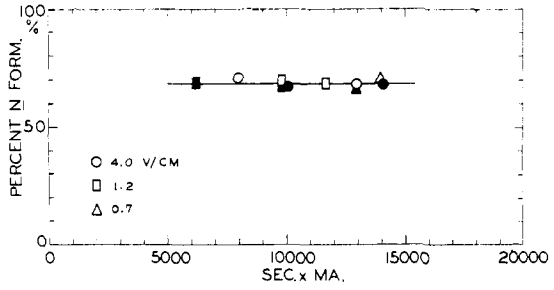

Fig. 8.—Percentage composition as a function of the product of current density and time of electrophoresis.[35] Conditions were the same as in Fig. 7. Open symbols are for ascending and filled symbols for descending boundaries.

tate the same result was obtained at 4.0 and 8.0 v./cm. Therefore it can be stated that mobilities and areas are independent of time and of current density over the range 0.7 to 8.0 v./cm.

Figure 9 gives another example which is more pertinent to the present work since it involves a study in urea. This experiment is the more striking because of the fact that N and F forms here have opposite sign of charge. Again the boundary positions are seen to be strict linear functions of the time of electrophoresis. We have now re-examined a great many of our earlier runs for linearity of boundary positions *versus* time and find linearity to hold in the vast majority of the cases. In systems containing only chloride ion near the mid-point of the *p*H transition region, *i.e.*, near *p*H 4, slight curvature was occasionally seen in the early stages of electrophoresis only. That this curvature is to be attributed to *p*H gradients seems amply substantiated by the fact that systems containing acetate, hence weakly buffered, give linear plots.

In our opinion these results amply substantiate our thesis that both boundary areas and boundary mobilities may be meaningfully interpreted in terms of the equilibrium constituents. As to why such a result should be obtained in spite of the relatively fast rate of the isomerization process, we have nothing further to add beyond the points raised previously.[4,36]

(36) This paradox has been discussed further by J. R. Cann, Abstr. 132nd Meeting American Chemical Society, September, 1957. His results indicate the half-time of equilibration to be of the order 5 hr. in the electrophoresis cell but less than 0.5 hr. otherwise, indicating some stabilizing force to arise in electrophoresis.

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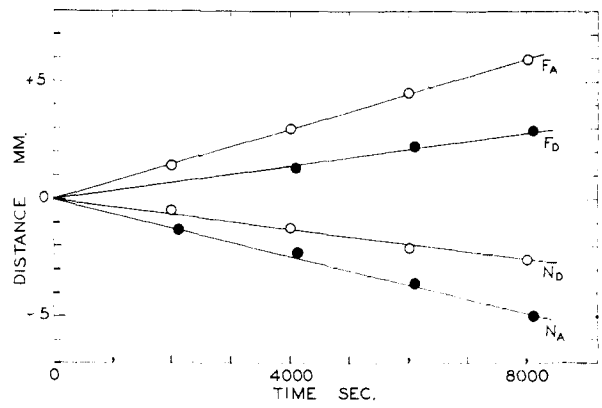

Fig. 9.—Distance migrated as a function of time, BPA at *p*H 4.92 in 0.02 ionic strength chloride plus 2 *M* urea, 0°. Under these conditions N and F forms, which are present in approximately equal proportions, move in opposite directions. Open circles refer to the rising limb of the cell (*i.e.* ascending F and descending N boundaries), filled circles to the descending limb.

By way of summary, the present results are taken as strong substantiating evidence for our general thesis, presented in previous communications, that two electrophoretically resolvable forms of BPA exist in acid solution and that the equilibrium between these forms is intimately related to the anomaly which exists in the *p*H titration curve of this protein. In presence of urea the equilibrium situation may be summarized by the equation

$$N + m\text{Urea} + n\text{H}^+ \rightleftarrows F + y\text{Cl}^-$$

Here m appears to be approximately equal to three; n is two below *p*H 5, zero from *p*H 5 to 6.7, and again approximately two above *p*H 6.7. The coefficient in chloride, y, appears to be approximately six in the region where n is zero at 0.02 ionic strength but appears to approach zero at higher ionic strength. The situation is obviously complex but its solution would appear to be essential to an ultimate understanding of the unusual properties of this protein.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF CHICAGO]

Formation of Keto-pyruvate in the Dehydrogenation Catalyzed by Yeast Lactic Oxidase[1]

BY ABRAHAM MARCUS[2] AND BIRGIT VENNESLAND

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With the help of deuterium, it is shown that when lactate is oxidized to pyruvate in the presence of yeast lactic oxidase there is no labilization of the hydrogen atoms of the methyl group.

