e., about 430 millivolts more positive than the hydrogen electrode (see table 1).

The direct experimental demonstration of acrylic acid as a hydrogen acceptor in the propionic acid fermentation is, however, still the missing link. Furthermore, the demonstration of an enzyme like fumarase which is able to attach water to acrylic acid or to remove water from lactic acid is still lacking. A direct reduction of the hydroxyl group in lactic acid, giving propionic acid, is possible and is known in organic chemistry but requires drastic treatment. The reduction of a hydroxyl group involves cleavage:



Aside from the reduction of peroxides, reductive cleavage is observed in a few cases in biological processes: e.g., the reduction of S—S compounds to 2SH or the reductive cleavage of the proline ring in *Clostridium sporogenes* (265).

The reduction of glycine and other amino acids to fatty acids (Stickland) with the liberation of ammonia is of particular interest in this connection. Is ammonia liberated before the reduction, in analogy to an anhydride formation, or is ammonia liberated during the reduction by a cleavage? The first possibility is realized in the demonstration of aspartase (246, 286), an enzyme catalyzing the reaction

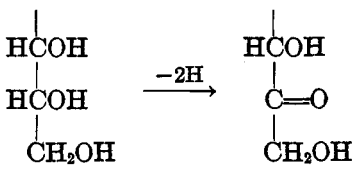


The equilibrium malic acid $\rightleftharpoons$ fumaric acid and the replacement of propionic acid by succinic acid in the presence of carbonate are two facts which make it very desirable to find the corresponding equilibrium: lactic acid $\rightleftharpoons$ acrylic acid. Some old experiments of Dakin (56) indicate that such a reaction actually exists.

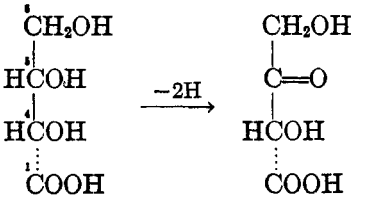
The formation of butyric acid from sugar by *Vibrion butyrique* (Pasteur) is discussed in the section dealing with the formation of fatty acids from carbohydrates.

The so-called aerobic ("oxidative") fermentations (20) are respirations rather than fermentations, since oxygen is the hydrogen acceptor. To this group belong the following: acetic acid "fermentation" (i.e., more or less complete accumulation of acetic acid formed by oxidation of alcohol), and citric and fumaric acid "fermentations" (the latter two occurring in molds).

The acetic acid bacteria are able to oxidize not only ethyl alcohol but also several polyalcohols: e.g., glycerol to dihydroxyacetone and sorbitol to sorbose (22). An explanation of why keto sugars are formed was given by Bertrand, who demonstrated the importance of a definite configuration of the alcohol groups.



This phenomenon was later illustrated by Kluyver and Leeuw (136), who were able to isolate the calcium salt of 5-ketogluconic acid in the oxidation of gluconic acid by an acetic acid bacterium ("Vinegar bacterium").

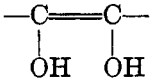


The oxidation of gluconic acid to 2-ketogluconic acid, which spontaneously is converted into carbon dioxide and a pentose, has also been observed (135). This last reaction is similar to the oxidation of 6-phosphohexonic acid to carbon dioxide[12] and ribose phosphate, a reaction observed in yeast juice by Lipmann (171) and later by Warburg (296) and Dickens (59).

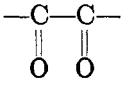
Space does not permit a discussion of the mechanism of citric acid formation in molds, although this reaction is of general interest because of the important rôle which citric acid seems to play in animal tissue respiration.

IV. THE MECHANISM OF HYDROGEN (OR ELECTRON) TRANSFER

Hopkins, Euler, and other investigators have for a long time been aware that the transfer of hydrogen ($\epsilon + H^+$) from donor to acceptor does not take place directly but requires an electron-transfer system. Such electron-transfer systems capable of taking up and giving off electrons rapidly have also been observed and isolated. Hopkins discovered the tripeptide glutathione; in 1927 Szent-Györgyi isolated ascorbic acid, which was identified as vitamin C by King and by Szent-Györgyi. Hopkins' glutathione contains an —SH group which, when oxidized one step (minus ($\epsilon + H^+$)), dimerizes to the meriquinone S—S. Ascorbic acid, which is a dienol,



is oxidized to a diketone



[12] The formation of carbon dioxide is due to a spontaneous decarboxylation of 2-ketophosphogluconic acid.

Szent-Györgyi and coworkers later described the stimulation of respiration by traces of members of the fumaric acid system. None of these substances, however, was identical with the essential electron-transfer system in fermentation and respiration.

Harden and Young in 1905 (108, 109) separated a heat-stable factor of low molecular weight from the enzyme system of alcoholic fermentation; this factor was called the coenzyme. Meyerhof in 1918 (196) demonstrated that coenzymes could also be separated from respiration enzymes (dehydrogenases). Later, when separate steps in the fermentations were described (Neuberg, Embden); the coenzyme of the fermentation was separated into different factors. Lohmann (179) found that magnesium ion and adenylic acid (adenine pentose monophosphate), a substance which Embden and Zimmermann (67) isolated from muscle tissue, are the constituents of the coenzyme of the enzymes catalyzing phosphate transfer (see later). Auhagen (5) separated the coenzyme of the enzyme which catalyzes decarboxylation from pyruvic acid (Neuberg's carboxylase). Auhagen's coenzyme was called cocarboxylase. Euler and Myrbäck (76, 77) separated another coenzyme which was a necessary component in the oxidoreduction of the alcoholic fermentation; this coenzyme was called cozymase. They purified the cozymase to a high extent and demonstrated that it contains adenylic acid but that it is not identical with adenylic acid, since it contains other nitrogenous compounds (71).

Although Euler was able to demonstrate the need of cozymase for the oxido-reduction, the nature of the action remained obscure. Cozymase occurs in all kinds of tissues, although only in very small amounts; the substance seemed to be a complicated and labile substance, difficult to separate from other nucleotides.[13]

In the course of three years (1932–35) Warburg and his coworkers succeeded not only in isolating two of the most important codehydrogenases as pure substances and in clarifying the complete constitution of one of them, but they furthermore demonstrated that the action of these coenzymes is a transfer of hydrogen (electrons) and were even able to prove what part of the complicated molecule is involved in the biological transfer of hydrogen. Besides this brilliant work, Theorell in 1935 and Negelein in 1936 in Warburg's laboratory demonstrated that the so-called coenzymes combine reversibly with specific proteins; some of these proteins have been purified by Negelein to an extent which can only be compared with the crystalline enzymes isolated by Sumner and Northrop and Kunitz.

[13] A nucleotide is a compound built up of a nitrogen base, a sugar or an alcohol, and phosphoric acid. The name "nucleotide" is derived from the high concentration of these compounds in the nucleus of the cell (nucleic acids, nucleoproteins), particularly in the chromosomes.

A. Pyridine nucleotides, the electron-transferring component of respiration and fermentation

By 1934–35 Warburg and Christian (300) had finished their purification of the so-called respiration coenzyme ("Atmungs Coferment"). The chemical analysis showed that the coenzyme was a dinucleotide, containing the two nitrogen bases adenine and nicotinic acid amide (amide of pyridine-β-carboxylic acid), besides two sugar molecules (presumably riboses, according to Euler (71)) and three phosphates. Warburg and Christian, on the basis of the constitutional formula of the coenzyme, introduced the chemical name "Triphosphopyridine nucleotide" (the more correct name "triphosphopyridine-adenine nucleotide" seems too long). The essential constituent of the nucleotide is the pyridine derivative, a nitrogen base which at that time was completely new in enzyme biology. Warburg and Christian were able to demonstrate, partly by means of classical chemical methods and partly by ultraviolet spectroscopic methods, developed by Haas (100) in Warburg's Institute, the following fundamental reaction:

$$\text{sugar} + \text{"pyridine"} = \text{sugar acid} + \text{"dihydropyridine"} \qquad (1)$$

the sugar being hexose monophosphate; the "pyridine" being the triphosphopyridine nucleotide and its specific protein (in the old nomenclature called hexosemonophosphate dehydrogenase).

Negelein and Haas (221) found that the triphosphopyridine nucleotide combines with a protein specific for the substrate, in this case hexose monophosphate ("Robison ester"). This pyridine-protein is designated by Warburg as "Triphosphopyridinproteid" (Robison ester).

Warburg and Christian (296) have isolated another protein which, combined with the pyridine nucleotide, catalyzes the oxidation of a sugar acid, phosphohexonic acid, to phosphoketohexonic acid (*cf.* 171, 59). This enzyme is called "pyridine-proteid (phosphohexonic acid)"; the old term would be "phosphohexonic acid dehydrogenase."

The amount of metabolite oxidized by "pyridine" in equation 1 depends of course on the amount of "pyridine"; the reaction is actually stoichiometric. Warburg and Christian demonstrated that in the presence of oxygen and another hydrogen-transfer system, which is able to react in the oxidized form with dihydropyridine and in the reduced form with oxygen, the hydrogen from the metabolite is transferred to oxygen and in this case only extremely small amounts of the hydrogen-transferring substances are necessary for a rapid oxidation of large amounts of the metabolite. The system which is able to take hydrogen from "dihydropyridine" and give hydrogen to oxygen has also been isolated by Warburg and Christian and is called the yellow respiration enzyme or, using a more chemical terminology, the alloxazine-nucleoprotein (see later).

The catalytic oxidation of hexose monophosphate to phosphohexonic acid by the two nucleotide proteins can be characterized by the following three equations:

hexose monophosphate + "pyridine"

$$= \text{hexonic acid phosphate} + \text{"dihydropyridine"} \quad (2)$$

"dihydropyridine" + "alloxazine"

$$= \text{"pyridine"} + \text{"dihydroalloxazine"} \quad (3)$$

$$\text{"dihydroalloxazine"} + O_2 = \text{"alloxazine"} + H_2O_2 \quad (4)$$

In equation 1, hexose monophosphate and "pyridine" were used up, the former as a hydrogen donor, the latter as a hydrogen acceptor. In the presence of both nucleotide proteins, even in minute amounts, only hexose monophosphate and oxygen disappear.

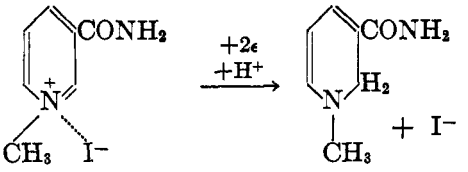
The coenzyme of the oxidoreduction in the fermentation, Euler's cozymase, is also a pyridine-proteid (diphosphopyridine nucleotide) transferring hydrogen from sugar to acetaldehyde and pyruvic acid. This was demonstrated at the same time by Warburg and Christian (295) and by Euler, Albers, and Schlenck (75). The only difference from the other pyridine compound is, as shown by Warburg and Christian, that the hydrogen transfer from triose phosphate through the diphosphopyridine nucleotide to pyruvic acid depends on the presence of inorganic phosphate and adenine nucleotide (adenosine diphosphate). This phosphate effect will be discussed in the next section.

In glycolysis the diphosphopyridine nucleotide transfers hydrogen from triose (as the phosphate ester) to pyruvic acid:

$$\text{triose} + \text{"pyridine"} = \text{glyceric acid} + \text{"dihydropyridine"} \quad (1)$$

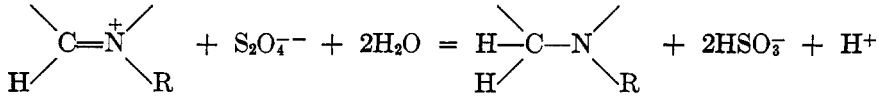
$$\text{pyruvic acid} + \text{"dihydropyridine"} = \text{lactic acid} + \text{"pyridine"} \quad (2)$$

Warburg and coworkers, having proved that the pyridine ring is the hydrogen-transferring system, started, in collaboration with Karrer (125), to find out whether the C=C or C=N double bond is the carrier of hydrogen removed from the metabolites. Karrer (124) synthesized the iodomethylate of nicotinic acid amide and, by reducing this substance with hydrosulfite, established the following reaction:



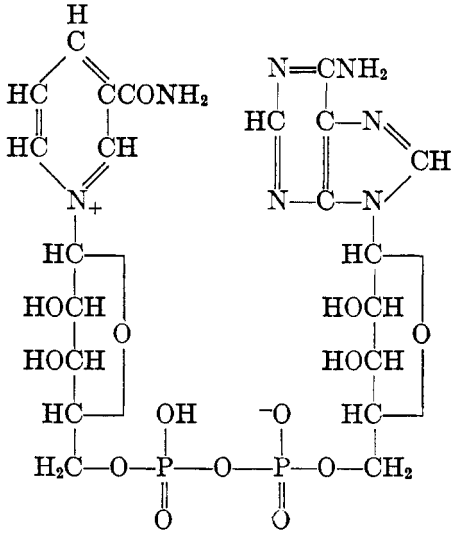
By reduction with sodium hydrosulfite ($Na_2S_2O_4$) a new acid equivalent (HI) arises besides sodium bisulfite. The $>C=\overset{+}{N}<$ group is therefore the oxidized form and the $>C-N<$ group the reduced form.

Warburg and Christian (295, 297) then demonstrated that reduction of the pyridine nucleotides with hydrosulfite gives rise to an extra acid group, owing to the removal of the quaternary nitrogen:

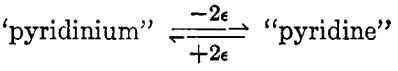


The extra acid will be taken up by the phosphate anion. Thus the pyridine nucleotides are quaternary nitrogen bases (pyridinium derivatives).

Furthermore, investigations of Euler and coworkers (78, 283) showed that alkali inactivation of the pyridine nucleotides yields adenosine diphosphate. On the basis of their investigations, Euler (71) illustrated the oxidized and reduced diphosphopyridine nucleotide in the following manner:



The redox potential of the system



is not exactly known. Clark (35), however, has calculated the potential in the presence of the pyridine-proteid. He obtained E_0' (pH 7) = -0.250

volt. Ball (6), using Clark's indicators and an enzyme system from milk, estimated the potential of the

$$\text{"pyridinium"} \underset{+2\epsilon}{\overset{-2\epsilon}{\rightleftharpoons}} \text{"pyridine"}$$

system at E_0' (pH 7) = −0.260 volt. Borsook (25) recently calculated E_0' from the detailed and careful equilibrium investigations of Euler and coworkers (glutamic acid–ketoglutaric acid system) and found E_0' (pH 7) to be −0.280 volt. This potential is from 180 to 150 millivolts more positive than the hydrogen electrode but is rather negative in comparison with the alloxazine system and particularly with the hemin system. Most of Clark's indicators (dyes) have potentials more positive than the pyridine system. The γ,γ-dipyridyl dyes, however, have potentials more negative than the pyridine system; E_0' (pH 7) for the dipyridyl dye methyl viologen is even 20 millivolts more negative than the hydrogen electrode.

Negelein and coworkers (221, 222) were able to isolate in a very pure state some of the proteins which are necessary for the pyridine catalysis. They furthermore showed, by means of very exact kinetic measurements, using the spectrophotometric method of Haas, that the pyridine nucleotide and the protein combine to a proteid able to dissociate. The reduced pyridine nucleotide ("dihydropyridine") forms a dissociable compound with the same protein ("dihydropyridine-proteid"). The pyridine-proteid is the compound which catalyzes the oxidation of the metabolite (or the reduction of "pyridine"). The velocity of this electron transfer is therefore proportional not only to the total amount of protein (E) but also to the ratio pyridine-proteid/proteids + protein (ρ_{ox}). On the basis of the reaction

$$AH_2 + \text{"pyridine"} \rightleftharpoons A + \text{"}H_2\text{-pyridine"}$$

calling the concentrations of "pyridine" and "H_2-pyridine" C_{ox} and C_{red}, the velocity of the reaction is:

$$\frac{dC_{ox}}{dt} = k \cdot E \cdot \rho_{ox}$$

where k is a velocity constant. If the reaction is reversible, the expression is:

$$\frac{dC_{ox}}{dt} = k_{ox} \cdot E \cdot \rho_{ox} - k_{red} \cdot E \cdot \rho_{red}$$

where k_{ox} is the velocity constant for the reaction from left to right and k_{red} for the reaction from right to left.

Considering the simple case where the reaction is only going from left

to right, Warburg illustrates the kinetics of the enzymatic oxidation–reduction in the following manner: In order to understand the nature of the enzymic pyridine reduction better, ρ_{ox} is expressed by the dissociation constants of the pyridine-proteid (D_{ox}) and of the dihydropyridine-proteid (D_{red}).

The dissociation of the pyridine-proteid is expressed in the following equation:

$$\text{pyridine } [C_{ox}] + \text{protein } [(1 - \rho_{ox} - \rho_{red})E] \rightleftharpoons \text{pyridine-proteid } [\rho_{ox} \cdot E]$$

The dissociation constant (D_{ox}) for this reaction is

$$D_{ox} = \frac{[C_{ox}] \cdot [(1 - \rho_{ox} - \rho_{red})E]}{[\rho_{ox} \cdot E]}$$

The dissociation constant of the dissociable dihydropyridine-proteid (D_{red}) is

$$D_{red} = \frac{[C_{red}] \cdot [(1 - \rho_{ox} - \rho_{red})E]}{[\rho_{red} \cdot E]}$$

From these two equations ρ_{ox} is obtained:

$$\rho_{ox} = \frac{C_{ox} \cdot D_{red}}{C_{ox}(D_{red} - D_{ox}) + D_{ox}(C + D_{red})}$$

where $C = C_{red} + C_{ox}$, i.e., the total concentration of nucleotide. The equation for ρ_{ox} used in the velocity equation gives:

$$-\frac{dC_{ox}}{dt} = k \cdot E \cdot \frac{C_{ox} \cdot D_{red}}{C_{ox}(D_{red} - D_{ox}) + D_{ox}(C + D_{red})}$$

These formulas illustrate the importance of D_{red} for the velocity of the reaction from left to right. Increasing D_{red}, which means easier removal of "dihydropyridine" from the protein, increases the velocity of the reaction from left to right ($-dC_{ox}/dt$). In the "pyridine-proteid (hexose monophosphate)," $D_{ox} = D_{red}$, which gives the simple equation:

$$-\frac{dC_{ox}}{dt} = C_{ox}\left[\frac{k \cdot E}{C + D}\right]$$

If the total concentration of nucleotide (C) is great in relation to D, the velocity equation is even simpler:

$$-\frac{dC_{ox}}{dt} = C_{ox}\left[\frac{k \cdot E}{C}\right]$$

If C is in great excess, the velocity of the reaction at $t = 0$ (i.e., $C_{ox} = C$) depends only on the total amount of protein:

$$-\frac{dC_{ox}}{dt} = k \cdot E$$

The independence of C indicates that the protein is completely saturated with "pyridine."

If $C = D$ the velocity at $t = 0$ is

$$\frac{dC_{ox}}{dt} = C\frac{kE}{2C} = \frac{kE}{2}$$

which means that the dissociation constant (D) is equal to that nucleotide concentration which saturates the protein (E) to the extent of 50 per cent (moles nucleotide > moles protein).

Negelein and coworkers have obtained excellent agreement between the theory and the experiments, which thus establishes the suggestion that the rate of oxidation of metabolites by "pyridine" and a special protein depends on the concentration of the dissociable compound: "pyridine"-proteid.

The rate of reduction of "pyridine" per unit of coenzyme is constant most of the time, provided that C_{ox} is great enough to saturate the protein completely. If the nucleotide concentration is increased to a very high extent the rate per milligram of nucleotide decreases, i.e., the efficiency decreases.

Negelein and Wulff (222) found that the protein catalyzing the oxidation of ethyl alcohol to acetaldehyde (or the reverse process) is 50 per cent saturated at a concentration of "dihydropyridine" one-third that of "pyridine." Furthermore, the catalytically active proteins form dissociable compounds with the substrates which are being oxidized or reduced. The idea of a substrate-enzyme compound was advanced as early as 1913 by Michaelis and Menten (209) on the basis of their studies of invertase action and has been very useful in the interpretation of enzyme kinetics.

I shall here present some of Negelein's values for the dissociation constants of protein–nucleotide compounds and for protein–substrate compounds.

	D OF PYRIDINE NUCLEOTIDES, THE SO-CALLED PROSTHETIC GROUP OF "PYRIDINE-ENZYMES" (D_{ox})	D OF DIHYDROPYRIDINE NUCLEOTIDES, THE SO-CALLED PROSTHETIC GROUP OF REDUCING "PYRIDINE-ENZYMES" (D_{red})
Hexosemonophosphate dehydrogenase	1.1×10^{-5}	1×10^{-5} [moles nucleotide / liter]
Alcohol dehydrogenase	9.5×10^{-5}	3.2×10^{-5} [moles nucleotide / liter]

The D values of the alloxazine nucleoproteins are at least one hundred times smaller than the D values of the pyridine nucleoproteins.

The dissociation constants of some coenzyme–enzyme complexes are presented below:

COENZYME-ENZYME COMPLEX	DISSOCIATION CONSTANT	REFERENCES
Alcohol dehydrogenase (diphosphopyridine-protein)	9.0×10^{-5}	(222)
d-Amino acid oxidase (flavinadenine-protein)	2.5×10^{-7}	(219)
Pyruvic acid dehydrogenase (thiaminpyrophosphate-protein)	2.7×10^{-6}	(175)
Glycogen phosphorylase (adenylic acid-glycogen-protein)	3.0×10^{-5}	(51)

In the equation

$$-\frac{dC_{ox}}{dt} = C\frac{kE}{2C} = \frac{kE}{2}$$

the velocity constant k can be calculated.

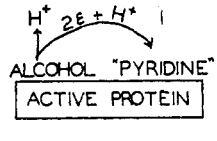
In the oxidation of hexose monophosphate by a "pyridine-proteid", $k_{(ox)} = 2.9 \times 10^4$ (1 min.), which means that 1 molecule of specific protein in 1 min. is able to bring 29,000 molecules of hexose monophosphate and pyridine nucleotide to reaction (221).

The velocity constant of the oxidation of alcohol by a "pyridine-proteid" is $k_{ox} = 1.7 \times 10^4$ (1 min.) (222).

