CCLXXXV. DISMUTATIONS AND OXIDOREDUCTIONS

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THE Embden-Meyerhof scheme of muscle glycolysis involves three oxidoreductions: (1) the dismutation of triosephosphate, (2) the oxidation of α -glycerophosphate by pyruvate and (3) the oxidation of triosephosphate by pyruvate. Many workers have confirmed that these oxidoreductions are catalysed by extracts of skeletal muscle. But the question of the nature of the enzymes and mechanisms involved in these reactions has received only scanty consideration. Euler *et al.* [1936] and Meyerhof & Ohlmeyer [1936] independently demonstrated that the presence of coenzyme I is necessary for the oxidoreductions to take place. Euler *et al.* presented spectroscopic evidence that the coenzyme functions in these reactions by undergoing a cycle of reduction by triosephosphate or α -glycerophosphate and of oxidation by pyruvate.

Woods & Green [quoted by Green, 1936] failed to detect the presence of dehydrogenase systems in actively glycolysing muscle extracts. The tests applied for the presence of triosephosphate, lactate and α -glycerophosphate dehydrogenases were: (1) reduction of methylene blue by the various substrates, and (2) reaction with molecular oxygen in presence of carriers. These negative findings presented a paradoxical situation. On the one hand, the evidence was complete that triosephosphate and α -glycerophosphate could be dehydrogenated to phosphoglycerate and that pyruvate could be hydrogenated to lactate. On the other hand, no evidence was forthcoming that the usual type of dehydrogenase system was responsible for these oxidoreductions.

The recent brilliant analysis by Dixon & Lutwak-Mann [1937] of the enzymic dismutation of aldehydes has brought to light the fact that two distinct types of enzyme exist which are capable of catalysing the oxidation of aldehydes. The aldehyde mutase of Parnas for example catalyses the dismutation of aldehydes to the corresponding alcohols and acids. Coenzyme I is an indispensable component of the oxidation system. Now although the mutase catalyses either the reduction of aldehyde to alcohol or the oxidation of aldehyde to acid, it fails to behave as either a typical alcohol or aldehyde dehydrogenase. That is to say, the mutase catalyses the oxidation of aldehyde by another molecule of aldehyde but apparently not by methylene blue, flavin, flavoprotein etc. The same holds true for the reduction of aldehyde to alcohol (actually the test is made for the reverse process). Dixon & Lutwak-Mann picture the aldehyde mutase as an enzyme with two active centres in close juxtaposition—the liaison agent being the coenzyme which is reduced at the one centre and oxidized at the other.

It was at once clear from this study of Dixon & Lutwak-Mann that enzymes of a type similar to the aldehyde mutase of liver must be responsible for the oxidoreductions observed in muscle extracts. In this communication the properties of three distinct mutases will be described, viz. the triosephosphate,

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triose and α -glycerophosphate mutases. They are all found in extracts of the skeletal muscle of rabbit.

The triosephosphate mutase catalyses the dismutation of triosephosphate to phosphoglycerate and α -glycerophosphate. This reaction does not proceed in absence of coenzyme I. In addition to this dismutation, the mutase catalyses the oxidation of triosephosphate by a whole series of α -ketonic acids, e.g. pyruvate, oxaloacetate, α -ketobutyrate and mesoxalate. For each molecule of triosephosphate oxidized to phosphoglycerate, 1 mol of ketonic acid is reduced to the corresponding hydroxy-acid. The aldehyde mutase of liver similarly catalyses not only the dismutation of aldehyde but also the oxidation of aldehydes by α -ketonic acids.

The triose mutase differs from the triosephosphate or aldehyde mutase in that it more slowly catalyses the dismutation of triose but rapidly catalyses the oxidation of triose by α -ketonic acids. Finally, the α -glycerophosphate mutase catalyses only the oxidation of the substrate by α -ketonic acids. No dismutation is possible.

Thus all the known mutases may or may not catalyse the dismutation of their respective substrates. The main property in common is that of utilizing α -ketonic acids as the oxidizing agent for the substance. Two other important similarities of these mutases are (1) the dependence upon coenzyme I and (2) the extreme sensitivity to low concentrations of iodoacetic acid. It is interesting to note that mutases are unique among oxidizing enzymes in their sensitivity to iodoacetic acid.