There are two well-defined and different types of enzymatic reactions whereby lactate may be oxidized to pyruvate. One type involves oxidation by a pyridine nucleotide in the presence of lactic dehydrogenase. The other type of reaction is mediated by a flavoprotein. Previous studies of the diphosphopyridine-nucleotide (DPN)-linked reaction catalyzed by lactic dehydrogenase have shown that the enzyme acts on the keto form of py-

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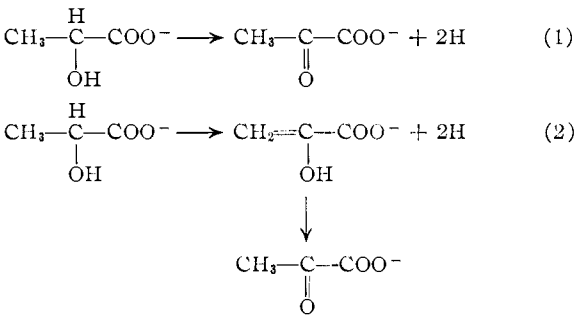
(2) Postdoctoral Fellow, United States Public Health Service (1956–1958).

ruvic acid.[3] The present experiments were designed to determine whether the oxidation of lactate to pyruvate by a flavoprotein also gives the keto form of pyruvate or whether an enol might be the first oxidation product with subsequent spontaneous ketonization. The two possible reaction sequences are



In the previous examination of the DPN-linked lactic dehydrogenase reaction, evidence for the direct reduction of ketopyruvate (equation 1, right to left) was obtained by reducing pyruvate to lactate in a medium of almost 100% D_2O.[3] The reducing agent, DPNH (*i.e.*, reduced DPN) had previously been shown to donate a hydrogen atom to the α-carbon atom of pyruvate.[4] The lactate formed in D_2O was isolated as the phenacyl derivative and analyzed for non-exchangeable D. In these experiments, all deuterium attached to oxygen is washed out during the isolation procedure. If the reduction occurred by a reversal of equation 2, the lactate must acquire a minimum of one atom of non-exchangeable D per molecule. This is because reduction of the enol involves the formation of two new C–H bonds (*i.e.*, one on the α-carbon atom and one on the β-carbon atom), and only one of these newly introduced H atoms can be donated by DPNH. The other must therefore be derived from the medium. Since the lactate contained only an insignificant amount of excess D, the reaction sequence of equation 2 was ruled out, and the conclusion was drawn that the reaction must occur by reversal of equation 1.

The flavoprotein selected for the present investigation was yeast lactic oxidase. This enzyme contains cytochrome b_2 as well as flavin and catalyzes oxidation of L-lactate by added oxidation–reduction dyes, cytochrome *c* or ferricyanide.[5–7] The reaction was carried out by oxidizing lactate in a medium of D_2O with ferricyanide as the oxidant. After complete reduction of the ferricyanide, the pyruvate which had been formed was immediately reduced by addition of DPNH and lactic dehydrogenase. The lactate formed by this second reaction was diluted with unlabelled carrier, isolated as phenacyl lactate and analyzed for D.

The results of two experiments are shown in Table I. The D content of the isolated phenacyl lactate was clearly far less than the one atom per molecule which must be obtained if the lactic oxidase reaction had converted the lactate to the enol form of pyruvate. The conclusion is that lactic oxidase, like lactic dehydrogenase, gives the keto form of pyruvate. The small amount of D incorporated into the lactate must be introduced by the non-enzymatic enolization of the pyruvate in D_2O.

TABLE I

DEUTERIUM CONTENT OF LACTATE

Expt. no.	Substrate	Dilution factor[a]	D content of lactate[b] Atom % excess	Atom D/ molecule
1	D,L-Lactate	22	0.014	0.04
2	L-Lactate	22	0.024	0.06

[a] Total lactate/lactate formed by reduction of pyruvate. [b] Analyzed as phenacyl lactate. Theory for one atom D per molecule = 8.33 atom % excess. Calculation for experiment 1: $0.014 \times 22/8.33 = 0.04$.