The velocity constant of the reduction of acetaldehyde by a "dihydropyridine-proteid" is (222)

$$k = 2.9 \times 10^4 \text{ (1 min.)}$$

All these studies have shown that the oxidation–reduction nucleotides and ordinary substrates display two properties in common: (*1*) hydrogen is transferred from the substrate to the nucleotide by an ordinary stoichiometrical reaction, and (*2*) both the substrate and the nucleotide form dissociable compounds with the catalytically active protein, e.g.:

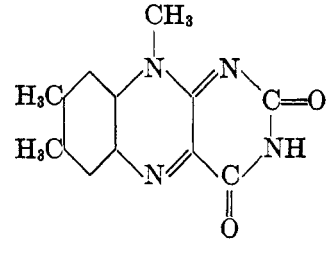


Owing to these facts, several biochemists prefer to classify the nucleotides as substrates. Several substrates like the fumaric acid system (271) and amino acids (140) are actually able to act as ϵ-transfer systems like the nucleotides. Although the analogies between substrates and nucleotides are of much importance, Warburg is justified in laying stress on the important differences between ordinary substrates and the nucleotides. The

alloxazine nucleotides and thiazole nucleotides (175), for instance, form proteids which (at pH 7) have dissociation constants far smaller than the substrate proteids. Another feature characteristic of the system of oxidation-reduction nucleotides is the appearance of semiquinones (210, 101), a phenomenon which will be discussed at the end of this section.

B. Alloxazine nucleotides

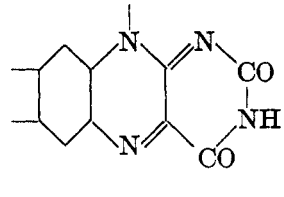
In 1932 Warburg and Christian (293) isolated a yellow enzyme protein compound which was decolorized by reduction; when the decolorized enzyme was reoxidized the yellow color returned. In alkaline solution and in light, the yellow dye was converted into another yellow dye which was soluble in chloroform; this substance was called lumiflavin. Warburg and Christian (294) isolated the lumiflavin ($C_{13}H_{12}N_4O_2$) and showed that urea is liberated by its alkaline hydrolysis. Stern and Holiday (263) showed that lumiflavin was an alloxazine with a methylated nitrogen, and Kuhn and coworkers (156) established that lumiflavin is trimethylalloxazine:



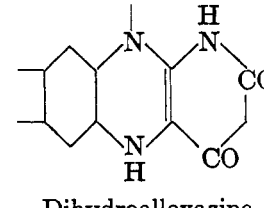
Lumiflavin

Hydrogen on a platinum catalyst reduces the lumiflavin to dihydroflavin, which is colorless; oxygen oxidizes the dihydroalloxazine to the yellow lumiflavin (294).

Warburg and Christian (294) obtained detailed absorption spectra for the yellow enzyme and for lumiflavin. The two spectra possess the essential resemblance. Since the spectrum of the yellow enzyme is an alloxazine spectrum, the hydrogen transfer by the yellow enzyme can be described as an action of the conjugated C=N units:



Alloxazine



Dihydroalloxazine

Kuhn and coworkers (157) in 1933–34 found that lumiflavin is a cleavage product of an alloxazine-ribityl compound (riboflavin), and in 1934 Theorell (275) discovered that the prosthetic group of the yellow enzyme is alloxazine-ribityl phosphate (riboflavin phosphate), i.e., a nucleotide. Kuhn and coworkers (1936) (154) succeeded in synthesizing the riboflavin phosphate.

Theorell in 1934 (276) succeeded in separating the alloxazine nucleotide and the specific protein without irreversible denaturation of the protein by dialysis for 72 hr. against cooled dilute hydrochloric acid; addition of alloxazine nucleotide to the protein gave resynthesis of the yellow enzyme. This was the first separation and reactivation of a respiration enzyme.

The alloxazine-proteid transfers electrons from dihydropyridine nucleotides to oxygen, thus completing systems which oxidize aldehydes to acids or alcohols to aldehydes by oxygen:

$$(\text{aldehyde} + H_2O) + \text{"pyridine"} = \text{carboxyl}^- + \text{"dihydropyridine"} \quad \text{(a)}$$

$$\text{"dihydropyridine"} + \text{"alloxazine"} = \text{"pyridine"} + \text{"dihydroalloxazine"} \quad \text{(b)}$$

$$\text{"dihydroalloxazine"} + O_2 = \text{"alloxazine"} + H_2O_2 \quad \text{(c)}$$

Like the pyridine nucleotides, the free alloxazine nucleotide is inactive; only the alloxazine nucleoprotein is active.

Theorell (279) proved that at low oxygen pressure the "dihydroalloxazine" can be reoxidized by the ferric ion of cytochrome *c*. F. G. Fischer (82) and Szent-Györgyi and coworkers (272, 158) showed that "dihydroalloxazine" can be reoxidized by fumaric acid in the presence of special enzymes.

In 1938 Warburg and coworkers (298) isolated some new yellow enzymes, the prosthetic group of which they showed to be alloxazine-adenine dinucleotides. These alloxazine dinucleotides have a striking resemblance to the constitution of pyridine-adenine nucleotides. A number of these new alloxazine-proteids were isolated at the same time in Warburg's Institute and by Straub (266) in Keilin's Institute. The experiments of Warburg and coworkers and those of Straub show that these new alloxazine-proteids are considerably more active than the old enzymes isolated in 1932. Combined with different proteins, the alloxazine dinucleotides are able to react not only with the pyridine nucleotide and with amino acids (298) but also with other systems; Corran, Green, and Straub (54) showed that one of the alloxazine-proteids isolated by Straub from heart muscle transfers hydrogen from "dihydropyridine" to methylene blue (and probably also to cytochrome) with an enormous velocity. Warburg points out that the

"old" alloxazine nucleotides might be cleavage products of the "new" dinucleotides.[13a]

Warburg and Christian (298) in their new research on the yellow enzyme introduced a very convenient method for the separation of alloxazine nucleotides from alloxazine nucleoproteins which have a very small dissociation constant: addition of $m/10$ hydrochloric acid to a cooled solution of the flavoprotein in 20 per cent ammonium sulfate solution liberates the alloxazine group, the ammonium sulfate protecting the protein against acid denaturation. Recombination experiments gave an excellent yield: 78 per cent of the protein and 95 per cent of the alloxazine dinucleotide remained active during the separation (298). The alloxazine nucleoproteins are dissociable only to an exceedingly small extent. The alanine

TABLE 2

Systems which reduce and oxidize alloxazine

REDUCING SYSTEM	ALLOXAZINE PROTEID SYSTEM	OXIDIZING SYSTEM
Triphosphopyridine nucleotide (malic acid)	Alloxazine mononucleotide (Warburg and Christian, 1932)	Oxygen Cytochrome *c* (Theorell) Fumaric acid (Szent-Györgyi)
d-Amino acids (*d*-alanine, *d*-proline, cysteine)	Alloxazine dinucleotide (Warburg and Christian, 1938)	Oxygen
Diphosphopyridine nucleotide	Alloxazine dinucleotide (Haas, 1938)	Methylene blue
Diphosphopyridine nucleotide	Alloxazine dinucleotide (Straub, 1938)	Methylene blue (cytochrome?)
Triphosphopyridine nucleotide	Alloxazine dinucleotide (Warburg and Christian, 1938)	Oxygen
Thiamin nucleotide (Lipmann, 1939)	Alloxazine dinucleotide (F. G. Fischer, 1939)	Oxygen Fumaric acid

"oxidase" has the highest dissociation constant: $K = 2.5 \times 10^{-7}$ moles per liter. A number of catalytically active alloxazine-proteids have been isolated; table 2 summarizes what is known about the systems which reduce and oxidize alloxazine.

Furthermore Ball (7), in Warburg's Institute, purified the xanthine "oxidase" and found that the alloxazine dinucleotide is one of the prosthetic groups of this enzyme. A similar observation was made by Gordon Green and Subrahmanyan (94) in purifying an aldehyde dehydrogenase from liver tissue. The xanthine oxidase and the aldehyde oxidase con-

[13a] Very recently, Haas, Horecker, and Hogness (J. Biol. Chem. **136**, 747(1940)) in some brilliant studies discovered a new, very active yellow enzyme, the prosthetic group of which is an alloxazine mononucleotide. The new yellow enzyme transfers electrons from triphosphopyridine nucleotide to cytochrome *c*.

tain another coenzyme besides the flavin component; the second coenzyme has not yet been identified.

Franke and Deffner (87) recently obtained a purified glucose dehydrogenase, the activity of which was found to be proportional to the content of alloxazine.

The normal redox potentials of free alloxazine nucleotides (211) amount to about −150 millivolts (pH 7), whereas the alloxazine-proteids have higher potentials, about −80 millivolts. The redox potential of the alanine "oxidase" is not known, but since the dihydroalloxazine forms a proteid which experimentally is undissociable, the potential will be raised to a considerable extent (*cf.* 36, 57).

It is well known that Warburg's discoveries of nicotinic acid and alloxazine as the essential compounds in redox enzymes were of fundamental importance for the numerous investigations of the action of these substances on growth. The result of these investigations was the identification of previously unknown growth factors (vitamins) with these two compounds.

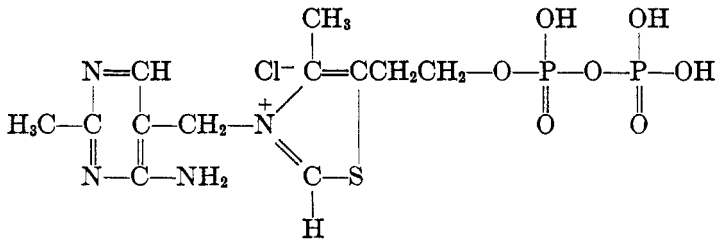
C. Thiamin nucleotides

The antiberiberi factor, vitamin B_1 (or aneurin), was identified by the work of Williams and of Windaus as a thiazole-pyrimidine derivative. In the case of B_1 the vitamin function was known long before the enzyme action.

Experiments of Peters and coworkers (244) showed that brain tissue from pigeons which have beriberi symptoms has a smaller oxygen consumption than normal brain tissue and exhibits an accumulation of acids. Peters and collaborators identified the acid accumulated in beriberi tissue as pyruvic acid and were furthermore able to demonstrate that lack of vitamin B_1 stopped or inhibited the further breakdown of pyruvic acid.

As mentioned before, the enzyme which catalyzes the decarboxylation of pyruvic acid to acetaldehyde and carbon dioxide, Neuberg's carboxylase, is a protein compound from which a prosthetic group, the so-called cocarboxylase, can be liberated (5).

In 1937 Lohmann and Schuster (184) identified Auhagen's cocarboxylase as vitamin B_1 pyrophosphate (thiamin pyrophosphate):



Thiamin nucleotide

Lohmann and Schuster resynthesized carboxylase by addition of thiamin pyrophosphate to the specific protein; thus they established that Neuberg's carboxylase, the enzyme which catalyzes the reaction

$$CH_3COCOOH = CH_3CHO + CO_2$$

is a thiamin nucleoprotein.

Krebs (147, 148), as well as Lipmann (173), discovered a new type of reaction, the dismutation of pyruvic acid:

$$2CH_3COCOOH + H_2O^{14} = CH_3CHOHCOOH + CH_3COOH + CO_2$$

One molecule of pyruvic acid acts as hydrogen acceptor, the other together with water[14] as hydrogen donor, the first yielding lactic acid, the second acetic acid and carbon dioxide.

Lipmann (174) furthermore demonstrated that pyruvic acid can be oxidized in animal tissue as well as by lactic acid bacteria according to the equation:

$$CH_3COCOOH + H_2O^{14} + O_2 = CH_3COOH + CO_2 + H_2O_2$$

He was able to obtain the pyruvic acid dehydrogenase from dried lactic acid bacteria in a stable form. Treating this concentrated enzyme preparation in the same manner as Lohmann did, Lipmann inactivated the system and reactivated it by addition of very small amounts of Lohmann's pure thiamin pyrophosphate. Recently (176) he observed that a purified pyruvic acid dehydrogenase treated according to the method of Warburg and Christian (dilute hydrochloric acid in the presence of ammonium sulfate) is inactivated. Apparently the dehydrogenase contains another component besides thiamin phosphate. Lipmann was able to restore the pyruvic acid dehydrogenase with a small amount of Warburg's alloxazine dinucleotide and thiamin pyrophosphate. The pyruvic acid dehydrogenase therefore is composed of a substance of high molecular weight (presumably a protein) and two prosthetic groups, thiamin and alloxazine nucleotides; the thiamin system therefore presents a striking resemblance to the pyridine–alloxazine system. Lipmann's experiments furthermore show that phosphate and adenylic acid are necessary components of the system. This last observation will be discussed in the next section.

Recently Green, Herbert, and Subrahmanyan (98) have isolated the thiamin nucleoprotein. It contains 0.46 per cent of diphosphothiamin and 0.13 per cent of magnesium. In high salt concentrations carboxylase is a firmly bound conjugated protein, whereas in dilute salt solutions or in alkaline ammonium sulfate solutions it dissociates into protein, diphospho-

[14] Later discoveries of Lipmann (175) show that phosphate and not water creates the reductans proper (see section V).

thiamin, and magnesium. It is a fairly generally accepted view that the phosphate or pyrophosphate and the amino groups in nucleotides represent the structures which link the coenzymes to the specific protein. This view was originally based upon the observations of Kuhn and collaborators (154), who found that the specific protein of the yellow enzyme can act not only with the flavin phosphate but also with the unphosphorylated flavin. In the last case, however, a much larger amount of protein had to be used in order to get the same activity as with flavin phosphate. This difference in activity was explained as being due to the difference in binding groups. Flavin is bound to the protein by one group, the amino group; flavin phosphate is bound by two groups, the amino group and the phosphate group.[15]

The inhibition of certain dehydrogenases by phosphate has been explained as a competition between the phosphate of the active nucleotide and inorganic phosphate. The phosphate concentrations which inhibit these dehydrogenases are, however, rather large. Some recent experiments by Buchmann and Heegaard (32) are of considerable interest for this problem. These investigators worked with thiamin pyrophosphate (cocarboxylase) and the specific enzyme protein. The activation of the enzyme protein by cocarboxylase was strongly inhibited by very small amounts of thiazole pyrophosphate but not at all by free thiazole. The same investigators (112) have also been able to give evidence for the hypothesis that amino groups play an essential rôle in linking coenzymes to enzyme proteins. They showed that the activation of the carboxylase protein by cocarboxylase is inhibited strongly even by minute amounts (16γ per cubic centimeter) of the aminopyrimidine which is a constituent of the cocarboxylase. The corresponding deaminated pyrimidine (hydroxypyrimidine) exerts even in large amounts no inhibitory effect on the reaction between cocarboxylase and the carboxylase protein.

Green *et al.* (98) are of the opinion that magnesium, which they find as a constituent of thiamin nucleoprotein (carboxylase), plays a rôle in linking the nucleotide to the specific protein.

The rôle of the quaternary nitrogen in thiamin

Lipmann, besides studying the enzymatic pyruvic acid oxidation, made an attempt to clarify the nature of the thiamin action in the oxidation of pyruvic acid. It is known that pyridine and thiazole are very closely related compounds, displaying the same physical properties. This, in connection with the above mentioned resemblance of the pyruvate oxida-

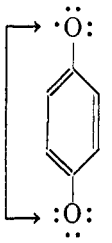
[15] Recent experiments by Warburg and Christian (299) demonstrate clearly the rôle of phosphate esterified to alcohol groups: 3-phosphotriose needs 1000 times less protein catalyst than free triose in order to be oxidized to glyceric acid.

tion to triose phosphate oxidation, led Lipmann to the assumption that the quaternary nitrogen in thiamin, as in the pyridinium compounds, accepts hydrogen from pyruvic acid and transfers it to the alloxazine system. Lipmann (172, 178) was actually able to reduce the quaternary nitrogen in thiamin and thiazole derivatives by hydrosulfite but the reduced product could not be reoxidized, presumably because of a secondary cleavage reaction (cf. 70). Later attempts (14, 264) to demonstrate a reversible oxidoreduction of thiamin have only established Lipmann's findings but have not provided further contributions to this theory.

D. The rôle of specific proteins in the formation of semiquinones

A gradual reduction of pyridine, thiamin, and flavin nucleotides with sodium hydrosulfite ($Na_2S_2O_4$) always yields intermediate, strongly colored products. These colored products, which again disappear when the reduction is complete, are supposed to be semiquinones (211, 212, 172).

Michaelis and collaborators (212) have demonstrated that one-step reduction, i.e., an uptake of only one electron, yielding a free radical, actually takes place for quite a number of dyes. The best possibilities for an accumulation of semiquinones, i.e., stabilization of a free radical, are in case the molecule involved has a symmetrical configuration, as, for instance, the ion:



A structure having one electron resonating between two equivalent structures represents a close analogy to the so-called three-electron bond (Pauling (240)). Since the quinones are ionized in strong alkali, the chances for an accumulation of the free radical are best in alkaline reactions.

The corresponding nitrogen compounds are able to accumulate free radicals at very acid pH values only; this is also the case with the alloxazines.

Haas (101) in Warburg's laboratory has made the important observation that when the alloxazine nucleoprotein is reduced at 0°C. by reduced triphosphopyridine nucleotide, a transitory intermediate red product is formed. This red compound has the same absorption spectrum as the

red radical that appears when free flavin is reduced by hydrosulfite at a pH less than 0. Thus the combination with the specific protein in neutral solutions seems to have the same effect as has a strong acid, displacing the equilibrium in favor of the radical.[16]

Michaelis is of the opinion that such a displacement of the equilibrium in favor of the radical might be one of the most essential actions of the catalytically active proteins of redox nucleotides (214, page 6):

> The following general principle may be postulated: In most organic compounds an oxidation of any valence-saturated compound to another such compound on a higher level of oxidation is a bivalent oxidation. The inertia of organic compounds toward oxidizing agents is due to the fact that the oxidation can proceed at a measurable speed only in two succesive univalent steps and consequently only if the intermediate radical can be formed. If the normal potential of the first step of oxidation is much higher than that of the second step the amount of the radical formed may be extremely slight and its concentration may be the limiting factor for the speed of oxidation. In many organic compounds this situation leads to a practical lack of reactivity at ordinary temperatures in absence of a catalyst. The rôle of an enzyme, then, is to displace the equilibria concerned in favor of the radical. A possible demonstration of this is Haas' experiment cited above: the combination with a specific protein and a coenzyme displaces the equilibrium in favor of the radical. . . .

Warburg expresses his opinion of the action of the specific protein in somewhat the same direction:

> Warum das Eiweiss so wirkt, ist heute das Problem der Fermentchemie. Zwei Gründe lassen sich zur Zeit anführen:
>
> 1. Wenn das Alloxazin an das Eiweiss gebunden wird, so wandert das Absorptionspektrum der Wirkungsgruppe um 20 mμ nach rot, was bedeutet, dass die Aktivierungsenergie des Alloxazins durch die Bindung an das Eiweiss kleiner, die Reaktionsfähigkeit des Farbstoffes also grösser wird.
>
> 2. In Lösungen von Alloxazinproteid und Pyridin-Nucleotid tritt unter gewissen Bedingungen eine Farbe auf, die nur von einer Verbindung der beiden Substanzen herrühren kann. Wahrscheinlich also sind die Reaktionen zwischen Alloxazin und hydrierten Pyridin innermolekulare Proteid-Reaktionen. Dann versteht man sofort, warum die nicht an Eiweiss gebundenen Flavin mit den hydrierten Pyridin-Nucleotiden nicht reagieren."

Both statements indicate that the task of the redox enzymes may be to decrease the extraordinarily high instability of the semiquinones of ordinary metabolites, thereby increasing the concentration of the proper reactive product. The electron acceptors, double bonds, seem to be independent of catalysts because they possess a certain degree of unsaturation,

[16] Pauling and Coryell (241) have found that hemoglobin contains four unpaired electrons per heme; oxyhemoglobin and carbon monoxide hemoglobin, however, contain no unpaired electrons. The oxygen molecule with two unpaired electrons in the free state accordingly undergoes a profound change in electronic structure on attachment to hemoglobin.

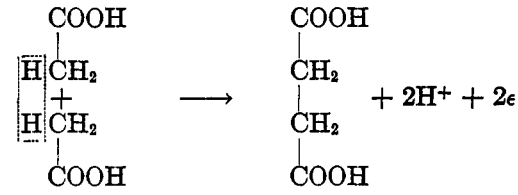
exhibiting paramagnetic properties (166). That much can be said today about the nature of specific protein catalysis in biological oxidation–reduction.

E. The fumaric acid system

Szent-Györgyi and collaborators (270) have shown that the dicarboxylic acids, succinic–fumaric acid and malic–oxaloacetic acid, when added to tissue systems in minute amounts (0.1 to 0.2 mg.) increase the oxygen uptake; the extra respiration exceeds several times the amount of oxygen necessary for a complete combustion of the small amount of dicarboxylic acids added. Szent-Györgyi therefore assumes that the dicarboxylic acids act as a hydrogen-transfer system like the nucleotides just mentioned. According to the potentials the malic acid–oxaloacetic acid system should work between the pyridine and the alloxazine system, and the succinic acid–fumaric acid system between alloxazine and cytochrome. Laki (158) showed that reactions of this kind actually take place.

Several metabolites seem, however, to be oxidized with the two nucleotides and the cytochrome system as the only hydrogen-transfer systems (279, 54). On the other hand, Annau and Erdös (4) showed that oxidation of pyruvic acid to acetic acid requires minute amounts of succinic acid (*cf.* also 8), and Colowick, Welch, and Cori (40) have recently demonstrated the importance of the succinic acid–fumaric acid system for the phosphorylation of glucose in kidney extracts.

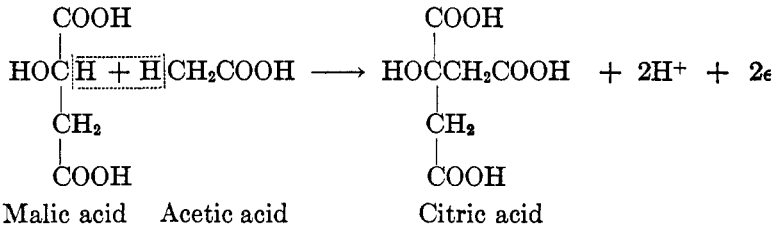
Thunberg (281), Krebs (151), and others assume that the fumaric acid system acts as carrier of acetic acid in the oxidation of this substance. An oxidative dimerization of two molecules of acetic acid gives succinic acid[17] (Thunberg):



The succinic acid then is oxidized to oxaloacetic acid and this substance is spontaneously decarboxylated to pyruvic acid, which when oxidized again gives acetic acid.

If a molecule of acetic acid is oxidatively condensed with malic acid, citric acid is formed.

[17] *Cf.* the oxidative dimerization of —SH compounds to S—S compounds.



Malic acid Acetic acid Citric acid

Simola (258, 125) recently demonstrated a very considerable formation of citric acid from pyruvic acid and malic acid, a formation which very likely corresponds to the oxidative condensation of acetic acid and malic acid. The oxidation of acetic acid and malic acid to citric acid is presumably of importance for the formation of glutamic acid (*cf.* section VII).