The reactions which have been considered in the present communication are the following:

- (1) Triosephosphate + triosephosphate $\longrightarrow$ phosphoglycerate + α -glycerophosphate
- (2) Triosephosphate + $\begin{cases} \text{pyruvate} \\ \text{oxaloacetate} \\ \alpha\text{-ketobutyrate} \\ \text{mesoxalate} \end{cases} \longrightarrow \text{phosphoglycerate} + \begin{cases} \text{lactate} \\ \text{malate} \\ \alpha\text{-hydroxybutyrate} \\ \text{tartronate} \end{cases}$
- (3) $\begin{cases} \text{Glyceraldehyde} \\ \text{Glycollic aldehyde} \end{cases} + \begin{cases} \beta\text{-indolepyruvate} \\ \text{pyruvate} \\ \text{oxaloacetate} \\ \alpha\text{-ketoisohexanoate} \\ p\text{-hydroxyphenylpyruvate} \\ \alpha\text{-ketobutyrate} \end{cases} \longrightarrow \begin{cases} \text{glycerate} \\ \text{glycollate} \end{cases} + \begin{cases} \beta\text{-indolelactate} \\ \text{lactate} \\ \text{malate} \\ \alpha\text{-hydroxyisohexanoate} \\ p\text{-hydroxyphenyllactate} \\ \alpha\text{-hydroxybutyrate} \end{cases}$
- (4) Acetaldehyde + $\begin{cases} \text{pyruvate} \\ \text{oxaloacetate} \\ \alpha\text{-ketoglutarate} \\ \text{mesoxalate} \\ \alpha\text{-ketobutyrate} \end{cases} \longrightarrow \text{acetic acid} + \begin{cases} \text{lactate} \\ \text{malate} \\ \alpha\text{-hydroxyglutarate} \\ \text{tartronate} \\ \alpha\text{-hydroxybutyrate} \end{cases}$
- (5) α -Glycerophosphate + $\begin{cases} \text{pyruvate} \\ \text{oxaloacetate} \\ \alpha\text{-ketoisohexanoate} \\ p\text{-hydroxyphenylpyruvate} \\ \alpha\text{-ketobutyrate} \\ \beta\text{-indolepyruvate} \end{cases} \longrightarrow \text{phosphoglycerate} + \begin{cases} \text{lactate} \\ \text{malate} \\ \alpha\text{-hydroxyisohexanoate} \\ p\text{-hydroxyphenyllactate} \\ \alpha\text{-hydroxybutyrate} \\ \beta\text{-indolelactate} \end{cases}$
- (6) $\begin{cases} \text{Lactate} \\ \text{Malate} \\ \alpha\text{-Hydroxybutyrate} \end{cases} + \text{phosphoglycerate} \xrightarrow{\text{cyanide}} \begin{cases} \text{pyruvate} \\ \text{oxaloacetate} \\ \alpha\text{-ketobutyrate} \end{cases} + \text{triosephosphate}$
- (7) β -Hydroxybutyrate + $\begin{cases} \alpha\text{-ketobutyrate} \\ p\text{-hydroxyphenylpyruvate} \\ \text{mesoxalate} \end{cases} \longrightarrow \text{acetoacetate} + \begin{cases} \alpha\text{-hydroxybutyrate} \\ p\text{-hydroxyphenyllactate} \\ \text{tartronate} \end{cases}$

Reactions of groups (1)–(6) are all catalysed by mutases. The reactions of group (7) require the β -hydroxybutyric dehydrogenase in addition to the mutase.

I. Preparation of the enzymes

The triose, triosephosphate and α -glycerophosphate mutases are best prepared from the skeletal muscles of rabbit. The muscles of a freshly killed animal are cooled by packing with ice and thoroughly minced. The mince is mixed with 2 vol. of iced water and allowed to stand for *ca.* 30 min. The mixture is squeezed through muslin. 2 vol. of cold acetone are then added to the filtrate. The precipitate is filtered immediately on Büchner funnels with suction and then washed with acetone and ether. If the washings are effected before the cake of precipitate has cracked, it is possible to pulverize the precipitate in a mortar and dry it within an hour *in vacuo* over liquid paraffin. The dried acetone powder (10 g.) is rubbed up with water (120 ml.) until a homogeneous paste is formed. The mixture is then dialysed for 15 hr. at 0° in cellophane sacs. The large amount of insoluble material is centrifuged off and discarded. The clear supernatant fluid contains the active enzymes. The activity is maintained for at least 10 days if the enzyme solution is kept at 0°. The enzymes are best kept in the form of the dry powder. The same method of preparation is applicable to tissues other than skeletal muscle. The aldehyde mutase can be similarly prepared from liver.