Frieden and Velick[8] have shown previously that ophio-L-amino acid oxidase oxidizes the amino acid L-leucine to the corresponding keto acid without the loss of hydrogen from the β-carbon atom. Thus both of the flavoprotein oxidations and all of the DPN-linked oxidations (*i.e.*, those catalyzed by alcohol dehydrogenase, lactic dehydrogenase, malic dehydrogenase and a β-OH-steroid dehydrogenase)[3,9] examined to date are similar in this respect, that there is no intermediary formation of an enol. As pointed out by Frieden and Velick,[8] this is in agreement with what has been generally assumed from chemical considerations.[10] The suggestion also has been made, however, that an enol is formed during the enzymatic oxidation of amino acids by flavoproteins.[11]

The absence of an enolic intermediate in the lactic oxidase reaction is of interest in connection with the fact that the enzyme has been shown to oxidize L-glycerate to hydroxypyruvate.[12] There is reason to predict that if this oxidation involved intermediary formation of an enol, the reaction products would be glycolaldehyde and CO_2 rather than hydroxypyruvate. This is because an enol of hydroxypyruvic acid (*i.e.*, CHOHCOHCOOH) would be expected to ketonize to hydroxymalonic semialdehyde (CHOCHOHCOOH) rather than to hydroxypyruvic acid ($CH_2OHCOCOOH$). The evidence rests on the inference that an enol of hydroxypyruvic acid is an intermediate in the spontaneous decarboxylation of α-keto-β-hydroxysuccinic acid, which decomposes spontaneously in aqueous solution to give two moles of CO_2 and one mole of glycolaldehyde per mole of the original β-keto acid.[13–15] The CO_2 has been shown to be de-

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rived from the two carboxyl groups, as expected.[15] It is reasonable to suppose that this decarboxylation occurs in two steps, in each of which a β-keto acid loses CO_2 to form an enol. The product of the first step would then be the enol of hydroxypyruvic acid. The fact that another molecule of CO_2 is lost shows that this enol does not ketonize to the relatively stable hydroxypyruvate but to hydroxymalonic semialdehyde which would be expected to undergo decarboxylation as observed.

Experimental

Material and Methods.—Potassium ferricyanide was recrystallized according to Folin.[16] DPNH was prepared as the salt of tris-(hydroxymethyl)-aminomethane, according to Loewus, *et al.*[17] A sample of the disodium salt of DPNH (Pabst) also was used. Lithium D,L-lactate was prepared by neutralizing boiling D,L-lactic acid (85%) with a saturated solution of lithium hydroxide and crystallizing from water–ethanol. The phenacyl derivative of the D,L-lactic acid was prepared according to Rather and Reid[18] and melted at 97–98°. Li-L-lactate and beef heart lactic dehydrogenase were the same as previously described.[4,19]

Lactic oxidase (cytochrome b_2) was prepared from dried baker's yeast according to the procedure of Boeri, *et al.*,[6] up through the last $(NH_4)_2SO_4$ fractionation. The preparation was dialyzed and lyophilized, with about 15% loss of activity. Protein was determined by the method of Lowry, *et al.*[20] The enzyme activity of the cruder fractions was determined by measuring the rate of methylene blue reduction.[21] The more purified fractions were assayed by measuring the rate of reduction of either ferricyanide (at 420 mμ) or of 2,6-dichlorophenol-indophenol (at 600 mμ). All measurements were made with a Beckman spectrophotometer. When measured in a cell of 1 cm. light path at 420 mμ, a solution containing 1 μmole of ferricyanide per ml. has an optical density (log I_0/I) of 1.03. The ferricyanide assay system was made up to contain 125 μmoles of phosphate buffer of *p*H 7.4, 2 μmoles of Versene (ethylenediaminetetraacetate), 400 μmoles of D,L-lactate, 2.0 μmoles of ferricyanide and water to make a total volume of 3.0 ml. The reaction was started by addition of enzyme. The reduction of ferricyanide was linear with time under these conditions. If 0.1 μmole of indophenol was substituted for the ferricyanide, the reduction was first order with respect to the indophenol. The lactic oxidase preparation employed in the experiments catalyzed the reduction of 1.1 μmoles of ferricyanide per minute per mg. protein at 20°. The half time for the reduction of 2,6-dichlorophenol–indophenol was 10 seconds per mg. protein (the first-order rate constant $K = 4.3$ min.$^{-1}$ mg.$^{-1}$).