F. Cytochrome and pheohemin

In 1925 Keilin (126) discovered some hemins which in the reduced form exhibit typical spectral lines. Such cell hemins were observed fifty years ago by McMunn but no attention was paid to his findings. Keilin, analyzing the cytochrome spectrum, came to the conclusion that three different cytochromes exist, which he called *a*, *b*, and *c*.

In 1936 Theorell (278) isolated cytochrome *c* as a pure substance. Cytochrome *c* is a hemin in combination with a basic protein. Recently Keilin and Hartree (127) described a very simple method for obtaining pure cytochrome *c* from heart muscle. The minced and washed heart muscle is treated with dilute trichloroacetic acid. A large amount of cytochrome *c* is liberated and can be precipitated at pH 3.5. By this method Keilin and Hartree were able to obtain the enormous yield of 1.5 g. of pure cytochrome *c* per kilogram of heart muscle.

Succinic acid seems to react directly with cytochrome *c* (273, 232). Theorell (279) showed that the reduced alloxazine nucleotide reacts directly with cytochrome *c*, giving alloxazine and reduced cytochrome *c* (*cf.* Haas *et al.* (1940)). This latter product cannot be oxidized directly by oxygen but only through Warburg's pheohemin enzyme (cytochrome oxidase; "Atmungsferment"). According to Ball (6), cytochrome *b* has the lowest potential and cytochrome *a* has the highest potential.[18]

G. The hemin catalysis

The hemin (iron porphyrin) catalysis was predicted by Warburg in 1923 (290) on the basis of some model experiments. Later Warburg demonstrated the occurrence of a hemin compound in yeast cells. This hemin compound is involved in the transfer of electrons from cytochrome

[18] The potentials are as follows: cytochrome *a*, +290 millivolts; cytochrome *c*, +270 millivolts; cytochrome *b*, −40 millivolts.

to oxygen. The reaction with oxygen is strongly inhibited by carbon monoxide, which combines with the reduced enzyme (Fe^{++}), and by cyanide, which combines with the oxidized enzyme (Fe^{+++}). The carbon monoxide–enzyme compound is split photochemically by certain wave lengths and hence is again available for oxygen. Following this principle Warburg obtained a detailed action-spectrum. This indirect spectrum was compared with the direct spectra of a number of different hemins. Warburg (291) identified the "oxygen-activating enzyme" as a pheohemin which with regard to its spectrum is an intermediate between red and green hemins. Keilin's so-called cytochrome a_3 (128) is perhaps identical with the oxygen-activating enzyme.

The importance of other heavy metals besides iron has been demonstrated in recent years. Kubowitz (152, 153) purified an enzyme which oxidizes polyphenols to quinones and showed that it is a copper protein. He furthermore showed, by the following ingenious arrangement, that copper ion is the prosthetic group of this enzyme: The copper enzyme was dialyzed against a dilute solution of cyanide which trapped the copper; the enzyme inactivated by this procedure was immediately reactivated by small amounts of copper ions.

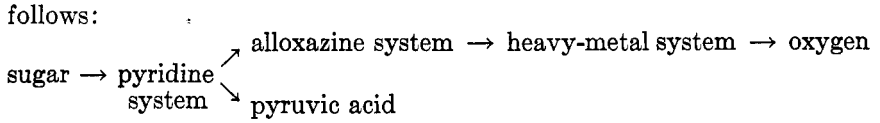
Keilin and collaborators purified other polyphenoloxidases and identified them as copper proteins. In a recent paper Keilin and Mann (129) have made the interesting discovery that ascorbic acid, which is an aliphatic dienol, is oxidized by polyphenoloxidases provided a trace (0.1 mg. per cubic centimeter) of catechol is added. Szent-Györgyi and collaborators (274) have recently discovered an enzyme which catalyzes the oxidation of dihydroxymaleic acid, which is a typical dienol. Swedin and Theorell (268) have purified this enzyme to a considerable degree; the enzyme seems to be a kind of peroxidase (*cf.* 267a, 268). The enzyme seems also to be a heavy-metal–protein compound. Szent-Györgyi is of the opinion that all dienols or diphenols form heavy-metal complexes, a suggestion which he was able to support in model experiments. A large number of dienols and diphenols actually form deeply colored iron complexes in water solution.

The heavy metals act presumably in all cases as electron-transfer systems, alternating between the ferro and ferri or cupro and cupri states.

The brilliant studies of Keilin and Mann (1940), showing that carboanhydrase is a zinc protein, should be mentioned here, although this subject is beyond the scope of this review.

H. The Pasteur reaction

The relation between fermentation and respiration can be illustrated as follows:



The alloxazine group of a yellow enzyme and the pyruvic acid compete for the reduced pyridine group; if oxygen is available, the alloxazine is kept in the oxidized form and will therefore be the strongest hydrogen acceptor. In the absence of oxygen (anerobic conditions), the alloxazine will be completely reduced very rapidly and the pyruvic acid formed will be the only hydrogen acceptor. This picture might serve as a simplified interpretation of the old phenomenon first observed by Pasteur (the so-called Pasteur effect), that in the presence of oxygen fermentation disappears or is depressed. The support for the hypothesis illustrated here is the observation of Lipmann (170) that the addition of large amounts of a very positive dye is able to regenerate the Pasteur effect in a system where this effect has disappeared.[19]

I wish, however, to point out that for several systems the illustration of respiration as a transfer of electrons through nucleotides and iron porphyrins is too simple.

As mentioned before in this review, small traces of succinic acid are necessary for the oxidation of pyruvic acid and glucose in animal tissue. Whether the succinic acid–fumaric acid system is interposed as an electron-transfer system between the alloxazine and iron porphyrin systems, as claimed by Szent-Györgyi, or whether it acts in another manner is a problem of great interest, also for the discussion of the Pasteur effect.

Tumor tissue (289), damaged tissue (289), and some tissue cultures (169) exhibit, in spite of a high respiration, a considerable aerobic glycolysis.

Colowick, Kalckar, and Cori (39) observed an aerobic glycolysis in kidney extract. The aerobic glycolysis (from glucose) disappears under anaerobic conditions, since glucose is not phosphorylated under these conditions. This aerobic "extract glycolysis" from glucose is probably due to an overproduction of hexose diphosphate (see section V), which by the action of glycolytic enzymes is converted into lactic acid. The authors discuss the possibility that the aerobic glycolysis in damaged cells is attributed to a too one-sided application of the oxidative energy on glucose phosphorylation which, in the presence of glycolytic enzymes, leads to lactic acid formation.

V. THE SIGNIFICANCE OF PHOSPHORYLATION IN OXIDATION–REDUCTION

A. The transfer of phosphate

As pointed out in section IV, alcoholic and lactic acid fermentation can be described as a hydrogen transfer by "pyridine" from an aldotriose to the carbonyl group of acetaldehyde or pyruvic acid. One complication, however, has so far not been mentioned in this review (except in the equa-

[19] Michaelis and Smythe (213) are of the opinion that the dyes inhibit primarily the phosphorylation of glucose, particularly the formation of hexose diphosphate.

tion of the dismutation of triose phosphate), that is, the problem dealing with the active form of the hydrogen donor. Whereas the hydrogen acceptor simply is acetaldehyde or pyruvic acid, the hydrogen donor in several cases is a much more complex system, the nature of which has not been revealed until recently; in fermentations the hydrogen donor is not triose but a phosphorylated triose.

The importance of phosphate for the alcoholic fermentation was discovered in 1905 by Harden and Young (108, 109). These investigators proved that in cell-free fermentation of glucose (yeast juice), inorganic phosphate disappeared by an esterification with glucose; the accumulated ester was hexose-1,6-diphosphate. Moreover, they observed a close relationship between the phosphate esterified and the amounts of carbon dioxide and alcohol formed. One half of the sugar utilized was split into carbon dioxide and alcohol and the other half was esterified to hexose diphosphate. This relation is expressed in the so-called Harden–Young equation:

$$2C_6H_{12}O_6 + 2Na_2HPO_4 = 2CO_2 + 2C_2H_5OH + 2H_2O + C_6H_{10}O_4(Na_2PO_4)_2$$

Later Robison (248) isolated hexose monophosphate from fermentation mixtures and showed it to consist of 60 per cent glucose-6-phosphate and 40 per cent fructose-6-phosphate. Usually dried autolyzed yeast accumulates much more hexose diphosphate than hexose monophosphate during fermentation, but investigations by Kluyver and Struyk (138) showed that the ratio between these two esters can readily be changed in favor of hexose monophosphate by dilution, and Smythe (260) showed that the yield of hexose monophosphate can be raised if certain redox dyes, for instance, rosinduline, are added. Addition of even very small amounts of arsenate (10^{-5} to 10^{-6} mole) entirely prevents the accumulation of hexose diphosphate and makes the fermentation independent of the addition of phosphate (110).

Meyerhof and collaborators (198) demonstrated the significance of the sugar phosphoric acid esters in the glycolysis in muscle tissue. Furthermore, Meyerhof and Lohmann and Parnas and Ostern and their coworkers have been able to demonstrate the mechanism of the phosphate transfer, a process which is closely connected with the fermentations in yeast and muscle tissue. Two phosphate esters are particularly important for the phosphate transfer in the metabolism of muscle tissue: adenylic acid, isolated in 1927 by Embden and Zimmermann (67), and creatine phosphate, discovered and isolated by Eggleton (62) and by Fiske and Subarrow (85) in 1927–29. The function of these two esters has been revealed by the investigations of Lohmann, Parnas and Lundsgaard, and others.

Adenylpyrophosphate (adenosine triphosphate) is in enzymatic equilibrium with creatine according to the equation (181, 160):

adenosine triphosphate + creatine $\rightleftharpoons$ adenosine diphosphate + creatine phosphate

Since the equilibrium constant is not far from 1, the free-energy change involved in this reaction is very small.

According to Euler and Adler (72) and to Ostern (233), the adenosine triphosphate is able to transfer one or two of its phosphate groups to sugar or to hexose monophosphate:

adenosine triphosphate + hexose monophosphate $\rightarrow$ adenosine diphosphate + hexose diphosphate

These processes cannot be reversed experimentally and give rise to a considerable liberation of free energy.

Furthermore, 2 moles of inorganic phosphate can be liberated from adenylpyrophosphate by an enzyme, adenylpyrophosphatase (117). For every mole of orthophosphate liberated from adenylpyrophosphate, 11,000 calories are set free as heat (ΔH) (198). Creatine phosphate is dephosphorylated only through the adenosine phosphate system, which can be considered as a phosphate-transfer system, since a minute amount of this nucleotide is able to dephosphorylate a large amount of creatine phosphate.

Adenosine triphosphate and creatine phosphate act exclusively as phosphate donors. Adenosine diphosphate can act both as phosphate donor and phosphate acceptor. Adenosine monophosphate (adenylic acid), hexose, and hexose monophosphate act as phosphate acceptors.[20] Adenosine monophosphate or diphosphate can be phosphorylated to triphosphate, not only by creatine phosphate but also by products formed in the fermentation of sugar. These phosphate donors,—phosphoglyceryl phosphate, phospho(enolic)pyruvate, and acetyl phosphate,—will be described in this section.

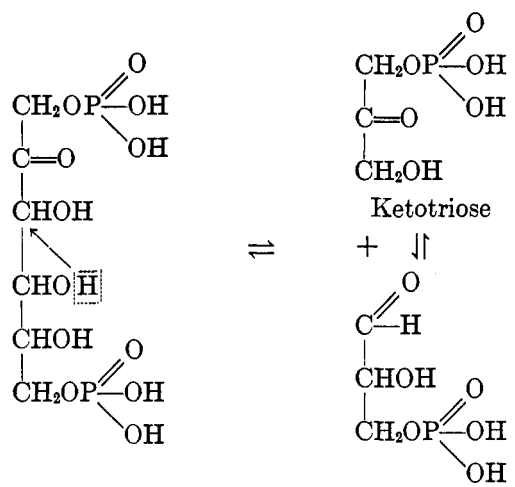
The classical work of Neuberg (224, 225) has been of very great importance for our understanding of fermentations as oxidation–reductions. The modern concept of the fermentation of phosphorylated sugars, however, was created by Embden and collaborators in 1933 (65) and developed by Meyerhof and coworkers (199). Owing to these brilliant investigations, every single step in the chain of reactions has been demonstrated and every single intermediate product has been isolated and identified. A further proof that the phosphate esters isolated are of physiological importance is the great rapidity with which these esters are converted into lactic acid

[20] Ostern *et al.* (234) have shown that in yeast systems adenosine (adenine-pentose) can be phosphorylated to the three different phosphorylation steps.

or into alcohol and carbon dioxide when small amounts of the right hydrogen and phosphate acceptors are present.

The mechanism of phosphate transfer connected with the fermentation has been cleared up by investigations of the Lemberg (23a) and the Cambridge schools (218). In a description of the Embden–Meyerhof scheme of fermentation it seems advisable to distinguish between three main phases: (*1*) Preparation of the actual hydrogen donor ("active sugar"), i.e., phosphorylation; (*2*) the oxidoreduction process proper; and (*3*) the regeneration of the hydrogen acceptor, i.e., anhydride formation and dephosphorylation.

1. Phosphorylation of sugars. In yeast, glucose (or fructose) is phosphorylated by adenosine triphosphate to hexose-6-phosphate and hexose-1,6-diphosphate (fructose diphosphate). In muscle tissue or tissue extracts, glycogen or starch is phosphorylated,[21] but here also hexose diphosphate is formed. The hexose diphosphate then undergoes a cleavage[22] by a reversible enzymatic reaction into two molecules of triose phosphate esters:



The enzyme which catalyzes this cleavage (or the reverse, the so-called aldol condensation) occurs in all tissues. This enzyme was called aldolase by Meyerhof and Lohmann (202, 203). The aldo-ester can be converted into the keto-ester or *vice versa* by a specific enzyme (isomerase).

2. The oxidoreduction. Until recently phosphoglyceraldehyde hydrate

[21] The initial steps will be described in a later section.

[22] *Cf.* the cleavage of rhamnose by some bacteria into propylene aldehyde and glyceraldehyde. The propylene aldehyde is immediately reduced to propylene glycol (137).

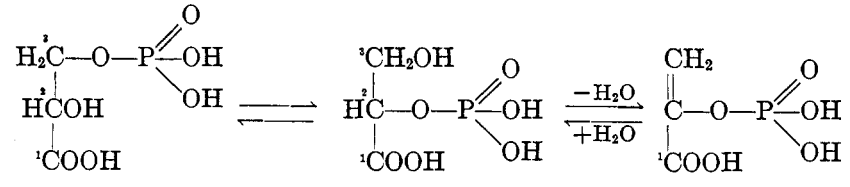
has been considered as the hydrogen donor proper, i.e., the substance which is dehydrogenated by the pyridine nucleotide; the latter then transfers the hydrogen to the carbonyl group of pyruvic acid or acetaldehyde. The end products of this oxidoreduction are phosphoglyceric acid and lactic acid or ethyl alcohol. This oxidoreduction, however, is not as simple as the scheme pictured in section III. Investigations from Needham's, Warburg's, and Meyerhof's laboratories (199) showed that simultaneously with the transfer of hydrogen from phosphotriose to the pyridine nucleotide, inorganic phosphate is taken up, yielding adenosine triphosphate. The primary acceptor of the inorganic phosphate was not known at that time, but it was shown that inorganic phosphate and adenosine diphosphate are necesary components in the oxidoreduction and that the inorganic phosphate is transferred to the adenosine diphosphate "by the energy of the oxido-reduction:"

phosphoglyce*raldehyde* + "pyridine"$^+$ + adenosine *di*phosphate
+ phosphate $\rightleftharpoons$ phosphoglyceric *acid*
+ "dihydropyridine" + adenosine *tri*phosphate

The reversibility of this process will be referred to later in this review. The coupling between the hydrogen and the phosphate transfer in this system is compulsory. However, in the presence of even very small amounts of arsenate (10^{-5} mole) the oxidoreduction proceeds without uptake of phosphate; no uptake of arsenate was detectable.

No phosphorylation has been observed in connection with the transfer of hydrogen from "dihydropyridine" to the carbonyl groups of pyruvic acid or of acetaldehyde (206).

3. The regeneration of the hydrogen acceptor. Several steps are necessary for this regeneration: anhydride formation, dephosphorylation, deenolization, and (in the alcoholic fermentation) decarboxylation (225). Meyerhof and Lohmann have demonstrated every single step; all the steps are catalyzed by specific enzymes and most of the step reactions have been shown to be reversible. The oxidation product of triose phosphate, 3-phosphoglyceric acid, is converted by a reversible enzyme reaction into the 2-phosphoglyceric acid, in which the phosphate group is esterified to the hydroxyl group of the middle carbon atom. The 2-phosphoglyceric acid undergoes a dehydration yielding phospho(enolic)pyruvic acid:



3-Phosphoglycerate 2-Phosphoglycerate Phospho(enolic)-pyruvate

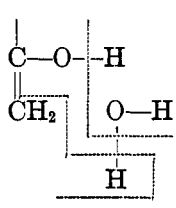
The phosphopyruvic acid has all the properties of an enolic ester (vinyl ester); i.e., it is hydrolyzed by small amounts of mercuric salts and by hypoiodite (183).

The phosphopyruvic acid is dephosphorylated by adenosine monophosphate or diphosphate (238), yielding free pyruvic acid, mainly in the keto-form (carbonyl) and adenosine triphosphate:

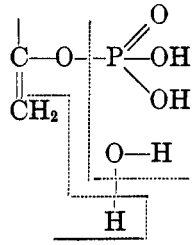
2 phospho(enolic)pyruvic acid + adenosine monophosphate

$\rightarrow$ 2 pyruvic acid + adenosine triphosphate

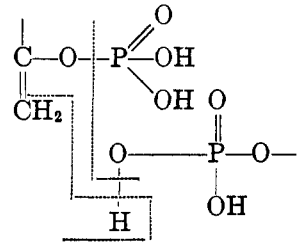
This reaction has so far not been demonstrated to be reversible. The ability of phospho(enolic)pyruvic acid to form pyrophosphate linkages must be ascribed to the simultaneous shift from an enolic to a keto structure:



(a) Conversion of enol-pyruvate to keto-pyruvate; $-\Delta F$ large.



(b) Conversion of phospho(enolic)-pyruvate to ketopyruvate + phosphate; $-\Delta F$ large.



(c) Formation of pyrophosphate linkage from phospho(enolic)-pyruvate; ΔF small.

B. The nature of the compulsory coupling between oxidoreduction and phosphorylation

The most complex step in the glycolysis is the oxidoreduction between triose phosphate and pyridine nucleotide, because this step requires the uptake of inorganic phosphate and the presence of adenosine diphosphate as an acceptor of this inorganic phosphate. The nature of this compulsory coupling between triose phosphate oxidation and phosphate uptake have remained completely obscure.

Recently, investigations from Warburg's laboratory (220, 299) have revealed the nature of the compulsory coupling between oxidoreduction and phosphorylation. Since these discoveries are most fundamental and of the greatest consequence for our understanding of energetic couplings, much attention will be paid to them in this review.

In 1939 Warburg and Christian (299) succeeded in the complete separation of different enzymes involved in the oxidoreduction of alcoholic

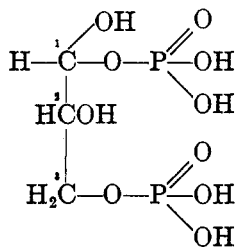
fermentation. This very high purification and separation of enzymes enabled Negelein and Brömel to observe and isolate a very important and interesting new ester, 1,3-diphosphoglyceric acid.

The following reversible reaction is supposed to take place:

glyceraldehyde-3-phosphate + phosphate

$$\rightleftharpoons \text{glyceraldehyde-1,3-diphosphate}$$

The new phosphate is linked to the aldehyde group[23] and the formula is as follows



i.e., the aldehyde phosphate replaces an aldehyde hydrate group. This diphosphotriose has never been isolated. If a small amount of Warburg and Christian's new crystalline enzyme is added to a solution of pyridine nucleotide and diphosphotriose, a rapid transfer of hydrogen from the triose to the "pyridine" takes place. The end products of this reaction are dihydropyridine nucleotide and diphosphoglyceric acid, which was isolated as the strychnine salt. The crystalline enzyme which catalyzes this oxidation is very active even in very small concentrations (0.8γ per cubic centimeter).

The oxidoreduction between diphosphotriose and pyridine is reversible:

1,3-diphosphoglyceraldehyde + "pyridine" $\rightleftharpoons$

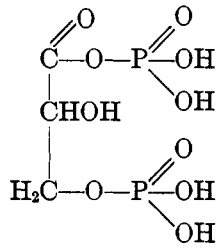
$$\text{1,3-diphosphoglyceric acid} + \text{"dihydropyridine"}$$

This is the oxidoreduction proper which is independent of inorganic phosphate and adenylic acid.

The aldehyde phosphate group of the diphosphotriose apparently represents the "active" sugar, since Warburg and Christian find that besides 1,3-diphosphotriose the 1-phosphotriose also is oxidized by the pyridine enzyme, although not nearly so rapidly; the well-known 3-phosphotriose is not oxidized.

[23] Apparently non-enzymatic as, for instance, the carbonyl sulfite reaction (*cf.* 175).

The 1,3-diphosphoglyceric acid apparently has the following formula (Negelein and Brömel):



The phosphate group linked to the carboxyl group is labile, although no enzyme seems to catalyze the liberation of phosphate (mineralization) from this carboxyl phosphate. Just like phospho(enolic)pyruvic acid and creatine phosphate, the phosphate of the carboxyl phosphate is transferred to adenosine monophosphate or diphosphate by a specific enzyme. Whether the phosphate of the "glyceryl phosphate" first has to pass the pyridine nucleotide or dihydropyridine nucleotide before entering the adenine nucleotide is not known, but the pyridine nucleotide is definitely not involved in the primary uptake of inorganic phosphate. Addition of arsenate abolishes the compulsory coupling between oxidoreduction and phosphorylation.

Warburg suggests a series of step reactions. In the presence of arsenate the reactions are as follows:

3-phosphotriose + arsenate $\rightleftharpoons$ 1-arseno-3-phosphotriose

1-arseno-3-phosphotriose + "pyridine" $\rightleftharpoons$

1-arseno-3-phosphoglyceric acid + "dihydropyridine"

1-arseno-3-phosphoglyceric acid $\rightarrow$ arsenate + 3-phosphoglyceric acid

The last equation expresses the fact that the arsenate is liberated from carboxyl arsenate spontaneously and rapidly enough to replace phosphate in the oxidoreduction.