II. Methods of estimation

Since practically all the reactions catalysed by these mutases involve either the production or disappearance of acid, the course of these reactions was followed manometrically by measuring the production or disappearance of CO₂. The enzyme solutions were mixed with bicarbonate and equilibrated with a mixture of 95% N₂ and 5% CO₂. The reactions were carried out at 38°. The retention of CO₂ by the proteins of the enzyme solution introduced a very small correction (less than 5%) and this was ignored.

To determine whether the increase in CO₂ pressure was due to acid formation or the actual production of CO₂, the following two methods were used with the Barcroft differential manometer. Both cups were filled with enzyme, coenzyme and bicarbonate solution, but the reactants were added only to one cup. The reaction was allowed to proceed to completion and the increase in CO₂ pressure determined. Strong acid was then introduced simultaneously into the two cups. If acid were produced, the manometric reading returned to the zero reading, whereas if CO₂ were produced, the manometric reading was unchanged. Another method involved opening the taps after the increase in CO₂ pressure had been recorded and allowing the manometric fluid to return to the zero reading. The taps were then closed and strong acid was introduced into both cups. If CO₂ were formed the manometric fluid remained stationary, whereas if acid were formed the manometric reading coincided with the reading at the end of the reaction (the sign of course being opposite).

Lactic acid was estimated by the method of Friedemann & Graesser [1933], and in cases in which the stereochemical form of lactic acid had to be ascertained, by the enzymic method of Dewan & Green [1937]. Malic acid was determined either polarimetrically by the uranium acetate method of Dakin [1924] or by oxidation with KMnO₄ and estimation as acetaldehyde. Rapoport's [1937] naphthoresorcinol method for estimation of glyceric acid was found to be very satisfactory. Inorganic phosphate was determined by the method of Fiske & Subbarow [1925]. α -Glycerophosphate was estimated manometrically with the use of the specific dehydrogenase from rabbit muscle [cf. Green, 1936]. The details of similar procedures are described by Dewan & Green [1937].

III. *Oxidation of triosephosphate by pyruvate*

A solution of muscle mutase in presence of hexosediphosphate and bicarbonate produces a large amount of CO_2 . This production is only slightly increased by addition of pyruvate or coenzyme I (cf. Table I). It is not hexosediphosphate

Table I. *The effect of coenzyme and pyruvate on the CO_2 production from hexosediphosphate*

The amounts of reagents were: 1 ml. enzyme, 1 ml. 0.15% coenzyme I, 0.4 ml. $M/10$ hexosediphosphate, 0.1 ml. M pyruvate and 0.4 ml. $M/2$ NaHCO_3 . The total volume was 4.0 ml. The hexosediphosphate was introduced from a Keilin cup after temperature equilibration.

	$\mu\text{l. CO}_2$ in 10 min.
Mutase + hexosediphosphate	200
Mutase + hexosediphosphate + pyruvate	264
Mutase + hexosediphosphate + pyruvate + coenzyme	330
As above without mutase	0
Mutase + coenzyme—no substrate	30

as such which is oxidized. Meyerhof & Lohmann [1934] have shown clearly that there is present in muscle extracts an enzyme, zymohexase, which catalyses the reaction

It is preferable to use hexosediphosphate as an instantaneous source of triosephosphate since the latter is extremely unstable.

In presence of $M/100$ fluoride, CO_2 production from hexosediphosphate is reduced considerably. But addition of pyruvate restores the CO_2 production practically to the level in absence of fluoride (cf. Table II). It is well known that

Table II. *The effect of pyruvate on the CO_2 production from hexosediphosphate in presence of NaF*

Details as in Table I

	$\mu\text{l. CO}_2$ in 15 min.
Mutase + $M/40$ NaF + coenzyme	20
Mutase + NaF + coenzyme + hexosediphosphate	96
Mutase + NaF + coenzyme + hexosediphosphate + pyruvate	363
Mutase + coenzyme + hexosediphosphate + pyruvate	400
Mutase + NaF + coenzyme + pyruvate	20

hexosediphosphate gives rise to pyruvic acid via phosphoglyceric acid. This reaction is inhibited by fluoride. The action of pyruvic acid as an oxidant for triosephosphate therefore can only be demonstrated when the production of pyruvic acid is inhibited by fluoride. The residual CO_2 production by hexosediphosphate in presence of fluoride is due to the dismutation of triosephosphate (cf. § V).