Experiment 1.—The reaction mixture contained 120 μmoles of phosphate buffer of *p*H 7.0, 2 μmoles of Versene, 378 μmoles of lithium D,L-lactate, 77.2 μmoles of potassium ferricyanide and 9 mg. of yeast lactic oxidase in a total volume of 2.0 ml. of 99% D_2O. The *p*H was maintained at 7.0 by addition of small amounts of $NaHCO_3$ during the course of the reaction. After 32 minutes at room temperature, the ferricyanide was reduced completely. Then about 80 μmoles of DPNH was added, followed by 0.05 ml. of an $(NH_4)_2SO_4$ suspension of heart lactic dehydrogenase.[19] After 5 min. when the reduction of pyruvate was complete, the reaction mixture was heated for 1.5 minutes at 100° to inactivate the enzymes. The optical density at 340 mμ of suitable aliquots of the reaction mixture was determined before and after the lactic dehydrogenase reaction. From the change, the amount of DPNH oxidized was calculated[4,19] to be 38.7 μmoles. This is a measure of the amount of pyruvate formed in the lactic oxidase reaction, in agreement with the value of 38.6 μmoles calculated from the amount of ferricyanide reduced. It was essential that the ferricyanide be completely reduced in the first reaction, prior to addition of DPNH. If this was the case, the second reaction, *i.e.*, the reduction of pyruvate by DPNH, proceeded smoothly and could be measured accurately, as shown by the good agreement between the ferricyanide reduced initially by lactate, and the DPNH subsequently oxidized by pyruvate. If the ferricyanide was not completely reduced, it oxidized the DPNH directly both by a non-enzymatic reaction and by an enzymatic reaction catalyzed by a very active DPNH diaphorase in the lactic oxidase preparation. (As a further complication, there is a change of optical density at 340 mμ accompanying the reduction of ferricyanide to ferrocyanide.)

After the final heat inactivation of the enzymes, 475 μmoles of unlabelled lithium D,L-lactate was added, to give a total of 475 + 378 = 853 μmoles of lactate, out of which $^1/_{22}$ or 38.7 μmoles had been oxidized to pyruvate and rereduced to lactate. The extraction of the lactate with ether, conversion to the phenacyl derivative and analysis of the phenacyl lactate for D, were then carried out as previously described.[4,19] It is pertinent to note that the enzyme is specific for L-lactate but that D,L-lactate may be used as described in the experiment since the L-lactate oxidized by lactic oxidase is regenerated in the reduction catalyzed by lactic dehydrogenase, which is also specific for L-lactate. The final solution thus contains equal amounts of the D- and L-isomer, regardless of what proportion has been oxidized and rereduced in the enzymatic reactions.

Experiment 2.—The reaction mixture was similar to that of experiment 1 except that 254 μmoles of lithium L-lactate and 89.6 μmoles of ferricyanide were added. The results of the DPNH analyses showed that 45.7 μmoles of pyruvate was reduced in the lactic dehydrogenase reaction, in good agreement with the 89.6/2 = 44.8 μmoles calculated from the amount of ferricyanide reduced. The subsequent procedures were identical with those of experiment 1, except that L-lactate was used as diluent.

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[CONTRIBUTION NO. 2230 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

Interactions of Amino Acids with Deoxyribonucleic Acid (DNA)

BY CHRISTINE D. JARDETZKY[1]

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A study of the binding of various amino acids to DNA was conducted using the technique at equilibrium dialysis. Only the basic amino acids and peptides were found to interact with DNA in solutions of low ionic strength. An approximate binding constant was derived from the data after applying a correction for the Donnan effect.

The fact that nucleic acids are thought to be templates for protein synthesis stimulated the investigation of the interactions of (DNA) with various amino acids. These interactions may depend not only on the negative charge of the phos-

(1) Postdoctoral research fellow of the American Cancer Society.