In case phosphate is taken up, the corresponding reactions are:

3-phosphotriose + phosphate $\rightleftharpoons$ 1,3-phosphotriose (a)

1,3-diphosphotriose + "pyridine" $\rightleftharpoons$

1,3-diphosphoglyceric acid + "dihydropyridine" (b)

1,3-diphosphoglyceric acid + adenosine diphosphate $\rightleftharpoons$

3-phosphoglyceric acid + adenosine triphosphate (c)

adenosine triphosphate $\rightarrow$ { inorganic phosphate / creatine phosphate / glucose phosphate / hexose diphosphate } (d)

In Lebedew juice both phosphate groups contained in diphosphoglyceric acid are transferred *via* the adenine nucleotide to glucose, fructose, or hexose monophosphate. In living yeast one half of the phosphate is transferred to hexose; the other half is liberated (mineralized). The new C_3-diphosphates are also formed in muscle tissue; in this system the phosphates are transferred to creatine.

The isolation of a carboxyl phosphate able to phosphorylate adenine nucleotides has already led to a new fundamental observation. Lipmann (174), working with pyruvic acid dehydrogenase from lactic acid bacteria, observed that the oxidation of pyruvic acid to carbon dioxide and acetic acid requires inorganic phosphate or arsenate. No phosphate uptake was observable by the ordinary methods. The new interpretation of the phosphate uptake in the alcoholic fermentation led Lipmann (176) to suggest a formation of acetyl phosphate as the primary product of the enzymatic pyruvic acid oxidation. The formation of acetyl phosphate from pyruvic acid could not be demonstrated at that time, but Lipmann prepared acetyl phosphate synthetically and showed that in the presence of dried lactic acid bacteria phosphate is transferred from acetyl phosphate to adenylic acid according to the reaction:

$$2\ \text{acetyl phosphate}^{----} + \text{adenosine monophosphate}^{--} \rightarrow$$
$$2\ \text{acetic acid}^{--} + \text{adenosine triphosphate}^{----}$$

The demonstration of this reaction is very important, because it again illustrates an extraordinary property of carboxyl phosphate,—the ability to phosphorylate the adenylic acid system. More recently Lipmann (177) has observed the formation of a very labile phosphoric ester in the bacterial oxidation of pyruvic acid. The properties of this labile ester correspond actually to those of acetyl phosphate.[24]

As mentioned before, the pyrophosphate linkages in the adenine polyphosphates represent 11,000 calories (ΔH) which, besides carboxyl phosphates, only can be derived from guanidine phosphates and from phospho(enolic)pyruvic acid. The free energy of ordinary phosphoric esters (glycerophosphate, 6-phosphohexoses) can be calculated from rough estimations of equilibrium constants of hydrolysis of such esters. This calculation gives ΔF a value of about 1000 to 2000 calories.

The free energy of the aldehydehydrate-phosphate linkages can hardly exceed 1000 to 2000 calories, since the phosphorylation of triose in position 1 apparently takes place with inorganic phosphate (Warburg and Chris-

[24] Lipmann points out that the large liberation of energy in the hydrolysis of acetyl phosphate can also be used to acetylate compounds, for instance, choline. The name "phosphorylacetate" indicates that the ester can function as acetate donor (*cf.* 175).

tian). This means that an oxidation of an aldehyde-phosphate group to a carboxyl phosphate represents a conversion of a phosphoric ester with a potential energy around 1000 calories to a phosphoric ester having a potential energy of the order of magnitude of 10,000 calories.

As a consequence of this great increase in the free energy (great $+ \Delta F$) of the phosphate linkages in the conversion of an aldehyde-phosphate into a carboxyl phosphate, the fall in free energy of the *total* group is much smaller than the corresponding free-energy decrease between free aldehyde (+ water) and carboxyl groups. This again means that the normal potential of the system

$$\text{aldehyde-phosphate}^{--} \rightleftharpoons \text{carboxyl phosphate}^{--} + 2H^+ + 2\epsilon$$

is around 9 to 10,000 calories (= approximately 200 millivolts) higher than the normal potential of the system

$$\text{aldehyde} + H_2O \rightleftharpoons \text{carboxylate}^- + 3H^+ + 2\epsilon$$

An increase of 200 millivolts from the potential of the free aldehyde–carboxyl system will give a potential very near that of the pyridine system. The potential of

$$\text{aldehyde} + H_2O \rightleftharpoons \text{carboxylate}^- + 3H^+ + 2\epsilon$$

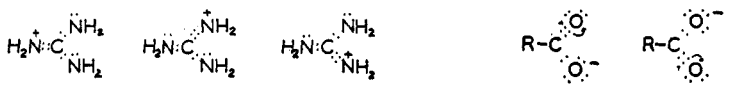
calculated from thermal data (Parks and Huffman (237), Borsook (26)) is 40 to 50 millivolts more negative than the hydrogen electrode, i.e., E_0' (pH 7) = approximately -460 millivolts. This is also in agreement with some experiments of Green *et al.* (99), who found that glyceraldehyde in the presence of a specific enzyme (mutase) reduced benzyl viologen completely.

Warburg and Christian's recent observations show that the potential of the aldehyde-phosphate $\rightleftharpoons$ carboxyl phosphate system amounts to nearly the same as the "dihydropyridine" $\rightleftharpoons$ "pyridine" system (i.e., -250 to -300 millivolts). If the ratio of oxidant to reductant of the pyridine system is greater than 1, which probably is the case under physiological conditions, diphosphotriose will easily be oxidized by "pyridine."

C. The relation between the electronic structure of phosphoric esters and their thermodynamic properties

Large free-energy decreases of reactions, as observed, for instance, in the hydrolysis of pyrophosphates, guanidine phosphates, carboxyl phosphates, and enolic phosphates, means that the stability of the products of reaction is much greater than that of the reactants. The modern physical and structural chemists lay stress on the phenomenon of resonance as a large factor in explaining the stability of molecular groupings. Molecules

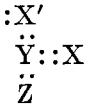
like the guanidinium ion and carboxylate ion can be described as resonating between two or three symmetrical structures.



Guanidinium ion Carboxylate ion

It is principally this resonance, stabilizing the guanidinium ion, which makes guanidine a base approximately 10^7 times stronger than ammonia, and this resonance preferentially stabilizing the carboxylate ion which makes carboxylic acids so much stronger than alcohols in acidity.

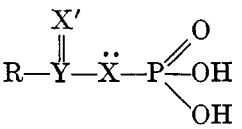
In general, molecular groups which have the following configuration exhibit high resonance:



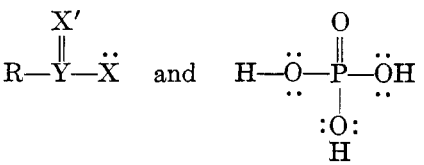
where the two X groups are the same or are closely alike in electron-attracting powers. Resonance is still more pronounced if Z is the same as X′ with an unshared pair of electrons.

It is one of the fundamentals of thermodynamics that the maximal amount of free energy is liberated in a reaction converting a molecular group of particularly low stability (or high energy) to one of particularly high stability (or low energy). As we have seen, the carboxylate ion, guanidinium ion (also ions of monosubstituted guanidines like creatine), etc. represent resonating structures of particularly high stability. Carboxyl phosphates, guanidine phosphates, enolic phosphates, and pyrophosphates share the same thermodynamic characteristic: hydrolysis of the phosphate ester linkage liberates much more energy (five to ten times more) than hydrolysis of hydroxy phosphate esters.

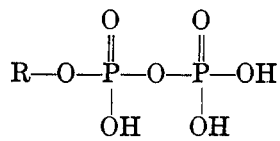
The phosphatic esters which are rich in energy actually display some common essential features in their structural composition. In all these esters we find the configuration:



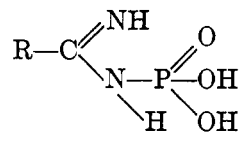
which can be hydrolyzed to give the configurations:



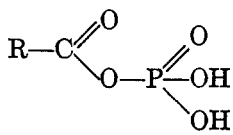
Ester linkages of high energy are as follows:



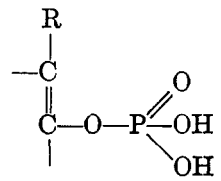
Organic pyrophosphate



Guanidino phosphate



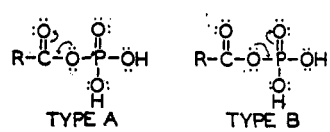
Carboxyl phosphate



Enolic phosphate

Only in the last compound, enolic phosphate, does X′ differ from X, a difference which tends to decrease the resonance contribution to the stability of the ester and the hydrolysis product. In this case the hydrolysis product is stabilized by tantomeric shift to the more stable keto-form, pyruvic acid.

The configurations of all the phosphoric esters displaying high esterification energy, and only these, show one important characteristic feature which will be referred to as opposing resonance.[25] Since the phosphate molecule on the right exhibits resonance between the different hydroxyl or amino groups and the P=O linkage and groupings on the left also exhibit an analogous resonance, the bridge of the ester linkage (—O— or —N—) is influenced by opposing resonance between the organic group (carboxylate ion, guanidino ion) and the phosphate group, each making demands on the same atom for the independent resonating systems.



TYPE A TYPE B

Resonance of type A therefore opposes that of type B, leading to less resonance energy for the groups combined as ester than for them when independent. The simultaneous elimination of two resonating structures makes carboxyl phosphates relatively very unstable. This instability of the ester in connection with the very high stability of the hydrolyzed product is responsible for the large liberation of free energy when this

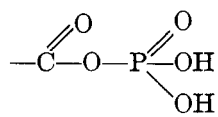
[25] Personal communication from C. D. Coryell.

kind of phosphoric ester is hydrolyzed. The great liberation of free energy by the hydrolysis of acetic acid anhydride is also attributed to opposed resonance of this type in connection with the high resonance of the two carboxylate ions. Negelein and Brömel (220) have investigated the ultraviolet spectrum of 1,3-diphosphoglyceric acid and have actually found a bond characteristic of the spectrum of acetic acid anhydride. Recently Lynen (190) found the same absorption band (mμ 217) for synthetically prepared acetyl phosphate. The phosphoric ester linkages with alcohol (hydroxyl) groups or aldehyde-hydrate groups (glycerophosphate, ordinary hexose phosphates, and monophosphotriose) do not exhibit opposing resonance and this, together with the lack of a resonating configuration of the free hydroxyl or aldehyde groups, will give a much smaller free-energy decrease upon the hydrolysis of this kind of phosphoric ester.

Warburg and Christian's separation of the enzymes involved in the biological oxidation of triose phosphate may also be of essential importance in obtaining values of the free energies of energy-rich phosphoric esters like carboxyl phosphate and adenylpyrophosphate. The possibilities of obtaining thermal data (heat of formation and heat capacity) for biologically important phosphoric esters are very small, because of the high requirements in purity and amount of substance. Thermal data for the inorganic phosphates exist but are very few and inaccurate. Latimer (159) estimates the pyrophosphate as "several tenths of a volt." More accurate thermal data of inorganic pyrophosphate would undoubtedly also be of interest in the study of adenylpyrophosphate. So far we know only the ΔH of the splitting of phosphate from adenylpyrophosphate. A large ΔF of such dephosphorylations makes, of course, direct equilibrium study impossible. These may, however, be applied to energy-poor phosphoric esters like carbonyl phosphates. Finally, free-energy changes can be measured by the potentiometric method which could, after the separation of all the "step enzymes" in the coupled oxidation of triose phosphate, be applied to the measurement of the free energy of some important phosphoric esters.

Assuming that the potentials of the systems of the triose phosphate oxidations are well defined, the following principles could be applied to obtain values for the free energy of the ester linkages in carboxyl phosphate and adenylpyrophosphate.

For the free energy of the ester linkage in



compare the redox potentials (ΔF) of the systems:[26]

$$\text{glyceraldehyde-3-phosphate} + \text{phosphate}^{--} \underset{+2\epsilon}{\overset{-2\epsilon}{\rightleftharpoons}} \text{phosphoglyceryl phosphate}^{--} \quad \text{(a)}$$

$$\text{glyceraldehyde-3-phosphate} + \text{arsenate}^{--} \xrightarrow{-2\epsilon} \text{phosphoglycerate}^{-} + \text{arsenate}^{--} \quad \text{(b)}$$

The difference between the redox potentials (E_0', pH 7) in the presence of phosphate and in the presence of arsenate would permit a calculation of the free energy of the glyceryl phosphate linkage. The difference between the two redox potentials might very well be about 250 to 300 millivolts.

The free energy of the pyrophosphate linkages in adenosine triphosphate can be obtained from the free energy of the glyceryl phosphate ester linkage by correcting for the ΔF of the enzymatic reaction:

$$\text{phosphoglyceryl phosphate}^{--} + \text{adenosine } \textit{di}\text{phosphate}^{---} \rightleftharpoons \text{phosphoglycerate}^{-} + \text{adenosine } \textit{tri}\text{phosphate}^{----}$$

This ΔF, which is supposed to be small, could be obtained by direct equilibrium determinations. However, the free energy of the pyrophosphate linkages in adenylpyrophosphate might also be obtained directly by a potentiometric method according to the following principle: Suppose the normal potential of the total system:

$$\text{glyceraldehyde-3-phosphate} + \text{phosphate}^{--} \underset{+2\epsilon}{\overset{-2\epsilon}{\rightleftharpoons}} \text{phosphoglyceryl phosphate}^{--}$$

$$\text{phosphoglyceryl phosphate}^{--} + \text{adenosine diphosphate}^{---} \rightleftharpoons \text{phosphoglycerate}^{-} + \text{adenosine triphosphate}^{----}$$

is obtained and compared with the potential of the same system plus adenylpyrophosphatase (the enzyme which catalyzes dephosphorylation from adenosine triphosphate), giving rise to the extra reaction:

$$\text{adenosine triphosphate}^{----} \rightarrow \text{adenosine diphosphate}^{---} + \text{phosphate}^{--}$$

[26] The ΔF of the reaction

$$\text{carbonyl} + \text{phosphate} \rightleftharpoons \text{carbonyl phosphate}$$

is not known, since the existence of carbonyl phosphate cannot be demonstrated by chemical methods. ΔF is probably very small (15 to 30 millivolts) and is of no importance for the present problem. Notice the appearance of a new acid equivalent in reaction b.

then the difference between the two potentials would give the ΔF of the dephosphorylation of one pyrophosphate linkage in the adenosine triphosphate. Assuming that the ΔF of the adenylpyrophosphate dephosphorylation is of the same order of magnitude as the ΔH, i.e., approximately 10,000 calories per mole of phosphate, then the redox potential of the system which oxidizes phosphotriose to phosphoglycerate and phosphorylates adenosine diphosphate to adenosine triphosphate should drop approximately 250 millivolts when adenylphosphatase[27] is added. The theory developed here is also illustrated in figure 2 of section X.

The clarification of the coupling between the triose phosphate oxidation and the uptake of inorganic phosphate represents one of the greatest advances in modern biology. The nature of the energetic coupling of living systems has always been wrapped in a shroud of mystery. For the first time since this recent discovery of the Warburg school, a complete description of a biological coupling is possible. By following this new line a clarification of other energetic couplings is to be expected.

D. Coupling between respiration and phosphorylation

A coupling between respiration and phosphorylation has been observed in hemolyzates of red blood corpuscles. Lennerstrand and Runnstroem (165) observed such a coupling in dry yeast preparations and found that triose phosphate was oxidized to phosphoglyceric acid.

Furthermore, phosphorylations coupled to respiration have been observed in animal tissue. This was first shown in minced kidney cortex and extract from kidney cortex (119). Such a system shaken with oxygen shows an intensive respiration. If phosphate and glucose are present and fluoride is added in order to stop dephosphorylation, most or all of the inorganic phosphate disappears in $\frac{1}{2}$ to 1 hr. The phosphoric ester which accumulates is mainly fructose-1,6-diphosphate. Fructose-6-phosphate added is phosphorylated to fructose-1,6-diphosphate. A new kind of esterification, so far only observed in kidney cortex, is the rapid phosphorylation of glycerol in kidney extracts; the ester formed was identified as the levorotatory α-glycerophosphate (123). Adenylic acid is phosphorylated rapidly to adenylpyrophosphate, an observation which corresponds to that of Engelhardt (68) and of Dische (60), made in experiments with red cells. The coupled phosphorylation and oxidation of fumaric or malic acid to phosphopyruvic acid has been mentioned and discussed in a previous section in this review.

All these phosphorylations depend on the rate of respiration; an inhibition of the respiration by cyanide gives an equal decrease in the phos-

[27] Only that fraction of the enzyme which splits one phosphate from the adenosine triphosphate. Such an enzyme can very easily be obtained from lobster muscles.

phorylation. Phloridzin inhibits the phosphorylation much more than the respiration.

Addition of 5 to 10 mg. of citric acid, glutamic acid, or another dicarboxylic acid, in some cases also alanine, approximately doubles the respiration, and the increase in phosphorylation of sugars, glycerol, or adenylic acid may be even larger.

Recent experiments of Colowick, Welch, and Cori (40) with dialyzed extracts show that the coupled phosphorylation system needs the following components: magnesium ion, succinic acid (about 0.1 mg. per cubic centimeter is sufficient), adenylic acid ($M/1000$ is a sufficient concentration), and pyridine nucleotide (cozymase). These experiments showed furthermore that extracts which had been dialyzed and aged for 24 hr. at 5°C., and therefore had oxidized succinic acid only one level to fumaric acid, still were able to phosphorylate glucose. Recent investigations of Belitzer and Tsibakova (18) on minced heart muscle also indicate that the step succinate $\xrightarrow{-2\epsilon}$ fumarate can give rise to phosphorylation.

These observations are of importance, because the succinic acid–fumaric acid system is quite different from the other systems known to be coupled with phosphorylations. The succinic acid oxidation is a desaturation, like the oxidation of saturated fatty acids. How the phosphate can enter such a system is not yet understood; perhaps an uptake of phosphate during the oxidation yields phosphomalic acid instead of fumaric acid.

Succinic acid dehydrogenase from kidney seems, however, not to be dependent on phosphate, since an intensive oxidation occurs even at very low phosphate concentrations ($M/3000$). Whether the succinic acid is oxidized directly by cytochrome *c* or whether the dehydrogenase possesses a prosthetic group (for instance, a flavin nucleotide) is so far an unsolved problem. The normal potential of the succinate–fumarate system, E_0' (pH 7), is approximately 0; the normal potential of cytochrome *c* is +270. This great potential difference between the two systems is probably of importance for the ability of the succinate oxidation to cause phosphorylation.

Furthermore, Colowick, Welch, and Cori (41) observed that if glucose is added to an extract (in the absence of fluoride) it is not oxidized; however, in the presence of 0.2 mg. of succinic acid a very intense oxidation takes place. This observation corresponds to that of Annau and Erdös (4) and of Banga, Ochoa, and Peters (8) that pyruvic acid only is oxidized in the presence of succinic acid.

Recent experiments by Colowick, Kalckar, and Cori (39) show a quantitative relation between the combustion of glucose (complete oxidation to carbon dioxide) and phosphorylation. Cell-free, dialyzed extracts of heart muscle are occasionally able to oxidize glucose completely. For every mole of glucose oxidized to carbon dioxide, an additional 5 to 6 moles of

glucose disappear, 5 moles of which are accumulated as fructose diphosphate. These quantitative relationships indicate strongly that at least ten, if not all twelve, steps in the glucose combustion can give rise to phosphate uptake.

Experiments with kidney and heart extracts show that the oxidation of citric acid and glutamic acid can give considerably more phosphorylation than oxidation of succinate.

In some experiments with heart extract, the oxidation of pyruvate (but not of succinate) gave remarkably high yields of phosphorylation. Per millimole of oxygen consumed, 4 millimoles of phosphate were taken up. Belitzer and Tsibakova and Ochoa have observed just as high ratios of P/O_2 or even higher, and these authors also point out that the high ratio P/O_2 may be explained by an additional uptake of phosphate when the hydrogen passes from one hydrogen-transfer system to another. Belitzer and Tsibakova's experiments exclude any anaerobic dismutations as a source of phosphorylation. The assumption that a transfer of hydrogen from one transfer system to the next (for instance, from the pyridine to the alloxazine nucleotide) is able to provide energy for phosphorylations deserves attention. There is no reason to assume that the step metabolite $\rightarrow$ "pyridine" (or "thiamine") is the only step in the long chain of hydrogen transfer which can provide energy for phosphorylations.

The large accumulation of hexose diphosphate in heart extracts, even in the absence of fluoride, when glucose or pyruvate are oxidized is presumably attributed to the lack of phosphatases.

This accumulation of hexose diphosphate in cell-free heart muscle extracts which burn glucose is actually the analog to the Harden–Young reaction in cell-free yeast juice. Hexose diphosphate also is accumulated in kidney extracts, which oxidizes glucose and pyruvate to carbon dioxide. The accumulation is, however, much smaller than in heart extracts and the main part of the glucose which disappears in addition to the combustion is converted into lactic acid. The lactic acid formation is not able to phosphorylate extra glucose.

The lactic acid formation from glucose in kidney extracts is therefore, at least in most cases, an exclusively aerobic phenomenon. The ratio moles of glucose utilized/moles of glucose oxidized is frequently much less than 6, presumably because kidney extracts contain large amounts of adenylpyrophosphatase.

In the living cell this ratio is also much smaller than 6, since only a small fraction of the phosphate taken up in the oxidation of metabolites needs to be transferred to glucose[28] for the autocatalysis of the respiration; the main part is presumably connected with the specific cellular structures.

[28] Since the hexokinase was separated from a conversion enzyme, i.e., the enzyme which catalyzes the conversion of 1-phosphoglucose to 6-phosphoglucose (see section VIII), the primary sugar ester formed is 6-phosphoglucose.

The formation of hexose diphosphate or of ordinary hexose monophosphate (6-phosphoglucose) from adenylpyrophosphate (polyphosphorylated by oxidation) and glucose or fructose represents a considerable fall in free energy, since a pyrophosphate linkage contains more than 10,000 calories, whereas alcohol–phosphate linkages represent hardly more than 1000 calories. It is likely, however, that in the living cell the energy of the pyrophosphate is utilized to a much greater extent. 1-Phosphoglucose (Cori ester, *cf.* section VIII, D), the precursor of polysaccharides, is probably formed from adenylpyrophosphate.

The yeast enzyme, hexokinase, which was discovered by Meyerhof in 1927, catalyzes the transfer of phosphate from adenylpyrophosphate to glucose or fructose. This enzyme system has recently been studied by the author in collaboration with Colowick (Colowick and Kalckar, 1940–41; experiments to be published).