The participation of a coenzyme in the oxidation of triosephosphate by pyruvate can easily be demonstrated with a mutase solution which has been treated several times with charcoal. On addition of coenzyme I, a fivefold increase in CO_2 production from hexosediphosphate and pyruvate takes place:

	$\mu\text{l. CO}_2$ in 10 min.
Mutase (charcoal-treated) + hexosediphosphate + pyruvate	55
As above with 1 ml. 0.15% coenzyme I	298

Charcoal removes from the mutase solutions traces of coenzyme which defy dialysis. There is evidence that the residual coenzyme is combined with protein. Although the amount of this residual coenzyme is very small indeed, it is sufficient to allow the triosephosphate mutase to work almost at maximum velocity.

From equation (8),

we should expect that for every mol of CO_2 produced from bicarbonate, there should be 1 mol of lactate and 1 mol of phosphoglycerate. Table III is a summary

Table III. *Oxidation of triosephosphate by pyruvate*

Lactic acid was estimated as acetaldehyde by the method of Friedemann & Graesser [1933]; phosphoglyceric acid as difficultly hydrolysable phosphate.

	Observed	Theory	% theory
Manometric CO_2 ($\mu\text{l.}$)	249	264	94
Lactic acid formed (expressed as $\mu\text{l. CO}_2$)	227	264	86
Phosphoglyceric acid formed (expressed as $\mu\text{l. CO}_2$)	233	264	88

of the quantitative relations. The source of CO_2 is the oxidation of triosephosphate to the corresponding acid. The reduction of pyruvate to lactate involves no change in acidic groups. Control experiments have shown that the CO_2 evolution is due to the production of acid and not to CO_2 as such.

Fig. 1. CO_2 equivalence for oxidation of triosephosphate by pyruvate.

Each manometer contained 1 ml. enzyme, 0.5 ml. 0.15% coenzyme I, 0.1 ml. M pyruvate and 0.4 ml. $M/2$ NaHCO_3 . 1 ml. of the hexosediphosphate solution was equivalent to 528 $\mu\text{l. CO}_2$.

Fig. 1 shows the time-velocity relations for the production of CO_2 from small quantities of hexosediphosphate. The rate of reaction between hexosediphosphate (i.e. triosephosphate) and pyruvate is only slightly dependent on the concentration of hexosediphosphate (cf. Table IV). Another point of interest in connexion with the kinetics is that the reaction proceeds with the same velocity in

a mixture of 95% O₂ and 5% CO₂ as in the usual mixture of 95% N₂ and 5% CO₂.

Table IV. *Effect of the concentration of hexosediphosphate on the rate of CO₂ production*

The mutase system contained 1 ml. enzyme and 1 ml. 0.15% coenzyme. The amount of pyruvate added was 0.1 ml. of *M* solution.

	$\mu\text{l. CO}_2$ in 5 min.
Mutase system + pyruvate	6
Mutase system + 0.05 ml. <i>M</i> /10 hexosediphosphate	100
Mutase system + 0.1 ml. <i>M</i> /10 hexosediphosphate	128
Mutase system + 0.2 ml. <i>M</i> /10 hexosediphosphate	148
Mutase system + 0.3 ml. <i>M</i> /10 hexosediphosphate	172

IV. *Oxidation of triosephosphate by oxaloacetate*

A mixture of mutase, hexosediphosphate, coenzyme and fluoride hardly produces any CO₂. Addition of oxaloacetate leads to a vigorous evolution:

	$\mu\text{l. CO}_2$ in 15 min.
Mutase + NaF + coenzyme + hexosediphosphate	25
As above with oxaloacetate (5 mg.)	208
Mutase + oxaloacetate	5
Oxaloacetate with no mutase	0

Although under certain conditions oxaloacetate is known to be unstable yielding pyruvate and CO₂, control experiments with the enzyme and oxaloacetate alone failed to show any demonstrable production of CO₂. Furthermore, the CO₂ produced by the complete system was found to be due entirely to acid formation. From equation (9),

we should expect that for each mol of CO₂ liberated from bicarbonate, there should be 1 mol of malate and 1 mol of phosphoglycerate. Table V shows that the results of the various quantitative estimations agree reasonably well with theory.