These studies show that the enzyme (kept as an ammonium sulfate precipitate) in the presence of magnesium ions, adenosine triphosphate, and hexoses (glucose or fructose), catalyzes the reaction:

adenosine *tri*phosphate + hexose → adenosine *di*phosphate + 6-phosphoglucose[28]

The reaction, which proceeds with considerable rapidity, can be followed not only by chemical methods but also manometrically, since one acid equivalent is liberated when an alcohol phosphate replaces a pyrophosphate. In this system only one pyrophosphate linkage is split; however, the remaining pyrophosphate is utilized (for hexose phosphorylation) if a heat-stable protein isolated from muscle tissue is added to the enzyme system. In the presence of hexokinase, magnesium ions, and the heat-stable protein, adenosine *di*phosphate is able to react with hexose according to the equation:

adenosine diphosphate + hexose → adenylic acid + 6-phosphoglucose

The heat-stable protein which, in addition to the yeast enzyme, is necessary for the last-mentioned reaction, occurs in muscle extracts but neither in liver nor in kidney extracts. The active protein is precipitated by ammonium sulfate (and can be purified by fractionation), by sodium sulfate, and by trichloroacetic acid. The trichloroacetic acid precipitate is soluble in alkali, and the solution exhibits full activity. The protein is inactivated when kept in alkaline solution but is completely reactivated by reduced glutathione or cysteine. The activity of the protein is not decreased by 15 min. boiling in 0.1 *N* hydrochloric acid. Pepsin hydrolyzes the protein and the activity disappears.

The heat-stable protein is active in very small amounts; 1γ of purified

protein per cubic centimeter still exhibits considerable activity. Insulin is inactive in the enzyme test.

The function of the protein in the phosphate transfer from the organic pyrophosphate compound to hexose is not yet known. A potassium chloride–bicarbonate solution, used for the extraction of myosin, extracts several times more of the active protein than is obtained by an ordinary water extraction. This observation, together with the fact that the protein is absent in liver and kidney extracts, deserves attention (*cf.* section IX, D).

The distribution of the phosphate energy will undoubtedly be one of the major problems in the future.

VI. THE SYNTHESIS OF FATTY ACIDS FROM SUGARS

Little is known about the pathway of fatty acid formation from sugar. However, a good deal can be learned by studying some bacterial fermentations where fatty acids are formed. The best known of this kind of fermentation is the butyric acid fermentation.

A. The butyric acid and butanol fermentations

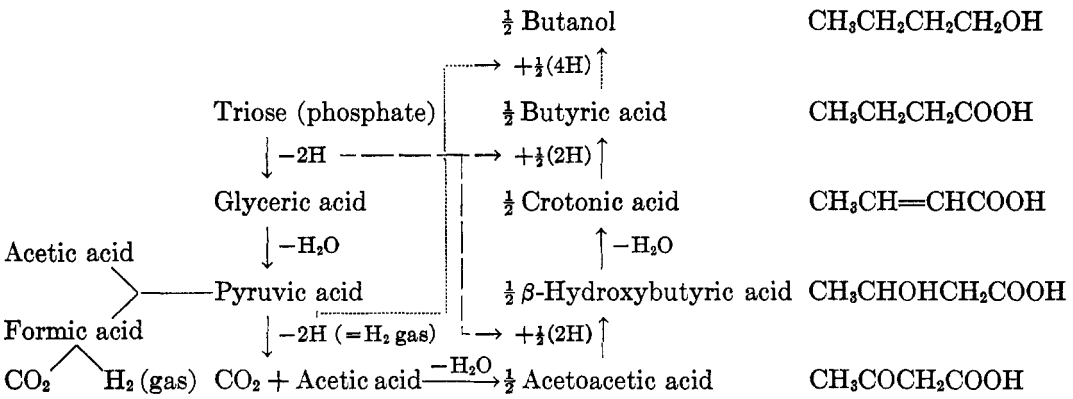
The anaerobic spore-form *Clostridium butyricum* ferments sugar according to the equation:

$$\text{glucose} = \text{butyric acid} + 2CO_2 + 2H_2$$

Besides these products, a varying amount of acetic acid is formed.

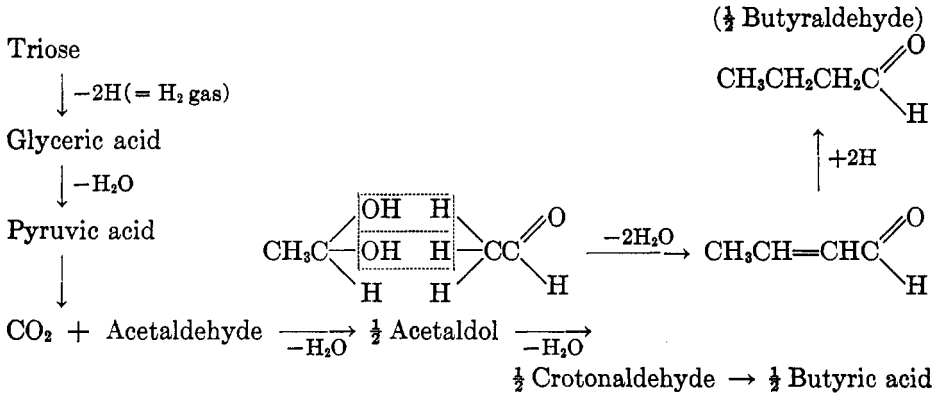
If butyl alcohol (butanol) is formed, no hydrogen gas arises, since the formation of butanol is equivalent to equal quantities of butyric acid and hydrogen gas.

The majority of investigations have been carried out on the fast growing *Clostridium acetobutyricum* (Fernbach). On the basis of these investigations, a reasonable scheme of the butyric acid fermentation would be as follows:



Most of the hydrogen gas presumably arises in the oxidation of pyruvic acid to acetic acid and carbon dioxide, because of the very low potential of this system. The condensation of acetic acid to acetoacetic acid has been demonstrated (247).[23a] Addition of acetic acid to a butanol fermentation mixture yields a quantitative amount of acetone but no butanol (118). This is due to the fact that all the hydrogen formed is used in the butanol fermentation; acetoacetic acid formed from the added acetic acid therefore accumulates and is spontaneously decarboxylated. The fact that added acetic acid or acetoacetic acid is not converted to butyric acid or butanol can therefore not be used as an objection against the theory of intermediate formation of acetoacetic acid in these fermentations. The reduction of acetoacetic acid to β-hydroxybutyric acid is a well-known reaction in animal tissues (96, 115). The reduction of crotonic acid to butyric acid has been demonstrated by Bernhauer (21).

Another group of investigators lays more stress on the condensation of acetaldehyde to acetaldol as the source of butyric acid. This theory is supported by two observations. Haehn's (103, 104) experiments with the mould *Endomyces vernalis* show that crotonaldehyde, the anhydride of acetaldol, is converted to fats. Kuhn and collaborators (155) have prepared stearic acid synthetically from crotonaldehyde. These two observations suggest that crotonaldehyde may be an essential intermediate, not only in the formation of palmitic acid but also in the butyric acid fermentation. The following scheme illustrates the crotonaldehyde theory:



It is not easy to say which of the two schemes best illustrates the butyric acid fermentation. The formation of acetoacetic acid and the reduction of this substance to β-hydroxybutyric acid are reactions which have been

[23a] A direct formation of acetoacetic acid from acetic acid is unlikely, because of the high stability of the carboxyl group. The acetoacetic acid is perhaps formed from acetyl phosphate or from acetopyruvic acid (*cf.* Krebs).

experimentally demonstrated in animal tissue. The formation of acetaldol from acetaldehyde has also been demonstrated, but the removal of the second molecule of water, yielding crotonaldehyde, is so far purely hypothetical. The addition of crotonaldehyde, on the other hand, gives rise to an increased formation of higher fatty acids. Feulgen's discovery of palmital (80), the aldehyde of palmitic acid, is of considerable interest in this discussion.

A reduction of crotonaldehyde to butyraldehyde is thermodynamically more likely than a reduction of butyric acid to butyraldehyde. Butyric acid, on the other hand, actually accumulates in the first phase of a butanol fermentation. If it is assumed that crotonaldehyde is reduced to butyraldehyde, an equilibrium between crotonaldehyde and butyric acid (far to the side of the latter substance) has to be postulated. Both schemes, however, illustrate very clearly the characteristic feature of a mixed assimilatory and dissimilatory fermentation: (*1*) The hydrogen acceptors are represented by the anhydrides of the first oxidation level (crotonaldehyde) or second oxidation level (acetoacetic acid), respectively, and, furthermore, by the anhydride of one of the reduction products (crotonic acid). (*2*) After a "concentration" of the oxygen at one end of the molecule (transformation of glyceric acid to pyruvic acid), one carbon is sacrificed as carbon dioxide and the other part (2C) is used for condensation.

The second scheme of butyric acid fermentation actually illustrates a sort of coupling between decarboxylation and a condensation, leading to fatty acid formation.

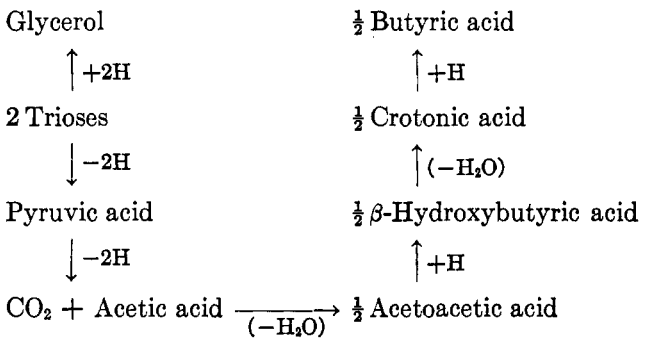
Fatty acid formation from sugar, regardless of whether it occurs in microörganisms or in animal tissues, is always connected with decarboxylation, simple or oxidative. Thiamin (vitamin B_1) is known to be of importance in the transformation of sugar into fatty acids (194). Transformation of glycerol into fatty acids gives rise to less carbon dioxide than the transformation of sugars to fatty acids. The most extreme illustration of this fact is van Niel's demonstration of the quantitative transformation of glycerol into propionic acid by propionic acid bacteria. This phenomenon has already been discussed.

The pathway of fatty acid formation from sugars in animal tissue is not known. The different possibilities have been discussed in a comprehensive review by Smedley McLean (259). The recent work of Schoenheimer and Rittenberg (256), using deuterium or radioactive carbon as tracers, will undoubtedly be able to throw considerable light on the pathway of fat formation in the animal organism.

Probably fatty acid formation from sugar in animal tissues is a mixed respiration and fermentation process. Since hydrogen gas is never formed

in animal tissues, this transformation can hardly be due to fermentation only.

If the triose is dismuted into glycerol and glyceric acid, sugar can be transformed into fatty acids by a pure fermentation without evolution of hydrogen.



The simultaneous formation of glycerol might be of importance for the formation of glycerides (fats).

B. The formation of higher fatty acids

The bacterial fermentative transformation of sugar into fatty acids, even the formation of caproic acid from alcohol (11), represents sources of energy for the growth of bacteria.

There is reason to believe that the transformation of sugar into fatty acid and carbon dioxide, occurring on a large scale in several animals, also represents a source of energy available for endergonic processes.

The understanding of the mechanism of fatty acid formation from sugar is closely connected with the knowledge of the oxidation of fatty acids. Knoop's hypothesis of β-oxidation has been of essential importance for the understanding of fatty acid oxidation. In this connection it is of interest that acetic acid condensation leads to a β-keto acid. According to the classical experiments of Knoop (139), every mole of fatty acid gives 1 mole of acetoacetic acid. Recent experiments (245, 23) show that in several cases more than 1 mole of acetoacetic acid can be formed from a molecule of the higher fatty acids, but these new observations are not in disagreement with the concept of β-oxidation.

Until recently the general opinion was that fatty acid dehydrogenases are strictly dependent on the integrity of the cell. This is not the case, since Leloir and Murry (163) and later Welch and Cori (301) found that homogenized liver tissue oxidizes butyric acid; the latter authors found a large increase in the oxygen consumption after addition of butyric acid to homogenized liver tissue. Furthermore, Konrad Land and collaborators

(143, 144) have recently been able to demonstrate a palmitic acid dehydrogenase in liver extract, which in the presence of methylene blue oxidizes palmitic acid to oleic acid. Adenylic acid (a pure product from Ostern's laboratory was used) is a necessary component of the palmitic acid dehydrogenase. These findings are of importance for the understanding of the nature of fatty acid dehydrogenases. The first step of fatty acid oxidation (desaturation) thermodynamically belongs to the hydrocarbon $\rightleftharpoons$ ethylene type, like succinic acid $\rightleftharpoons$ fumaric acid.

Schmidt (255) finds that the respiration in dialyzed heart muscle is increased considerably by the addition of phospholipids (purified).

The nature of fat formation, i.e., esters of glycerol (and phosphate) and carboxyl groups of fatty acids, is not known. The fundamental discovery of fatty aldehydes like palmital and their esterification with glycerophosphate (Feulgen (80)) may give the solution of the problem of fat formation. Oxidation of palmital-glycerol ester would yield a palmitin glyceride.

VII. THE SYNTHESIS OF NITROGEN COMPOUNDS

A. Assimilation of nitrogen

A few words about nitrogen fixation might be useful in this review.

The bacterial nitrogen fixation was discovered by Winogradsky in 1899 (305). Winogradsky was also the first to realize that the nitrogen fixation was a reduction of N≡N to ammonia or derivatives of ammonia. As pointed out by Burk and Horner (33), however, a reliable demonstration of ammonia formed from nitrogen is difficult, because the process is so slow that it is difficult to distinguish it from an autolysis of the bacteria (azobacteria). Bortels in 1931 (28) discovered that the reduction of nitrogen to ammonia is catalyzed by molybdenum (*cf.* the Haber process); how the molybdenum acts, as an electron-transfer system or in some other manner, has not yet been ascertained.

In contrast to the reduction of the double bond in oxygen (O=O), the reduction of the triple bond in nitrogen (N≡N) is an endergonic reaction (*cf.* Lewis (166)). As pointed out by Burk, the stable N≡N bond can be reduced only by hydrogen. It is not unlikely that the oxidation of pyruvic acid, because of the great negativity of the system

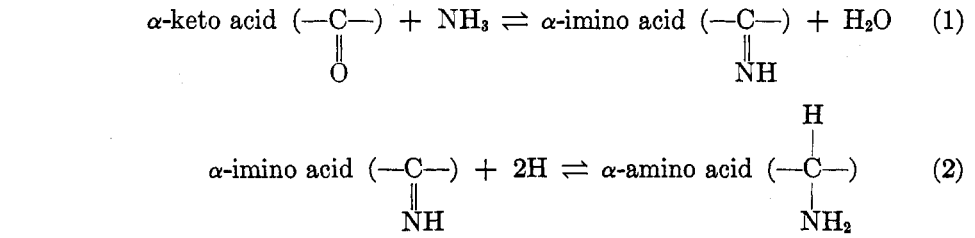
$$\text{pyruvate} + H_2O \rightarrow \text{acetate} + CO_2$$

is an important energy source for the reduction of nitrogen.

B. The formation of amino acids from sugars and related compounds

In 1925 Knoop and Oesterlin (141) showed that palladium and hydrogen can reduce keto acids and ammonia to amino acids. Neubauer (223), Embden, and others demonstrated the deamination of amino acids to the

corresponding keto acids. Knoop and Oesterlin in model experiments reduced α-keto acids with palladium and hydrogen in the presence of ammonium salts and obtained a high yield of the corresponding amino acids. They interpreted the reaction as follows:



(1)

(2)

Thus the imino acid replaces the keto acid as hydrogen acceptor; the labile nitrogen in the imino acid is "fixed" by the reduction to amino acid. That the oxidative deamination and the reductive amination are reactions of importance in living systems was established in 1937–38 by two discoveries,—(*1*) Warburg and Christian's purification of the *d*-amino oxidase, and (*2*) Adler and Euler's demonstration of the enzymatic reduction of α-ketoglutaric acid and ammonia to glutamic acid.

1. The d-amino oxidase was first described by Krebs (146), who observed that a long series of *d*-amino acids (the optically active amino acids in proteins are mainly *l*-amino acids) are oxidized if added to water extracts of kidney cortex or liver tissue. Warburg and Christian (298) purified Krebs' oxidase and separated it into a protein and a nucleotide which was identified as a flavin-adenine nucleotide (*cf.* section IV). The alloxazine ring of the nucleotide accepts the hydrogen from the amino group, and the hydroalloxazine formed gives the hydrogen to oxygen, which is reduced to hydrogen peroxide. Negelein and Brömel (219) have shown that the hydrogen peroxide in the pure enzyme preparations free from catalase oxidizes the pyruvic acid formed from alanine to acetic acid and carbon dioxide. Proline and valine also are oxidized by the pure *d*-amino oxidase.

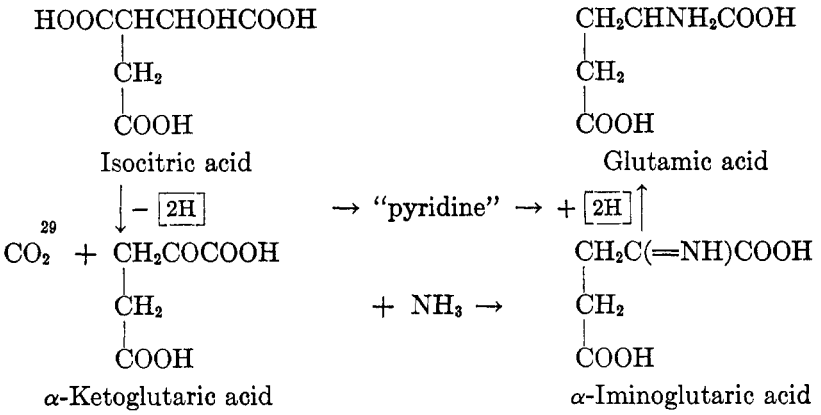
2. The glutamic acid–ketoglutaric acid system. Adler (1, 2), in Euler's laboratory, demonstrated that Knoop and Oesterlin's reductions of keto acids in the presence of ammonia to amino acids also occur in biological systems. Euler and coworkers (73) showed that glutamic acid is oxidized by a pyridine nucleotide (Warburg's coenzyme) to α-ketoglutaric acid and ammonia.

Adler prepared the hydropyridine nucleotide in large amounts and succeeded, in the presence of the specific glutamic acid dehydrogenase, in reducing α-ketoglutaric acid and ammonia to glutamic acid.

If the pyridine nucleotide is kept reduced by a hydrogen-donor system (e.g., alcohol-aldehyde + specific protein enzyme), catalytic amounts of

pyridine nucleotides are able to reduce large amounts of ketoglutaric acid and ammonia to glutamic acid.

Adler, Euler, Günther, and Plass (3) recently, in a very interesting paper, succeeded in converting citric acid (or isocitric acid) quantitatively to glutamic acid:



The conversion of isocitric acid and ammonia into glutamic acid is actually a "fermentation." The isocitric acid is formed from citric acid through the anhydride *cis*-aconitic acid (191, 192).

The position of citric acid in metabolism is not clear. A citric acid dehydrogenase (better "isocitric acid dehydrogenase") was first demonstrated by Thunberg. According to Krebs and Johnson (151), citric acid is formed by a condensation between oxaloacetic acid and an oxidation product of sugar, presumably acetic acid.

Simola and collaborators (258, 125) recently have been able to demonstrate extensive formation of citric acid in animal tissue *in vivo* as well as *in vitro* when pyruvic and malic acids were added. This might be due to a formation of acetic acid and oxaloacetic acid which immediately condense. It is not unlikely that the formation of citric acid is connected with the complex oxidation of acetic acid.

Krebs and Cohen (150) have recently shown an interesting dismutation of α-ketoglutaric acid and ammonia into glutamic acid, succinic acid, and carbon dioxide. In the absence of ammonia the dismutation proceeds to a much smaller extent. Apparently the iminoglutaric acid is a better hydrogen acceptor than the ketoglutamic acid is (*cf.* Krebs). The step

$$\text{ketoglutaric acid} + H_2O - 2H = \text{succinic acid} + CO_2$$

corresponds to the oxidative decarboxylation of pyruvic acid to acetic acid and carbon dioxide.

[29] Spontaneous decarboxylation (*cf.* acetoacetic acid).

The system

$$\text{glutamic acid} \rightleftharpoons \text{ketoglutaric acid} + NH_3 + 2H^+ + 2\epsilon$$

is a reversible redox system with an E_0' (pH 7) = −0.50 millivolt (Borsook (26)). Wurmser and Fillitti-Wurmser (310) found that the system

$$\text{alanine} \rightleftharpoons \text{pyruvic acid} + NH_4^+ + 2H^+ + 2\epsilon$$

is also a reversible redox system with an $E_0' = -0.48$ volt. A reductive formation of alanine from pyruvic acid and ammonia is thus possible. Glycolysis in the presence of ammonia would therefore yield alanine instead of lactic acid.

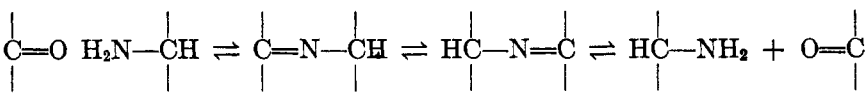
C. Transamination

Braunstein and Kritzman (31) made the important discovery that ketodicarboxylic acids and α-amino acids or *vice versa* react in the presence of specific enzymes (aminopherases) in such a manner that the amino group is transferred to the keto group, for instance:

$$\text{glutamic acid} + \text{pyruvic acid} \rightleftharpoons \alpha\text{-ketoglutaric acid} + \text{alanine}$$

One of the reacting components, either the keto acid or the amino acid, has to contain two acid groups as, for instance, glutamic acid or aspartic acid (the corresponding keto acids, phosphoserine and homocysteinic acid, are also active (30)).

Braunstein and Kritzman interpret the transamination as a condensation, forming a Schiff base:



Only *l*-amino acids are transaminated. According to Braunstein, the transamination is the only way in which *l*-amino acids are oxidized. The Braunstein enzyme aminopherase is found in large amounts in all animal tissue; the glutamic acid represents the most important amino-transfer system.

D. The aspartase system

Quastel and Woolf (246) and Virtanen and Tornanen (286), working with *B. coli*, demonstrated a new type of amination–deamination:

$$\text{aspartic acid} \rightleftharpoons \text{fumaric acid} + NH_3$$

The reaction is related to the equilibrium

$$\text{malic acid} \rightleftharpoons \text{fumaric acid} + H_2O$$

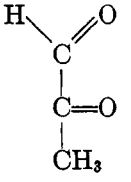
The ΔF values for these equilibrium reactions are less than 1000 calories.

Possibly deaminations related to the aspartase reaction are responsible for the reduction of amino acids to fatty acids in certain strains of *Clostridia* (Stickland). The unsaturated compound formed by deamination is the hydrogen acceptor proper.

E. The formation of peptides from amino acids

Peptides can be formed directly from amino acids if the latter are present in very high concentrations. The equilibrium lies far to the side of hydrolysis (*cf.* phosphoric esters in the presence of phosphatases). There is reason to believe that peptide formation in tissues is always coupled with oxidoreduction just like phosphorylations.

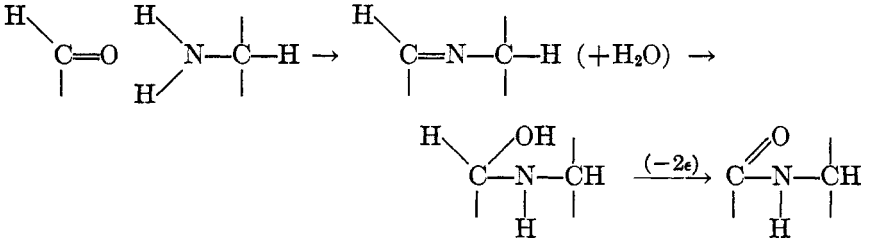
Linderström-Lang (168) points out that a primary reaction between a carbonyl group and an amino group followed by an oxidation of this linkage yields a peptide bond. He calls attention to the fact that, in order to obtain a typical polypeptide of α-amino acids, the amino group has to react with a dicarbonyl compound like methylglyoxal, where reductive amination of the 2-keto groups makes complete the addition of a new amino acid residue to the polypeptide chain.



Methylglyoxal

Methylglyoxal was discovered by Neuberg (226) in toluene-treated glucose-fermenting yeast. Methylglyoxal is formed by non-enzymatic dephosphorylation of triose phosphate (202).

The oxidation of a carbonyl-amino compound to a peptide shows some resemblance to an oxidation of an aldehyde (*cf.* also xanthine oxidation):



It is of importance to notice that both the condensation and the oxidation are exergonic reactions, indicating that peptide linkages are rather stable structures. The stability of the peptide structure able to resonate between

the amide and the enolic configurations is also pointed out by Pauling and Niemann (242).

The author of this review is inclined to think that carboxyl esters, like carboxyl phosphates (which yield pyrophosphate), glycerides (fats), and peptides are formed from the corresponding carbonyl esters by oxidation. This mechanism has been established in the case of carboxyl phosphate formation (Warburg, Negelein, Lipmann), is likely in the case of glycerides (Feulgen), and it may very well be that the "potential" energy of the carbonyl group is the driving force also in the peptide formation.

VIII. THE SYNTHESIS OF MONO- AND POLY-HEXOSES

A. Photosynthesis

In the green plant, sugars are formed from carbon dioxide and water with light as the energy source. Carbon dioxide is the hydrogen acceptor and water the hydrogen donor. Since the potential difference between the hydrogen donor and the hydrogen acceptor is 1200 to 1300 millivolts (= 56,000 calories) to the wrong side, the hydrogen donor belonging to the system of high potential and the hydrogen acceptor to the system of low potential, a supply of at least 56,000 calories per mole from an outside source of energy is necessary. This energy source is the light.

In the field of photosynthesis three main lines have been followed: (*1*) The number of quanta absorbed per mole of oxygen developed from water (the so-called quantum yield); (*2*) the constitution and mode of action of chlorophyll; (*3*) the intermediate reactions in the formation of sugar from carbon dioxide and of oxygen from water.

The chlorophyll of green plants was isolated by Willstätter and Stoll in 1910–12; these two investigators worked out the constitution of chlorophyll. Chlorophyll is a porphyrin compound in combination with magnesium. The chlorophyll of the photosynthetic active bacteria is very closely related to that of the green plants. Investigations of Hans Fischer *et al.* showed that the vinyl group, $CH_2{=}CH{-}$, in plant chlorophyll is replaced by an acetyl group, $CH_3CO{-}$, in bacterial chlorophyll. We have, however, only very limited knowledge about the mode of operation of chlorophyll, mainly because photosynthesis so far cannot be separated from the cell structure.

It seems certain that chlorophyll and not the carotinoids is involved directly in the light reaction, i.e., in the absorption of light. It has been suggested that chlorophyll transfers hydrogen, an assumption which seems reasonable but so far has no experimental basis.

The third problem, the pathway of sugar formation from carbon dioxide and water, has until recently not been treated experimentally, only specu-

latively. The work of van Niel, Gaffron, and others with the photosynthetic purple bacteria and the work of Ruben, Perlmann, and collaborators with radioactive carbon isotopes have given us the basis for an understanding of the reaction.

van Niel (228) found that the bacterial photosynthesis in some respects differs from that of the green plants: (*1*) no oxygen is formed; (*2*) the bacteria can live and grow without oxygen, provided light is present; (*3*) the bacteria can grow in the dark, provided oxygen is present; (*4*) several of the bacteria require hydrogen sulfide as well as carbon dioxide. van Niel found the following equations:

$$1 \text{ mole } H_2S \rightarrow 1 \text{ mole S per } 0.5 \text{ mole } CO_2 \text{ to organic material}$$

$$1 \text{ mole } H_2S \rightarrow 1 \text{ mole } H_2SO_4 \text{ per } 2 \text{ moles } CO_2 \text{ to organic matter}$$

Does hydrogen sulfide replace water or does it replace ordinary metabolites? A decisive answer to this question was given by Gaffron's demonstration of a bacterial photosynthesis without hydrogen sulfide. He (89) found that in some purple bacteria photosynthesis of hydrogen sulfide takes place in the absence of hydrogen sulfide; in these cases fatty acids replace hydrogen sulfide as hydrogen donor. Gaffron found the following relationships:

Utilization of 1 mole of C_3H_7COOH: 1 mole of carbon dioxide reduced

Utilization of 1 mole of $C_5H_{11}COOH$: 2 moles of carbon dioxide reduced

Utilization of 1 mole of $C_7H_{15}COOH$: 3 moles of carbon dioxide reduced

Gaffron also found that hydrogen can replace hydrogen sulfide. In the light of Gaffron's findings, van Niel (230) emphasizes the importance of Winogradsky's old experiments with sulfur bacteria, where in the dark hydrogen sulfide is oxidized to sulfur (chemosynthesis). van Niel considers both oxidation of hydrogen sulfide and reduction of carbon dioxide as typical dark reactions also in the case of the purple bacteria and assumes that both in the purple bacteria and in the green plant the oxidation of water represents the process which requires light energy. As a research hypothesis van Niel draws the following picture of the two sorts of photosynthesis:

Both in the plant and in bacterial photosynthesis water is dehydrogenated, giving rise to a reduction of carbon dioxide and a formation of a kind of oxide or oxidation product, perhaps a peroxide. In the plant photosynthesis, the oxidation product formed is spontaneously split into a stable oxide and free oxygen; in the bacterial photosynthesis, the oxide formed is stable and has to be reduced by hydrogen donors like hydrogen sulfide or fatty acids, and consequently no oxygen is formed in this case.

Both the spontaneous liberation of oxygen and the reduction by hydrogen donors restore the group as acceptor of a new molecule of water. van Niel emphasizes that, although for the time being the chances in obtaining the photochemical reaction in cell-free extracts are very small, the chances for an observation of some of the dark reactions in cell-free extracts are much better.

B. Chemosynthesis

Chemosynthesis includes a number of reactions where carbon dioxide is reduced by inorganic or organic compounds; these reactions are independent of light.

The interesting reactions were discovered as long ago as 1890 in the brilliant microbiological investigations by Winogradsky (303, 304). He succeeded in isolating some soil bacteria capable of oxidizing ammonia to nitrite (*Nitrosomonas*) and nitrite to nitrate (nitrobacteria (19)). Winogradsky observed that, in proportion to the oxidation of ammonia, carbon dioxide was reduced to organic compounds according to the following relation: 35 moles of ammonia are oxidized to nitrous acid for 1 mole of carbon dioxide reduced. Meyerhof in 1916 (195) confirmed Winogradsky's equation and found that 105 moles of nitrous acid were oxidized to nitric acid for 1 mole of carbon dioxide reduced. Thus three times as many moles of CO_2 are oxidized in the one-step oxidation of nitrous acid to nitric acid as in the three-step oxidation of ammonia to nitrous acid.

Winogradsky also demonstrated the oxidation of hydrogen sulfide to sulfur in the so-called sulfur bacteria. Carbon dioxide also functions as the hydrogen acceptor in this oxidation.

Woods (309) recently discovered the reversible reduction of carbon dioxide to formic acid by hydrogen gas, and Barker (10) observed the oxidation of ethyl alcohol to acetic acid with the proportional reduction of carbon dioxide to methane. That the methane formed is derived from carbon dioxide has recently (12) been proved directly by means of radioactive carbon dioxide (C^xO_2), which yields radioactive methane (C^xH_4).

Reactions like the oxidation of ammonia or of hydrogen sulfide by carbon dioxide may look very reasonable from a purely chemical point of view but not from a thermodynamic point of view. The normal potentials of redox systems like ammonia–hydroxylamine or hydrogen sulfide–sulfur are considerably higher than that of the redox system carbon dioxide–formic acid. A very clear illustration of this thermodynamic paradox is the oxidation of alcohol to acetic acid with a stoichiometrical reduction of carbon dioxide to methane. Barker (10) observed the following relationship:

$$2CH_3CH_2OH + CO_2 = 2CH_3COOH + CH_4 + 2H_2O$$

However, E_0' (pH 7) of the hydrogen-donor system

$$\text{alcohol} \rightleftharpoons \text{acetaldehyde} + 2H^+ + 2\epsilon$$

is -160 millivolts. E_0' (pH 7) of the hydrogen-acceptor system

$$CO_2 + 2H^+ + 2\epsilon \rightleftharpoons \text{formic acid}$$

is -430 millivolts. How can the negative system serve as hydrogen acceptor for a much more positive system? The explanation must be that the first reduction step of carbon dioxide is carried out by small amounts of strong reducing agents in the cells (for instance, carbonyl groups). The later steps of carbon dioxide reduction,—for instance, of the the reduction of formaldehyde to methyl alcohol or of this substance to methane,—can very well be carried out by alcohol. As soon as acetaldehyde is formed, a reduction of carbon dioxide to formic acid by this substance takes place. The later reductions can be performed directly by alcohol. The interpretation of oxidoreductions between ammonia or hydrogen sulfide and carbon dioxide may be explained in the same manner; the final reduction product of carbon dioxide in these redox processes is, however, not known.

Other kinds of chemosynthesis,—for instance, the reversible reduction of carbon dioxide with hydrogen gas, yielding formic acid, or the uptake of carbonates in the propionic acid fermentation, yielding succinic acid,—have been described in previous sections in this review.

C. The formation of sugar from lactic acid and related compounds

The resynthesis of sugar from lactic acid formed by working muscles (*d*-lactic acid) was first demonstrated by Embden and coworkers (66) in perfusion experiments with mammalian livers. Not only monohexoses but also polyhexoses (glycogen) were formed from lactic acid.

Meyerhof (197) demonstrated a formation of glycogen from lactic acid in muscle tissue, but the liver seems to be the most important organ in carrying out the synthesis of sugar from lactic acid (66, 189). The synthesis of sugar from lactic acid always requires oxygen consumption.

Two main hypotheses have been advanced in order to explain the pathway of sugar formation from lactic acid. Meyerhof supposed that one part (about 20 to 25 per cent) of the lactic acid was completely oxidized by a respiration process which furnishes energy to the resynthesis of sugar from the other part.

Kluyver in 1931 (135) advanced a different hypothesis. According to him, all the lactic acid formed is oxidized in two steps: the first step yields pyruvic acid, which is decarboxylated to carbon dioxide and acetaldehyde; the second step consists in the oxidation of acetaldehyde to glycolaldehyde.

The latter oxidation has been demonstrated in model experiments by Conant and Tongberg (42), who used ceric solution as an oxidizing agent; the glycolaldehyde formed was assumed to be polymerized to sugar. Alkaline solutions of glycolaldehyde actually show slow polymerization of glycolaldehyde into sugar. At the time when Kluyver first discussed this problem, nothing was known about energetic couplings between oxidation–reduction and phosphorylation. Kluyver's theory actually gives a simple and rational explanation of a resynthesis: one part of the molecule is sacrificed as carbon dioxide and the other part is oxidized and polymerized. This coupling is very closely related to the formation of fatty acid from sugar, a process which Kluyver was one of the first to recognize as a mixed dissimilation and assimilation.

Although Kluyver's hypothesis of sugar formation seemed to be the more attractive of the two hypotheses, it has never been supported by enzymatic experiments. Furthermore, the decarboxylation of pyruvic acid into acetaldehyde and carbon dioxide does not occur either in animal tissue or in a number of microörganisms. If carboxylase occurred in animal tissue, ethyl alcohol and not lactic acid would be formed as a result of anaerobic sugar oxidation.

Recent experiments of Hastings, Kistiakowsky, *et al.* (111) with lactic acid containing radioactive carbon in the carboxyl group show that the glycogen formed from such labelled lactic acid contains radioactive carbon only to a very small extent. A much greater amount of the radioactive carbon was found in the carbon dioxide. These recent findings may be a support of the Conant–Kluyver theory. Nevertheless, the lack of carboxylase in animal tissue remains a strong objection against the theory. The rather high radioactivity in the carbon dioxide might also be explained by the new type of decarboxylation–carboxylation recently discovered by Carson and Ruben (34) (see section III, propionic acid fermentation).

Modern enzyme chemistry has rendered much support to Meyerhof's theory. In 1937 Meyerhof and collaborators (207) and at the same time Green, Needham, and Dewan (99) in Cambridge succeeded in the demonstration of what might be called a reverse fermentation. Lactic acid and phosphoglyceric acid in the presence of the pyridine nucleoprotein but without oxygen gave pyruvic acid and phosphotriose; the last was trapped with cyanide or semicarbazide. Meyerhof and coworkers were able to demonstrate this reverse fermentation without trapping the triose, provided they added large amounts of adenylpyrophosphate, and they made the interesting discovery that the added adenylpyrophosphate was dephosphorylated stoichiometrically, so that for every mole of phosphoglyceric acid reduced to phosphotriose 1 mole of phosphate was liberated.

Working with reduced "pyridine," Meyerhof succeeded in the demonstration of a dephosphorylation of adenylpyrophosphate coupled with the reaction

"hydropyridine" + phosphoglyceric acid $\rightleftharpoons$ "pyridine" + phosphotriose

The reverse reaction:

phosphotriose + "pyridine" $\rightleftharpoons$ phosphoglyceric acid + "hydropyridine"

is coupled with a phosphorylation of adenylic acid to adenylpyrophosphate (206). Meyerhof explained these two facts in the following way: the oxidoreduction of glycolysis furnishes energy for the phosphorylation of adenylic acid, whereas the dephosphorylation of adenylpyrophosphate furnishes energy to the reverse oxidoreduction, i.e., for the sugar formation from lactic acid. Under natural conditions adenylpyrophosphate occurs only in small amounts and therefore has to be rephosphorylated by energy-furnishing redox processes; this rephosphorylation of adenylic acid is one of the important tasks of the respiration. Although the clear demonstration of these step reactions, proceeding in the reverse direction of those in the glycolysis, represented the first step towards a chemical understanding of sugar synthesis from lactic acid, two problems remained still unanswered. The first was the formation of phosphoglyceric acid from pyruvic acid; the second the nature of the coupling between dephosphorylation and oxidoreduction. The first problem is partly solved, the other completely.

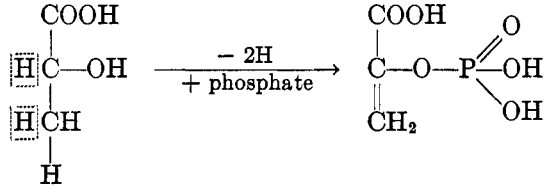
Formation of sugar from lactic acid is a reverse glycolysis, which means that the hydroxyl group of lactic acid is the hydrogen donor and the carboxyl group of phosphoglyceric acid the hydrogen acceptor; the latter substance is regenerated steadily from the precursor, phosphopyruvic acid, by the addition of water. The missing link in the scheme is the phosphorylation of pyruvic acid to phosphopyruvic acid; this process has never been demonstrated experimentally. Furthermore Meyerhof and collaborators, (204) on the basis of some experiments with radioactive phosphate, assume that the well-known reaction

phosphopyruvic acid + adenosine diphosphate $\rightarrow$ pyruvic acid

+ adenosine triphosphate

goes in one direction only. Of importance for this problem, however, is the formation of phosphopyruvic acid from malic or fumaric acid added to kidney extracts (Kalckar (123)). Fumaric acid or malic acid was oxidized in the presence of fluoride and gave rise to an accumulation of phosphopyruvic acid which could only be formed by an oxidation of malic acid, since fluoride prevents a formation of this ester from sugar. Ferd-

man (79) has recently been able to demonstrate a corresponding formation of phosphopyruvic acid from lactic acid in muscle tissue. Presumably phosphopyruvic acid is formed by an oxidation of lactic acid by the following reaction:



Lactic acid Phospho(enolic)pyruvic acid

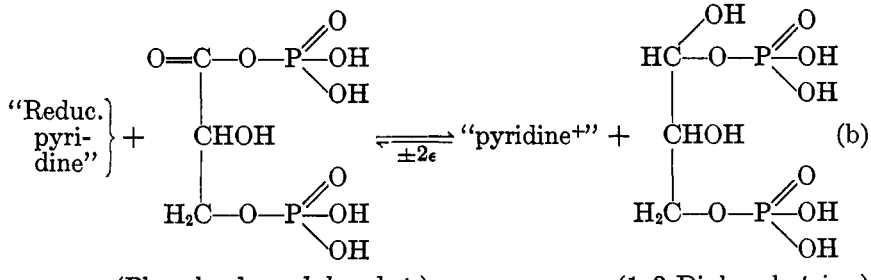
Whether the phosphate enters the lactic acid or, simultaneously with the oxidation, enters the oxidation product, phospho(enolic)pyruvic acid, is unknown. A corresponding reaction with a preliminary formation of phosphoöxaloacetic acid is probably the mechanism of the phosphopyruvic acid formation from malic and fumaric acids. Addition of phosphate instead of water to fumaric acid would yield phosphomalic acid (*cf.* Lipmann (175)).

The nature of the coupling between the reduction of phosphoglyceric acid and adenylpyrophosphate dephosphorylation was not understood until Warburg and his pupils (299, 220) separated the different enzymes and obtained the new labile 1,3-diphosphoglyceric acid (*cf.* section V). The diphosphoglyceric acid reacts with the adenine nucleotide in the reversible reaction

*di*phosphoglyceric acid + adenosine *di*phosphate ⇌ *mono*phosphoglycerate + adenosine *tri*phosphate

The *di*phosphoglyceric acid accepts the electrons from the reduced "pyridine." The diphosphotriose formed splits to monophosphotriose and phosphate. The reverse glycolysis proceeds therefore as follows:

lactate⁻ + "pyridine"⁺ $\rightleftharpoons[\pm 2\epsilon]$ pyruvate⁻ + "reduc. pyridine" (a)



(Phospho*glycerylphosphate*) (1,3-Diphospho*triose*)

The phosphorylation of the carboxyl group by adenylpyrophosphate makes a reduction of this group thermodynamically much easier, since the potential of the aldehyde–carboxyl system after a phosphorylation is raised approximately 200 millivolts; the reasons for such a shift in the potential have been given in section V. Thus a phosphorylation of the carboxyl group creates a relatively good hydrogen acceptor. The carboxyl phosphate is reduced to aldehyde-phosphate, which immediately liberates the phosphate. The result of these reactions is that adenylpyrophosphate is dephosphorylated and monophosphoglyceric acid is reduced to monophosphotriose; this over-all reaction is identical with Meyerhof's equation.

The reduction of carboxyl groups to carbonyl groups is thermodynamically difficult to carry out in biological systems, because in such systems the carbonyl–carboxyl redox system is the strongest reducing system of all. According to Wieland's theories (302), aldehydes before dehydrogenation take up water to form aldehyde hydrates, which become the hydrogen donor proper; a reverse reaction, i.e., a reduction of carboxyl to aldehyde hydrate, is thermodynamically very unlikely (E_0' approximately -450 millivolts). According to Warburg (299), the aldehyde group of triose (and according to Lipmann (175) the carbonyl group in pyruvic acid) takes up phosphoric acid (carbonyl phosphate) before biological dehydrogenation; the carbonyl ester thus formed represents the hydrogen donor proper. A reverse reaction, i.e., reduction of the carboxyl phosphate of diphosphoglyceric acid to carbonyl phosphate by the reduced pyridine nucleotide, is thermodynamically a very likely reaction.

Thus replacement of water by phosphate converts processes which would have been highly irreversible into more reversible[30] processes and thereby prevents scattering of energy as heat. We meet the same feature again in the cleavage of polysaccharides which can be split by water (diastatic hydrolysis, irreversible) or by phosphate (Cori's phosphorolysis, reversible); these last reactions will be described in the next section.

D. The synthesis of polyhexoses from monohexoses

The liver and the muscles are able to build up polyhexoses, mainly glycogen, from the monohexoses, glucose and fructose, provided oxygen is present. Until very recently the formation of glycogen has been demonstrated only in an intact liver perfused with oxygenated blood or in liver slides shaken in oxygenated saline solution. It was a general belief among physiologists that, although reactions like phosphate esterifications can be demonstrated in cell-free extracts (or even with crystalline proteins), the formation of glycogen is a biological manifestation which is so closely

[30] Reversible in a thermodynamic sense.

connected with the living intact cell structure that one would be very unlikely to find glycogen formation in extracts, not to speak of purified enzyme systems. Owing to the brilliant work of Cori and Cori and collaborators, the mechanism of the breakdown and the formation of glycogen has now been recognized.

In order to understand the mechanism of glycogen formation, one has to be acquainted with the investigations on the breakdown of glycogen. For a long time it has been the general belief that the only biological way of breaking down polyhexoses (starch, glycogen, dextrins) was an ordinary hydrolysis catalyzed by certain enzymes, called amylases or diastases. These enzymes which occur in the digestive tract were also considered responsible for the liberation of glucose (blood sugar) from the liver; the liver, however, does not contain diastase in large amounts, and probably all activity is due to diastase in the blood plasma. Since diastase, even in the presence of very high amounts of glucose, cannot catalyze glycoside formation, the formation of glycogen from glucose cannot be due to a reverse diastase action.