Table V. *Oxidation of triosephosphate by oxaloacetate*

Malic acid was estimated as acetaldehyde by the method of Friedemann & Graesser [1933]. 1 mg. of lactic acid is equivalent to 3.26 mg. malic acid.

	Observed	Theory	% theory
Manometric CO ₂ ($\mu\text{l.}$)	1300	1320	98
CO ₂ produced from bicarbonate, i.e. due to acid formation ($\mu\text{l.}$)	1400	1320	106
Malic acid formed (expressed as $\mu\text{l. CO}_2$)	1267	1320	96
Phosphoglyceric acid formed (expressed as $\mu\text{l. CO}_2$)	1148	1320	87

Malic acid was estimated in three ways: (1) by oxidation with permanganate as for lactic acid (using another conversion factor however), (2) polarimetrically by the uranium acetate method, and (3) manometrically by the enzymic method. Methods (1) and (3) yielded results in good agreement with the manometric CO₂. The results obtained by method (2) were consistently low (*ca.* 60% of the theory). Since this method is the only one of the three which distinguishes between lactic and malic acid, it was of importance to ascertain the cause of the discrepancy. First, we found that known amounts of *l*-malic acid added to the trichloroacetic acid filtrate of the enzyme solution showed a rotation only 75% of the theory. There is apparently some substance present in the deproteinized solution which interferes with the estimation of malic acid. Secondly, we

observed that the natural phosphoglyceric acid in presence of uranium acetate showed a very high rotation opposite in sign to that of *l*-malic acid ($[\alpha]_D$ ca. 150°). Since the optically active *d*-phosphoglyceric acid was present with *l*-malic acid in equimolecular proportions after the reaction had terminated, the low polarimetric results for the malic acid estimation were considered to be due in part to this complication. By adjustment of the *pH* it was found possible to reduce this interfering action of phosphoglyceric acid. In 5% acetic acid solution, malic acid easily formed the uranium complex whereas phosphoglyceric acid did not. The optimum condition for the latter is at neutrality. But even after reducing the interfering action of phosphoglyceric acid, the observed rotations were still 25% lower than the theoretical values calculated from the manometric data.

Szent-Györgyi [1936] and his co-workers have already shown that triose-phosphate rapidly reacts with oxaloacetate in minced tissue. Recently, Parnas & Szankowski [1937] have confirmed that this reaction takes place in frog muscle brei.

V. Dismutation of triosephosphate

By suppressing the production of pyruvic acid from phosphoglyceric acid by means of NaF, it is possible to study the dismutation of triosephosphate to *d*-glycerophosphate and phosphoglycerate. This dismutation is almost completely inhibited by the presence of any of the α -ketonic acids which can act as hydrogen acceptors. In other words, triosephosphate as oxidant cannot compete with oxidants such as pyruvate, oxaloacetate etc. The rate of the dismutation is only about a fifth of the rate when pyruvate acts as the oxidant.

From equation (10), there should be formed,

1 mol of α -glycerophosphate and phosphoglycerate for each mol of CO_2 liberated from bicarbonate. Table VI is a summary of the quantitative relations. Both

Table VI. *The dismutation of triosephosphate*

The estimation of α -glycerophosphate was carried out with the use of the specific enzyme which oxidizes only the (–) form [cf. Green, 1936]. The deproteinized extract to be estimated was treated in succession with strong alkali and acid to destroy the coenzyme, neutralized and concentrated to a small bulk. The oxygen uptake in presence of the enzyme was then determined. Each mol of α -glycerophosphate is equivalent to 1 atom of oxygen. The observed uptake is corrected for the small enzyme blank.

	Observed	Theory	% theory
Manometric CO_2 ($\mu\text{l.}$)	248	264	94
Total difficultly hydrolysable phosphate (expressed as $\mu\text{l. CO}_2$)	559	528	106
α -Glycerophosphate (expressed as $\mu\text{l. CO}_2$)	268	264	102

α -glycerophosphate and phosphoglycerate have difficultly hydrolysable phosphate groups. The difference between the total difficultly hydrolysable phosphate and the phosphate equivalent to α -glycerophosphate which can be determined independently represents the phosphate equivalent to phosphoglycerate. Fig. 2 shows the time-velocity curve for the production of CO_2 during the dismutation of triosephosphate.