In 1935 Cori and Cori (36) found that glycogen in intact muscles takes up inorganic phosphate, and the same year Parnas and Ostern (238) found the same phenomenon in aged and dialyzed muscle extract; the product formed was found to be a 6-monophosphate. Since the oxidative enzymes are inactivated or removed, the uptake of phosphate is not coupled with an oxidation. The product formed by the phosphorylation is the well-known mixture containing 70 per cent of glucose-6-monophosphate and 30 per cent of fructose-6-monophosphate (Embden ester). In 1936 Cori and Cori (47) made the important discovery that the primary product of the glycogen phosphorylation in muscle extract is glucose-1-phosphate, i.e., a hexose phosphate phosphorylated on the aldehyde group.

The glucose phosphate (Cori ester) exhibits the following properties: no reduction in a non-enzymatic system, since the aldehyde group is phosphorylated; strong dextrorotation, $[\alpha]_D^{20} = +120°$; and high lability to acid hydrolysis (5 min. boiling in 1 *N* hydrochloric acid liberates all the phosphate). Cori, Colowick, and Cori (46) furthermore succeeded in a chemical synthesis of glucose-1-phosphate. Glucose was acetylated and brominated; the bromoacetylglucose was heated with silver orthophosphate. The triacetylglucose monophosphate thus formed was subjected to a mild acid hydrolysis, yielding 1 mole of glucose-1-phosphate per mole of triacetylglucose monophosphate. Both the 1-ester isolated from muscles and the chemically prepared 1-ester are rapidly converted to glucose-6-phosphate by a specific enzyme occurring in all tissue (Cori (44)). The enzyme requires small amounts of magnesium ions. The conversion

of the 1-ester to the 6-ester is irreversible, i.e., the addition of the 6-ester to this enzyme does not yield the 1-ester.[31]

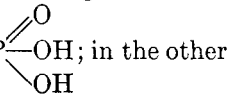
The discovery of glucose-1-phosphate represents the introduction to a new chapter in the study of carbohydrate metabolism.

Cori, Cori, and Schmidt (48, 52) and Kiessling (130) in 1939 succeeded in the separation of the enzyme which converts glycogen into the 1-ester from the enzyme which converts the 1-ester into the 6-ester. The purification of this enzyme bore great fruit, because it enabled both Kiessling and Cori and collaborators to demonstrate a new and very interesting enzymatic equilibrium reaction:

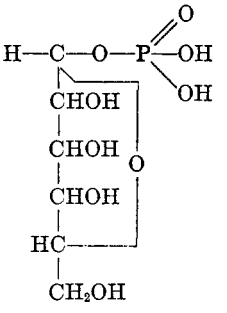
$$\text{glycogen} + n(H_3PO_4) \rightleftharpoons n(\text{glucose-1-phosphate})$$

where the equilibrium is very much to the side of glycogen; about 80 per cent of the 1-ester is converted into glycogen. The chemically prepared 1-ester is just as active as the enzymatically prepared 1-ester. Adenylic acid acts as coenzyme (*cf.* section IV). The equilibrium constant is dependent on the concentrations of inorganic phosphate and 1-ester but is independent of the glycogen concentration, presumably because glycogen is a substance of high molecular weight or is in colloidal dispersion acting essentially as a solid saturating body.

Cori (45), as well as Parnas, point out that the rôle of phosphate in the cleavage of glycogen to glucose-1-phosphate corresponds to that of water in the hydrolysis of glycogen into glucose. In the first case the glycoside linkages are split by HO—P(=O)—OH; in the other by HO—H. The cleavage



[31]



Glucose-1-phosphate (Cori ester)

It is worth while to notice that the phosphate in the glucose-1-phosphate is linked to a carbon atom in the "Haworth ring." The hydroxyl group of the 1-carbon atom is an acid group which forms salts, for instance, calcium glucosate.

of glycogen by phosphate is therefore called phosphorolysis in analogy to hydrolysis, the splitting by water. The replacement of water by phosphate makes the reaction reversible (*cf.* the oxidation of glyceraldehyde).

Experiments made by Cori and Cori (50) with purified enzyme preparations from brain and muscle tissues showed two interesting new phenomena: (*1*) The enzyme purified from muscle tissue gives rise to the formation of a polyhexose, which, like starch, gives a blue color reaction with iodine and shows the same Debye diagram as potato starch. (*2*) In the polymerization of 1-phosphoglucose to glycogen, the addition of glycogen is necessary to start the reaction.

Cori and Cori (51) recently analyzed this glycogen activation in detail; they found 1.4 mg. of glycogen per 100 cc. of reaction mixture sufficient to give a prompt abolishment of the lag period. Muscle phosphorylase activated by 10 mg. per cent glycogen exhibits a marked decrease in activity after 20 min., in spite of the fact that a large amount of newly formed starch (140 mg. per cent) is present. The newly formed starch therefore has much less "autocatalytic" effect than the glycogen added. This phenomenon, together with the fact that the starch formed by muscle phosphorylase is firmly bound to the proteins, indicates that the starch blocks the surface of the enzyme and limits the amount of polysaccharide which can be synthesized per enzyme molecule. The polysaccharide formed by liver and brain is closely related to ordinary glycogen, i.e., gives a brown color with iodine, is not firmly bound to the proteins, and has an autocatalytic effect on polysaccharide formation. The rate of polysaccharide formation is raised considerably by increasing the amounts of glycogen added. Cori and Cori find that the effect of added glycogen corresponds to that of a coenzyme. In an experiment which contained 15 mg. of protein per 100 cc., the concentration of glycogen which gives one half of maximal velocity was 27.6 mg. per 100 cc. The authors point out that one molecule of glycogen (assuming for it a molecular weight $>250{,}000$) could hardly be bound to one enzyme molecule. Probably many enzyme molecules form one unit of glycogen or starch from units of twelve to eighteen glucose molecules.

The thermodynamics of the conversion of 1-phosphoglucose to 6-phosphoglucose is of considerable interest, since this reaction seems to be practically irreversible. This means that a phosphorylation of the aldehyde group requires at least a couple of thousand calories more than a phosphorylation of the 6-hydroxyl group. A direct phosphorylation of the aldehyde group with inorganic phosphate, which apparently takes place in the oxidation of glyceraldehyde by pyridine enzymes, seems hardly possible in glucose, presumably owing to the "Haworth ring."[32]

[32] *Cf.* also the missing reaction between hexoses and sulfite.

Perhaps the pyrosphosphate energy is of importance also for the formation of 1-phosphoglucose.

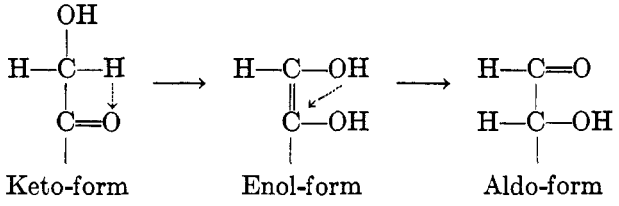
Thus the reaction which requires most energy is the phosphorylation of glucose to 1-phosphoglucose; the 1-ester thus formed is polymerized without cost of energy. It is important to bear this fact in mind, because the formation of polymerized products in living systems until very recently has been considered as synthesis "par excellence."

The Cori–Kiessling equilibrium is, as pointed out in the introduction, a clear illustration of a thermodynamically spontaneous polymerization. The "expensive" part is the formation of the precursor substance, in this case 1-phosphoglucose, which then polymerizes without further cost or even with a small liberation of energy.

A phosphorylation of glucose to glucose-1-phosphate has so far only been demonstrated indirectly in liver slices. Ostern and coworkers (236) have found in recent experiments with liver slices that calcium is necessary for the synthesis of glycogen from glucose. These investigators are inclined to think that the importance of calcium for glycogen formation is due to a depression of the phosphate concentration.

E. The conversion of fructose to glucose

The conversion of fructose to glucose takes place with great rapidity in the liver. The mechanism of this reaction is not entirely known. In alkaline solution a slight and very slow conversion from fructose to glucose takes place. It is generally assumed that the conversion of the ketohexose to the aldohexose has to pass an enolic step:



Keto-form Enol-form Aldo-form

The conversion of fructose to glucose in the liver is a rather complicated process which requires tissue respiration. If, however, the fructose is phosphorylated in the 6-position, the enzymatic conversion into glucose-6-phosphate is a simple reversible process which is catalyzed by a special enzyme (Lohmann (180)).

The equilibrium of the reaction:

$$\text{fructose-6-phosphate} \rightleftharpoons \text{glucose-6-phosphate}$$

is approximately two-thirds glucose ester and one-third fructose ester. Liver tissue dephosphorylates the glucose phosphate much faster than the

fructose phosphate (53, 92). The phosphorylation of fructose is the only step which requires respiration.

F. The action of dinitrophenol

Recent work in microbiology has brought to light many cases in which a metabolite added to bacteria has been synthesized to cell constituents simultaneously with an oxidation of another part of the added metabolite.

If, for instance, a certain amount of glucose is added to aerobic microorganisms, about one-third to one-half is converted to a cell constituent (either sugar, protein, or fatty acid). Clifton and Logan (38) made the interesting observation that the addition of dinitrophenol, even in such low concentrations as $m/16{,}000$, converts the metabolism of the bacteria to a pure combustion metabolism, i.e., the oxygen consumption corresponds to the complete oxidation of all the glucose added. Recently Douderoff (61), in van Niel's laboratory, made the important observation that pyruvic acid is accumulated in the presence of dinitrophenol; pyruvate was isolated as the dinitrophenylhydrazine compound. The accumulation of pyruvic acid in bacteria poisoned with dinitrophenol is surprising. The phenomenon indicates that phosphoglyceric acid formed in the normal metabolism is reduced to triose phosphate by pyruvic acid. An inhibition of this reduction would give a temporary accumulation of the equilibrium ester: phosphoglyceric acid $\rightleftharpoons$ phosphopyruvic acid.

IX. THE SIGNIFICANCE OF PHOSPHORYLATION IN LIVING CELLS

A. The occurrence of phosphorylation in living cells

Most studies on phosphorylations have been carried out in cell juices or extracts, where the lack of enzymes or the inhibition of enzymes leads to the accumulation of phosphoric esters in such amounts that they can be isolated. In tissue slices or intact organs the accumulation of phosphoric esters usually is smaller. In intact muscles very large amounts of creatine phosphate and adenylpyrophosphate occur, and in the introduction it has been described how this storage of phosphoric esters decreases rapidly during contractions. When working muscles are poisoned with iodoacetic acid ($m/10{,}000$) a marked increase in the amount of hexose phosphates takes place (Lundsgaard (185)). Cori and Cori (49) found that epinephrine, even in extremely small amounts, when added to muscles gives a marked increase in the amount of hexose monophosphate.

Distinct accumulations of hexose phosphates have also been demonstrated in the living yeast cell during the fermentation of sugar (McFarlane (193)), in microörganisms (306), and in the perfused intestine during the absorption of glucose or fructose (Lundsgaard (188)).

Even in cases where a formation of phosphoric esters cannot be observed, such a formation might very well take place. It must be borne in mind

that phosphoric esters are intermediate products in metabolism, just like ketone bodies or pyruvic acid, and can therefore not be expected to accumulate under normal conditions. This is also illustrated by the experiments with kidney extract with and without fluoride: in the presence of fluoride a large accumulation of hexose diphosphate occurs; in the absence of fluoride the accumulation is very small.

One of the objections against the assumption that hexose phosphates are oxidized also in intact cells is based on the observation that tissue slices (for instance, brain tissue) utilize glucose but neither hexose monophosphate nor hexose diphosphate. This phenomenon is, however, explained simply by the fact that only free sugars are able to pass the cell membrane. Thus the free sugar is the transport form,[33] and the phosphorylated sugar is the form in which it is oxidized. That sugar also can be oxidized in the unphosphorylated form appears from the experiments of Müller and of Franke and Deffner (87) with the glucose dehydrogenase from molds and those of Harrison with the glucose dehydrogenase from liver.

A number of investigators who held the view that phosphate does not play any rôle in the metabolism of intact cells referred to some important observations made by Nilsson and Alm in 1936.

Nilsson and Alm (231) describe the preparation and properties of a new sort of dried yeast which, since it is dried much faster (less autolysis) than the ordinary dried yeast (Lebedew yeast), contains more intact enzyme systems than the Lebedew yeast and therefore shows a metabolism much more like living yeast cells, although the membranes of this dried yeast are more or less digested. If glucose is added to ordinary slowly dried yeast (Lebedew yeast), the fermentation does not start immediately but only after a so-called induction period, presumably because of the slow formation of hexose diphosphate before any energy-spending oxidoreduction has started. Addition of minute amounts of hexose diphosphate and acetaldehyde starts the fermentation immediately (*cf.* Meyerhof (199)). Furthermore, Lebedew yeast accumulates hexose diphosphate according to Harden and Young's equation and requires the extra addition of orthophosphate as soon as all the inorganic phosphate has been esterified. Nilsson yeast, on the other hand, has only a very brief induction period, ferments glucose more rapidly, and exhibits no accumulation of phosphoric ester, thus being independent of the extra addition of inorganic phosphate, just like living intact yeast cells. Nilsson and Alm found, in addition, that inorganic phosphate, added to a suspension of quickly dried yeast (Nilsson yeast), gives no stimulation of the fermentation but even an inhibition which, however, does not appear before half of the glucose has been fermented. Furthermore, the addition of phosphate to Nilsson

[33] Peters *et al.* have shown that vitamin B_1 penetrates the cell membrane much more quickly than does vitamin B_1 pyrophosphate.

yeast gives an accumulation of hexose diphosphate in the first half of the fermentation period. When half of the glucose has been fermented, the accumulation of hexose diphosphate culminates. The rather complicated observations are illustrated in curves taken from one of Nilsson's publications (figure 1, from reference 231, page 259). The factor which is present in Nilsson yeast and which is necessary for the occurrence of an alcoholic fermentation of the same type as that of living yeast (i.e., according to the Gay–Lussac equation: glucose = 2 ethyl alcohol + 2 CO_2) is very thermolabile and is inhibited by inorganic phosphate.

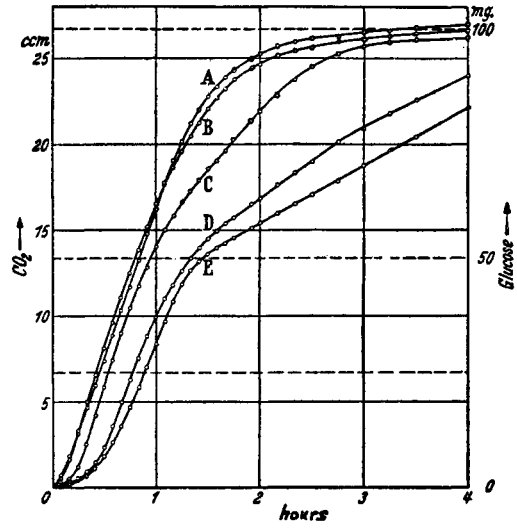


FIG. 1. Fermentation of glucose by Nilsson yeast. Total volume = 2 cc.; 200 mg. of dry yeast; 100 mg. of glucose. 0.67 molar phosphate (pH 6.4) added. 0.84 cc. of phosphate solution equivalent to 100 mg. of glucose. Temperature, 30°C. Curve A, without extra phosphate added; curve B, 0.25 cc. of 0.67 molar phosphate solution added; curve C, 0.50 cc. of 0.67 molar phosphate solution added; curve D, 0.75 cc. of 0.67 molar phosphate solution added; curve E, 1.00 cc. of 0.67 molar phosphate solution added.

Recently Lipmann observed a very similar phenomenon in maceration juice from bakers yeast; here the fermentation proceeds according to the Harden–Young equation until approximately 80 per cent of the phosphate is esterified; the fermentation then proceeds according to the Gay–Lussac equation without further decrease of the inorganic phosphate, until all the sugar is fermented.

B. The apparent absence of phosphorylation in living yeast

The phosphate effect discovered by Nilsson and Alm has, as mentioned

before, led a number of biologists to believe that phosphorylations do not occur in living cells. Nilsson himself did not interpret his observations in this direction, however. He assumed that sugar was monophosphorylated and then split into 1 mole of triose and 1 mole of triose phosphate; the unphosphorylated triose was assumed to be the substance which is fermented. Nilsson and Alm's observations can, however, also be interpreted in a quite different way, which has the great advantage of making it possible to correlate their findings with the modern concepts of sugar oxidation; in particular, with Negelein and Brömel's discovery of the diphosphoglyceric acid described in section V. Nilsson and Alm point out that the difference between the fermentations in quickly and in slowly dried yeast must be attributed to the presence of a phosphatase in the first kind of yeast and the absence of this phosphatase in the latter kind. The extraordinary thermolability of this phosphatase, in connection with its sensitivity to high phosphate concentrations, indicates the identity of the phosphatase with the so-called adenylpyrophosphatase, i.e., with the specific enzyme which catalyzes the liberation of orthophosphate from the organic phosphate ester. The enzyme was first observed in extracts of liver tissue (Jacobsen (117)), but occurs also in kidney and muscle tissue. This enzyme is not only inhibited by inorganic phosphate (13) but shows also an extraordinary sensitivity to even very moderate rises in the temperature (69). The assumption that adenylpyrophosphatase occurs in Nilsson yeast but is absent from Lebedew yeast would therefore be able to account for most of the facts.

Actually, Lebedew yeast and Lebedew juice do not contain adenylpyrophosphatase; it would be of considerable interest to investigate whether rapidly dried yeast contains adenylpyrophosphatase and if addition of this enzyme will convert the "juice fermentation" (characterized by the conversion of 50 per cent of the sugar to carbon dioxide and alcohol and the other 50 per cent to hexose diphosphate) into the fermentation type of the living yeast which converts 100 per cent of the sugar into carbon dioxide and alcohol.[34]

In any case, however, it must be obvious that the large accumulation of phosphoric esters observed in different extract systems is an artifact due to inhibition or lack of some enzyme systems.

C. The phosphate cycle

The classical cycles of Parnas and Meyerhof account for the phenomena observed in yeast juice of muscle extracts poisoned with fluoride. For every mole of triose phosphate converted into alcohol or lactic acid, 2

[34] In order to carry out such an experiment, the adenylpyrophosphatase preparations which are very impure have to be subjected to fractionation.

moles of phosphate are used to phosphorylate sugar, which means that 2 moles of triose phosphate arise per mole of triose phosphate oxidized. This gives an accumulation of 1 mole of triose phosphate per mole of lactic acid formed or 1 mole of hexose diphosphate per mole of glucose converted into alcohol and carbon dioxide, which actually is the Harden–Young equation.

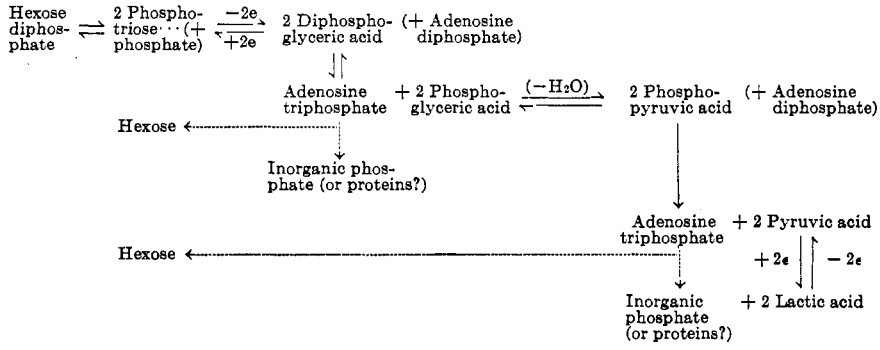
The lack of adenylpyrophosphatase in yeast juice means that adenylpyrophosphate can be dephosphorylated only by a phosphate acceptor, such as glucose.

The necessity of phosphate acceptors for the alcoholic fermentation and for the glycolysis in cell-free systems has been very clearly demonstrated in the brilliant experiments of Meyerhof.[35] These experiments are significant for the interpretation of a number of observations where cell-free systems ferment unphosphorylated sugars better than phosphorylated sugars. Meyerhof (199) showed that the reason why hexose diphosphate is not fermented by Warburg and Christian's purified fermentation system is that no phosphate acceptor is present. Geiger (90) found that brain extracts ferment glucose and fructose more than ten times faster than the corresponding mono- or di-phosphates which, however, are accumulated during the glycolysis of glucose and fructose. Recent experiments by Ochoa show, however, that brain extracts form large amounts of lactic acid, both from hexosemonophosphate and from hexosediphosphate. Warburg and Christian (296) observed an interesting effect of glucose and fructose on oxidation; the oxidation of phosphohexonic acid proceeds to completion only in the presence of fructose or glucose. The nature of the action of fructose is unknown.

It is very likely that in the living cell the same reactions occur as in extracts, with the difference that in living cells only 1 mole of phosphate is used for esterification of sugar per mole of triose phosphate oxidized; the other mole of phosphate is liberated as inorganic phosphate, presumably by the activity of adenylpyrophosphatase. This seems also to be the case in extracts of brain (Ochoa: J. Biol. Chem., in press). (See diagram at top of page 161.)

If all the phosphate goes back to hexose, 2 moles of hexose diphosphate are formed for every mole of hexose diphosphate fermented; this will give an accumulation of 1 mole of hexose diphosphate per mole of hexose diphosphate fermented, i.e., the type of fermentation taking place in Lebedew juice and expressed in Harden and Young's equation.

[35] Belitzer (17) has recently demonstrated a marked stimulation of the respiration of muscle pulp by creatine, during which creatine is phosphorylated to creatine phosphate. This is the first demonstration of what might indicate a compulsory coupling between respiration and phosphorylation in animal tissue.



D. The "break" in the phosphate cycle

Needham and Phillai (218) and Meyerhof and coworkers (205) have shown that half of the phosphate taken up in the oxidation can be transferred to creatine. The phosphate in the intact working muscle, however, is not only transferred to sugar and creatine but also liberated as inorganic phosphate, since muscular contraction leads to a great increase in the inorganic phosphate and decrease in phosphocreatine.

As pointed out by D. M. Needham (217) (*cf.* also Lundsgaard (187)), this break in the phosphate cycle very likely represents the transmission of the phosphorylation energy to the contractile system. As mentioned before, the dephosphorylation of adenylpyrophosphate represents a liberation of free energy which is larger than most of the biological step reactions (ΔH = 11,000 calories per phosphorus atom). It is most unlikely that such a large amount of energy should merely be scattered as heat, as would be the case if a plain liberation of inorganic orthophosphate took place.[35a] The only way in which pyrophosphate energy can be used is by a primary reaction of the adenylpyrophosphate with the contractile protein, myosin:[36] Since exhaustion of the adenylpyrophosphate storage in the iodoacetate-poisoned muscle is accompanied by rigor (Lundsgaard (185)), adenylpyrophosphate dephosphorylation may be coupled to the relaxation (recharging) of the myosin system. Thus, there is reason to believe that in the living cell adenylpyrophosphate is not dephosphorylated directly but through cellular structures, acting as phosphate-transfer systems.

[35a] *Cf.* the discussion of the sugar phosphorylation by pyrophosphate (section V, D).

[36] Of interest in this connection is the reaction between metaphosphate and albumin (Perlmann and Herrmann (243)). Albumin and metaphosphate form, at the acid side of the isoelectric point of the protein, a crystalline compound. The crystalline metaphosphate-protein is soluble in dilute salt solutions. The metaphosphate was found linked to the basic groups of the protein.

If we suppose that myosin in the contracted state acts as a phosphate acceptor and during the relaxation process as a phosphate donor, then we would get an illustration of how changes in cellular structures are able to "regulate" metabolic processes.

The coupled reactions might be illustrated as follows:

$$\text{triose phosphate} + \text{phosphate}^{--} \underset{\Delta F \text{ small}}{\overset{-2\epsilon}{\rightleftharpoons}} \overset{+2\epsilon}{} \text{phosphoglycerylphosphate}^{--} \quad (1)$$

$$\text{phosphoglycerylphosphate}^{--} + \text{adenosine diphosphate}^{---} \overset{\Delta F \text{ small}}{\rightleftharpoons} \text{phosphoglycerate}^{-} + \text{adenosine triphosphate}^{----} \quad (2)$$

$$\text{adenosine triphosphate}^{----} + \text{contracted myosin} \xrightarrow[\text{cal" energy}]{\Delta F \text{ small, but conversion of "chemical" to "mechani-}} \text{adenosine diphophate}^{---} + \text{phosphate}^{--} + \text{relaxed myosin} \quad (3)$$

$$\text{relaxed myosin} \xrightarrow{-\Delta F \text{ large}} \text{contracted myosin} \quad (4)$$

The more contracted the myosin, the greater the "consumption" of adenosine polyphosphates and the oxidation[37] of triose phosphate. Thus, according to these considerations, a contraction of myosin starts the oxidation of phosphotriose to pyruvate and of the latter to the acetate level.

The sudden increase in respiration (or, in the absence of oxygen, in lactic acid formation) succeeding a muscle contraction is an old observation. Recent studies of Millikan (216), who measured the rate of reduction of myoglobin in rest and during contraction, show that the increase in oxygen consumption appears less than $\frac{1}{5}$ second after the contraction starts.

The dephosphorylation of creatine phosphate, the other reaction which rephosphorylates adenylic acid, appears much later than the oxygen consumption. It must, however, be borne in mind that a sensitive method for measuring creatine phosphate hydrolysis, corresponding to Millikan's method of estimation of the rate of the oxygen consumption, does not exist.

The modern concept of biological oxidations and phosphorylations will probably be able to give a real chemical explanation of the fundamental biological phenomenon that changes in cellular structures as, for instance, the contraction and relaxation of myosin, are able to regulate oxidations.

If carbonyl groups were oxidized according to the old Wieland scheme

[37] *Cf.* the experiments of Belitzer (17), showing that the respiration of muscle pulp is stimulated considerably by addition of the phosphorus acceptor, creatine.

(i.e., by uptake of water, forming carbonyl hydrates which then were oxidized to the stable carboxylate structure), the free energy would be liberated during the oxidation (*cf.* table 1) and therefore scattered as heat. Such a reaction has a very high degree of irreversibility (167) and the rate of such a kind of oxidation must therefore remain unaffected by changes

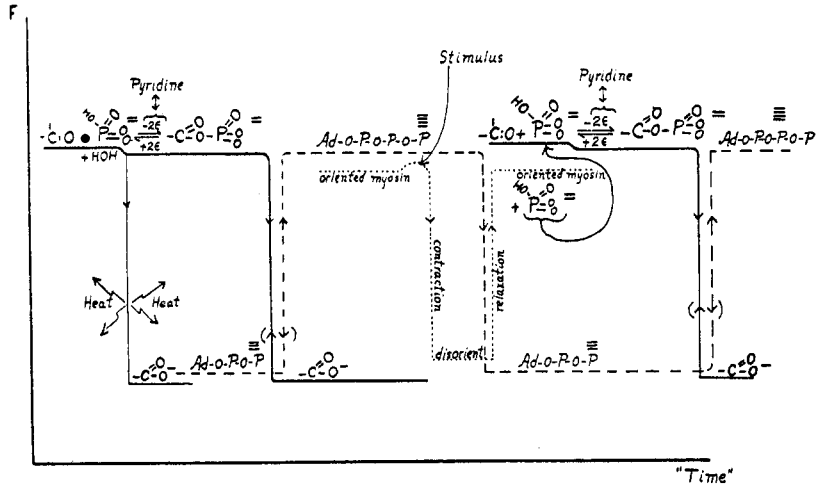


FIG. 2. This figure shows how metabolism and changes in myosin might be coupled. The balance shows only that when myosin in the muscle contracts and relaxes, carbonyl groups are oxidized. The single steps in this cellular coupling can be detected only by purification and separation of enzymes. $\diagdown C{=}O$, the carbonyl group of sugars or pyruvic acid; $-C(O^-)=O$, the carboxylate ion of sugar acids, or lower fatty acids; $-C(=O)-O-P(=O)(O^{--})-OH$, the carboxyl phosphate of glyceryl phosphate or acetyl phosphate; Ad—O—P—O—P^{---} represents adenosine diphosphate and Ad—O—P—O—P—O—P^{----} represents adenosine triphosphate (the number of negative charges is in accordance with Lohmann (182)); *F*, free energy (the relative changes in free energy are arbitrary). —, energy of metabolite; - - -, energy of adenine polyphosphate. The electrons ($\pm 2\epsilon$) are accepted by the pyridine nucleotide or furnished by the reduced pyridine nucleotide.

in cellular structure. One of the important trends at present in enzyme chemistry is toward the study of the complex dephosphorylations which occur during and after muscular contraction.

It is hardly necessary to emphasize how difficult and complicated an experimental demonstration of the restitution of myosin by adenylpyrophosphate (reaction 3) will be. Even if a phosphorylation of myosin *in*

vitro should be successfully demonstrated, we are far from an understanding of the relaxation process. This process, which is supposed to be accompanied by a liberation of phosphate and which represents the conversion of chemical into mechanical energy, must be connected with a secondary structure which stabilizes the "recharged" myosin.[37a] The stimulus is supposed to proceed in this secondary structure and to abolish the stabilizing effect, thus causing an immediate "discharge" in the myosin.

E. The mechanism of sugar absorption

It has been postulated that absorption of sugars in intestine and in kidney cortex requires a transitory phosphorylation of sugar (186, 282). A biological transformation of sugar into a sugar phosphoric acid ester would keep the sugar concentration in the cell at an extremely low level and would therefore permit a steady diffusion of sugar from the intestinal tube or kidney tubules into the cell. The sugar phosphoric acid ester was supposed to be rapidly dephosphorylated at the other end of the cell, making a rapid diffusion of the liberated glucose and phosphate into the blood stream possible. This scheme actually illustrates how sugar can be transferred from the intestine or kidney tubules to the blood, even in cases where the sugar concentration in the intestine or kidney tubules is lower than in the blood (120). It has for a long time been known that membranes which are able to transfer salt or organic compounds against a gradient of concentration require oxygen, apparently because the absorption is coupled with energy derived from cell respiration.

Is there any experimental support for the theory that the driving force in the absorption of sugars is connected with a phosphorylation–dephosphorylation cycle? The enzymes required for such reactions as phosphorylation–dephosphorylation actually occur in large amounts in intestinal mucosa and kidney cortex. In the animal organism three tissues contain large amounts of phosphatases,—the ossification centers in cartilage, the intestinal mucosa, and the kidney cortex. Robison (249) discovered the occurrence of large amounts of phosphatases in cartilage in the phase when the calcification appears. He very clearly demonstrated that the function of the phosphatases in this tissue is a precipitation of calcium phosphate from the soluble calcium hexosemonophosphate. In his monograph (249) Robison discusses the significance of the phosphatases in kidney and intestinal mucosa: . . . "the kidney phosphatase may be concerned in the normal secretion of phosphates in vivo . . .".

On the basis of two well-known facts, Lundsgaard (186) in 1933 advanced

[37a] The so-called "relaxed" or "recharged" myosin corresponds in many respects to stretched rubber; the heat capacity is decreased as a consequence of the increased orientation (*cf.* H. Mark and K. H. Meyer).

his hypothesis that phosphorylation was involved in the mechanism of sugar absorption. He started with the question whether there is any connection between (*1*) the occurrence of high phosphatase activity in both kidney cortex and intestinal mucosa and (*2*) the fact that these same two tissues absorb glucose.

A glycoside, phlorhizin, inhibits the absorption of glucose both in kidney and in intestinal mucosa and the same glycoside strongly inhibits the phosphorylation of polyhexoses in muscle extracts (186). Later the rapid phosphorylation of glucose and fructose in kidney cortex was discovered (119) and the action of phlorhizin on this system was demonstrated. The phosphorylation system in kidney cortex is actually very sensitive to phlorhizin ($M/500$ gives 80 to 90 per cent inhibition (120)). Recently Lundsgaard (188) in perfusion experiments demonstrated a marked accumulation of phosphoric esters in the intestinal mucosa when glucose and fructose are absorbed. The accumulation of phosphoric esters was greatest when fructose was absorbed. The classical work of Cori (43) showed that fructose is absorbed more slowly from the intestinal tract than glucose and galactose. Since fructose is rapidly phosphorylated, the slow absorption of fructose may be due to a delayed dephosphorylation (121). This assumption would at least explain the great accumulation of hexose diphosphate during an absorption of fructose. It is known that both fructose monophosphate and fructose diphosphate are dephosphorylated much more slowly than glucose monophosphate. Lundsgaard emphasizes, however, that so far we are unable to decide whether the accumulation of phosphate esters during sugar absorption is due to the absorption or merely represents an increased metabolism.

Since galactose is so rapidly absorbed from the intestine (43), it would be of great importance to demonstrate that a rapid enzymatic phosphorylation of galactose takes place in intestinal mucosa.

Lundsgaard's hypothesis is supported by microchemical studies of the kidney cortex, which show that glucose absorption (288), phlorhizin accumulation (63), and phosphatase activity (93) are confined to the same structure in the kidney cortex, the proximal tubules. Kidneys without a filtration system, i.e., aglomerular kidneys (for instance, from the toad fish), contain much less phosphatase than glomerular kidneys from closely related species (Kalckar, 1940; unpublished work).

X. COÖRDINATION BETWEEN OBSERVATIONS MADE *in vitro* AND *in vivo*

Is it possible to reconcile the results obtained from enzyme studies with those obtained from experiments on living cells?

The application of results obtained from *in vitro* experiments to the explanation of *in vivo* phenomena requires very careful consideration.

We have, however, numerous illustrations of how successful such attempts can be. As pointed out by Green (97), Keilin's studies of the cytochromes offer a classical example of a perfect reconciliation of *in vitro* and *in vivo* studies. The first studies of cytochrome by MacMunn and by Keilin were *in vivo* experiments, in which the absorption lines were observed in the wings of insects and in yeast cells. Later studies by Keilin, Theorell, and Ogston and Green showed that the properties of the isolated cytochrome *c* are identical with those of the cytochrome in the tissue. The demonstrations of flavin and nicotinic acid as the essential components of oxidation enzymes (Warburg and collaborators) added greatly to the significance of the identification of these two compounds as essential growth factors (vitamins).

The enzymatic experiments of D. M. Needham, Meyerhof, and others make it possible to account for almost every chemical process occurring in contracting muscles. The results of Lundsgaard's investigations of normal and iodoacetate-poisoned muscles are very well interpreted by the recent enzymatic work of Needham and Meyerhof.

The use of isotopes in *in vivo* experiments, introduced in 1924 by Hevesy, is of great value for a coördination of "vivo" and "vitro" results. This method is able to illuminate reactions which cannot be detected *in vivo* in any other way.

It has been stated earlier in this review that the use of radioactive carbon in enzymatic experiments may reveal the pathway of some important biological syntheses (photosynthesis; carbohydrate synthesis from lactic acid). The use of radioactive phosphorus may also be of value in harmonizing the results gathered from studies of muscle enzymes with those derived from investigations of intact muscle.

Hevesy and Rebbe (114) showed that radioactive inorganic phosphate injected into frogs has entered the creatine phosphate fraction after 3 hr. at 2°C. to an extent of 49 per cent and at 21°C. to an extent of 78 per cent.

Korzybski and Parnas (145) injected radioactive phosphate into mammals and found that an equal distribution of radioactive phosphate between inorganic phosphate, creatine phosphate, and adenylpyrophosphate in the muscle had been reached 60 min. after the injection. Hevesy and collaborators (113) find that the plasma phosphorus enters the muscles very slowly. After 3 hr. the plasma still contains twelve times more radioactivity per milligram of inorganic phosphorus than the inorganic phosphorus fraction in the muscles. The radioactive phosphorus which has entered the muscle appears very soon in the phosphorus of the esters. This may explain the result of some recent experiments by Sacks (253), which disagree with the investigations of Hevesy and Rebbe and those of Korzybski and Parnas. Sacks found that 2 hr. after the injection of

radioactive phosphorus the creatine phosphate and adenylpyrophosphate fractions contain only 10 to 15 per cent of radioactive phosphate (10 to 15 per cent saturation).

Sacks analyzes the muscles 2 hr. after the injection of radioactive phosphorus, using very strong preparations. At that time the difference in radioactivity per mg. of phosphorus between plasma and muscle must be very great (considerably greater than 12:1) and a small amount of blood in the muscle analyzed would increase the radioactivity in the so-called inorganic phosphorus fraction in muscle very much.[38] This might be the whole explanation of the disagreement between the results of Hevesy and of Korzybski and Parnas on the one side and those of Sacks on the other. It is at least obvious that the possibility of "plasma contamination" must be mentioned and accounted for.

If the strong radioactivity of the inorganic phosphorus is not due to "blood contamination," the question arises whether the radioactive phosphate is localized in the connective tissue or inside the muscle cell. It is at least obvious that Sacks is not justified in rejecting the Embden–Meyerhof scheme on the basis of his experiments.

XI. CONNECTIONS BETWEEN BIOLOGY AND PHYSICS

The aim of this review has been not only to collect and coördinate knowledge from very different fields, like animal physiology, microbiology, enzyme chemistry, organic and physical chemistry, but also to interpret all the fundamental biological phenomena from a dynamic point of view.

The recent revolutionary progress in our understanding of the coupling between oxidations and phosphorylations has led to new problems, particularly in the field of physical chemistry. It has been pointed out previously in this review that there exists a great lack of physical data for the different states of inorganic phosphate (ortho-, meta-, pyro-, and tri-phosphate). It would be of great importance for biologists to have exact physical data for pyrophosphate linkages, especially in the light of the recent discoveries of the formation of the latter from carboxyl phosphates. Furthermore, certain physical methods (for instance, Raman spectra) might be used to detect and estimate the very labile carbonyl phosphates which are supposed to be the reactive form in which carbonyl groups are oxidized in biological systems. The branch of physical chemistry which deals with the structure of chemical compounds can undoubtedly be of the greatest value for biological chemistry. The concept of resonance makes it possible to account qualitatively for a number of fundamental biochemical reactions. Quantitative data for the thermodynamic stability of different

[38] A muscle frozen during or just after a contraction contains, of course, much more blood than a resting muscle.

types of ester linkages could probably be obtained from experimental work in the field of structural chemistry.

In any case it would be a very great advantage for biological sciences if physicists and physical chemists would pay more attention to the fundamental well-defined chemical reactions which are the driving forces behind the various manifestations of life.

XII. SUMMARY

The fundamental reactions, oxidation and reduction, are defined as the removal of electrons (ϵ) and uptake of electrons, respectively. In organic systems oxidations are usually accompanied by proton (H^+) liberation, and reduction by proton uptake. Oxidations are endergonic ($+\Delta F$) and reductions are exergonic ($-\Delta F$) in redox systems which have positive normal potentials. In redox systems with a negative normal potential, oxidations are exergonic and reductions endergonic. Among the metabolites, fatty acids (paraffins, olefins) represent rather positive systems [E_0' (pH 7) slightly positive], while sugars and sugar acids (carbonyl (H_2O), carboxyl) represent strongly negative (reducing) systems.

Biological systems transfer electrons from negative redox systems (electron donors) to positive systems (electron acceptors); this electron transfer takes place in cellular respiration or fermentation.

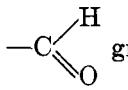
In respiration the electron acceptor is obtained from the environment in the form of oxygen; in fermentations the electron acceptor has to be formed from the electron (hydrogen) donor.

The transfer of electrons from donor to acceptor never takes place directly but always stepwise through one or several transfer systems. The larger the difference between the normal redox potentials (E_0') of electron donor and electron acceptor, the more electron-transfer systems are interposed.

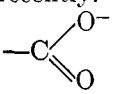
The nature of the electron-transfer systems is known; most essential in the electron-transfer system of fermentations is a pyridine compound in combination with a specific protein catalyst. α-Keto acids do not react with pyridine compounds but with thiazole compounds. In respiration, alloxazine and iron porphyrin compounds are interposed in addition to pyridine or thiazole compounds.

Electron acceptors (double bonds) represent reactive structures, exhibiting paramagnetic properties. Electron donors, i.e., ordinary metabolites, show no paramagnetic properties; these compounds have to be activated by some specific protein catalyst. The nature of the action of specific, catalytically active proteins is not known, but there is some evidence that they decrease the potential barrier between the valence-saturated compound and the reactive free radical (semiquinone).

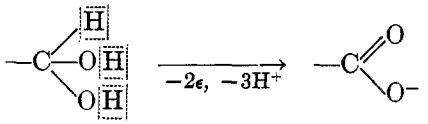
A number of electron donors (metabolites) cannot be oxidized unless orthophosphate is present; this is the case with triose phosphate and pyruvic acid. These oxidations were said to be "coupled" with a phosphate uptake, since the phosphate was found in organic compounds after the oxidation. The rôle of phosphate in the oxidation of carbonyl groups has been revealed recently. It was generally believed that oxidation of



groups to

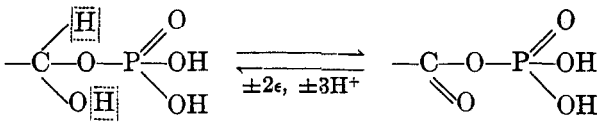


requires the uptake of water:



Carbonyl hydrate Carboxylate ion

Modern enzyme studies have shown that not water but phosphate is taken up in the biological carbonyl oxidations:

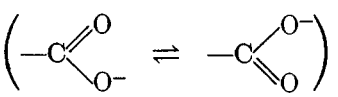


Carbonyl phosphate Carboxyl phosphate

Arsenate can replace phosphate. Two types of carboxyl phosphate have been discovered: glyceryl phosphate (isolated as 1,3-diphosphoglyceric acid) and acetyl phosphate. Both show the same absorption line (mμ 217) as acetic acid anhydride (acetyl acetate). The thermodynamic consequences of the new reactions are far reaching. Thermal data show that the reaction

$$\text{carbonyl} + H_2O \xrightarrow[-3H^+]{-2\epsilon} \text{carboxylate}^-$$

involves a large decrease in free energy, i.e., a large increase in stability, due to the formation of the resonating carboxylate structure



The ΔF of the reaction

$$\text{carbonyl phosphate}^{--} \xrightleftharpoons{\pm 2\epsilon,\ \pm 2H^+} \text{carboxyl phosphate}^{--}$$

is small, since this reaction has been shown to be easily reversed. Thus the replacement of water by phosphate in the carbonyl oxidation means

that the main part of the free energy of the carbonyl group is not released but is kept in certain acid anhydrides and transferred to structures where it has a chance to be transformed into mechanical work. It has been shown that the phosphate of carboxyl phosphates can be directly transferred by a specific enzyme to form pyrophosphate compounds (adenosine polyphosphates); this reaction is easily reversed, i.e., ΔF is small. The dephosphorylation of the pyrophosphate linkages is known to be one of the most strongly exergonic biochemical reactions. There is increasing evidence that the dephosphorylation of the pyrophosphate compound is linked with the relaxation of contracted myosin, i.e., the recharging of the discharged contractile system. The discharge may "regulate" the metabolism of sugar (carbonyl) in the following manner:

$$\text{carbonyl} + \text{phosphate}^{--} \rightleftharpoons \text{carboxyl phosphate}^{--} + 2\epsilon + 2H^{+} \quad (1)$$

$$\text{carboxyl phosphate}^{--} + \text{adenosine } \textit{di}\text{phosphate}^{---} \rightleftharpoons \text{carboxylate}^{-} + \text{adenosine } \textit{tri}\text{phosphate}^{----} \quad (2)$$

$$\text{adenosine } \textit{tri}\text{phosphate}^{----} + \text{"contracted" myosin} \rightarrow \text{adenosine } \textit{di}\text{phosphate}^{---} + \text{phosphate}^{--} + \text{"relaxed" myosin} + H^{+} \quad (3)$$

Since reactions 1 and 2 are reversible reactions, the enzymatic oxidation of carbonyl to carboxylate cannot proceed unless the irreversible reaction 3 takes place. Thus, the amount of "contracted" myosin regulates the oxidation of carbonyl compounds.

The replacement of water by phosphate has also been shown in another type of reaction: namely, the breakdown of polyhexoses (starch and glycogen). Certain enzymes in the digestive tract catalyze the splitting of glycoside linkages by water. These enzymes (diastases, amylases) hydrolyze starch and glycogen to glucose. The reaction is practically irreversible, i.e., addition of glucose to this enzyme does not yield any detectable amount of polyhexoses.

In the tissues glycogen is broken down not by a hydrolysis but by a phosphorolysis, i.e., phosphate breaks the glycoside linkages of the polyhexose, and 1-phosphoglucose is formed as a primary product. The reaction

$$\text{polyhexose} + \text{phosphate} \rightleftharpoons \text{1-phosphoglucose}$$

is very easily reversed; addition of 1-phosphoglucose to the specific enzyme (phosphorylase) immediately yields large amounts of polyhexose.

Thus, the replacement of water by phosphate converts strongly exergonic reactions like oxidation or the splitting of glycoside linkages, to nearly energy-neutral reactions.

We are now able to perceive clearly that the "expensive" steps in the

biological reduction of acids to aldehydes or in the biological polymerization of monohexoses to polyhexoses are not the reduction proper nor the polymerization but the formation of energy-rich phosphorylated precursors (i.e., carboxyl phosphate and 1-phosphoglucose, respectively).

The great progress made in the last three or four years is mainly attributable to the isolation and identification of these precursors and to the separation and isolation of the specific protein catalysts.

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