The enzymic method which was used to estimate α -glycerophosphate is applicable only to the laevorotatory form. Since the theoretical amount of α -glycerophosphate was found by this method, it is clear that only the (–) form is produced in the dismutation. Recently, Fischer & Baer [1937] have pointed

out that (–)- α -glycerophosphate gives rise on oxidation to the non-natural phosphoglycerate. Thus we are left with the dilemma that α -glycerophosphate on oxidation to phosphoglycerate changes from the *l*- to the *d*-stereochemical series.

Fig. 2. CO_2 equivalence for the dismutation of triosephosphate.

Each manometer contained 1 ml. enzyme, 0.5 ml. 0.15% coenzyme I, 0.4 ml. $M/2$ NaHCO_3 and 0.1 ml. M NaF . 1 ml. of the hexosediphosphate solution was equivalent to 1320 $\mu\text{l. CO}_2$.

VI. Oxidation of α -glycerophosphate by pyruvate

The triosephosphate mutase catalyses both the dismutation of triosephosphate and its oxidation by pyruvate and oxaloacetate. This mutase is distinct from the mutase which activates α -glycerophosphate. Euler *et al.* [1937] have already shown that the α -glycerophosphate mutase is fairly widely distributed in animal tissues and that it depends upon the collaboration of coenzyme I. They considered the enzyme from a different point of view, particularly with respect to the equilibrium between the substrate and coenzyme. The α -glycerophosphate dehydrogenase of the cytochrome class [cf. Green, 1936] is not identical with the mutase which attacks the same substrate.

The muscle mutase solution in presence of α -glycerophosphate and coenzyme I does not produce CO_2 from bicarbonate. On addition of pyruvate, evolution of CO_2 takes place (cf. Table VII). This reaction is very much slower than the corresponding one between triosephosphate and pyruvate. The α -glycerophosphate mutase is rather fragile and many of our muscle preparations failed to

Table VII. Oxidation of α -glycerophosphate by pyruvate

The quantities used were 1 ml. enzyme, 1 ml. 0.15% coenzyme, 0.1 ml. M pyruvate and 0.1 ml. M α -glycerophosphate.

	$\mu\text{l. CO}_2$ in 15 min.
Mutase + α -glycerophosphate + coenzyme	15
Mutase + α -glycerophosphate + pyruvate	35
Mutase + α -glycerophosphate + coenzyme + pyruvate	208
Mutase + coenzyme + pyruvate	15

show any activity. The rate of oxidation of α -glycerophosphate by pyruvate depends upon the concentration of coenzyme I.

If the reaction proceeds as in equations (11) and (12),

we should expect 2 mols of lactate and 1 mol of phosphoglycerate for each mol of CO_2 produced from bicarbonate. The α -glycerophosphate mutase presumably catalyses only reaction (9) whereas (10) is catalysed by the triosephosphate mutase. Since no way was known to us of resolving the two mutases, it was necessary to study the two consecutive reactions. The source of CO_2 is the oxidation of triosephosphate to phosphoglycerate. Table VIII is a summary of

Table VIII. *Oxidation of α -glycerophosphate by pyruvate*

The *dl*-form of α -glycerophosphate was used. Hence half the total difficultly hydrolysable phosphate represents the phosphate of phosphoglycerate. This assumption is valid since the whole of the (–) α -glycerophosphate was oxidized in this experiment as shown by the theoretical CO_2 production.

	Observed	Theory	% theory
Manometric CO_2 ($\mu\text{l.}$)	217	224	97
Difficultly hydrolysable phosphate formed (expressed as $\mu\text{l. CO}_2$)	244	224	109
Lactic acid formed (expressed as $\mu\text{l. CO}_2$)	475	448	106

Fig. 3. CO_2 equivalence for the oxidation of α -glycerophosphate by pyruvate.

Each manometer contained 2 ml. enzyme, 0.5 ml. coenzyme, 0.4 ml. $M/2 \text{ NaHCO}_3$ and 0.1 ml. $M/2 \text{ NaHCO}_3$. The total volume was 4 ml. The theoretical CO_2 production is calculated on the basis that only one isomeride is attacked.

the quantitative estimations. The α -glycerophosphate used was the racemic form. As shown in the curves of Fig. 3, only one isomeride is attacked, viz. the (–) enantiomorph.

Oxaloacetate reacts with α -glycerophosphate as rapidly as pyruvate:

	$\mu\text{l. CO}_2$ in 30 min.
Mutase + coenzyme + α -glycerophosphate	30
Mutase + pyruvate	228
Mutase + oxaloacetate	252

VII. Triose mutase

The muscle mutase solution in presence of *dl*-glyceraldehyde (Kahlbaum) produces no CO_2 from bicarbonate. On addition of coenzyme I a slow but steady evolution of CO_2 ensues. On further addition of pyruvate, the evolution becomes extremely rapid—in fact as fast as for the reaction between triose-phosphate and pyruvate (cf. Table IX). The slow reaction in absence of pyruvate

Table IX. *Oxidation of glyceraldehyde by pyruvate*

The quantities used per manometer were 2 ml. mutase, 1 ml. 0.15% coenzyme I, 0.1 ml. *M d*-glyceraldehyde and 0.1 ml. *M* pyruvate.

	$\mu\text{l. CO}_2$ in 5 min.
Mutase + coenzyme	18
Mutase + coenzyme + glyceraldehyde	60
Mutase + coenzyme + glyceraldehyde + pyruvate	360
Mutase + glyceraldehyde + pyruvate	10
Glyceraldehyde + pyruvate	0

is probably due to the dismutation of glyceraldehyde to the corresponding alcohol and acid.

Assuming that glyceraldehyde reacts with pyruvate according to equation (13),

we should expect 1 mol of glycerate and 1 mol of lactate to be produced for each mol of CO_2 . Table X shows that the quantitative estimations are in complete

Table X. *Oxidation of glyceraldehyde by pyruvate*

The glyceraldehyde tested was the *dl*-form. The theoretical CO_2 production is calculated on the assumption that only one isomeride is attacked. Glyceric acid was estimated by the method of Rapoport [1937].

	Observed	Theory	% theory
Manometric CO_2 ($\mu\text{l.}$)	250	248	101
Lactic acid (expressed as $\mu\text{l. CO}_2$)	211	248	85
Glyceric acid (expressed as $\mu\text{l. CO}_2$)	240	248	97

agreement with the predictions of equation (13). Glyceric acid was precipitated from the trichloroacetic acid filtrate of the reaction mixture by addition of basic lead acetate and two volumes of alcohol. After several hours at 0° , the precipitate was centrifuged, washed with 60% alcohol and decomposed with H_2S . Estimation of glyceric acid was then carried out by the method of Rapoport [1937]. When the enzymic reaction was inhibited with iodoacetic acid, the test for glyceric acid was negative. This and similar controls ruled out any possibility that the original reactants were being estimated as glyceric acid. Fig. 4 shows that only half the *dl*-glyceraldehyde is oxidized. This must mean that one isomeride is unattacked. Through the kindness of Prof. Fischer we were able to test a sample containing 80% *d*-glyceraldehyde and 20% *l*-glyceraldehyde. The results indicated that the *l*-component was the one which was not attacked.

The kinetics of the triose mutase are summarized in Figs. 5-8. The rate of oxidation of triose by pyruvate is proportional to the concentration of coenzyme I within the limit of the saturating concentration. It is interesting that the

Fig. 4. CO₂ equivalence for the oxidation of glyceraldehyde by pyruvate.

Each manometer contained 1 ml. enzyme, 1 ml. coenzyme, 0.1 ml. *M* pyruvate and 0.4 ml. *M*/2 NaHCO₃. Total volume was 4 ml.

endogenous coenzyme in the mutase solution, although sufficient to allow the triosephosphate-pyruvate reaction to take place at practically maximum velocity, is totally inadequate for the triose-pyruvate or the α -glycerophosphate-pyruvate reactions. It is possible, however, that the coenzyme is not present in an available form for these two mutases. The K_m of the mutase for the reducing substrate is *ca.* *M*/400 for the *dl*, i.e. *M*/800 for the *d*-form, and about the same value for the oxidizing substrate. The range of *pH* which can be covered with the use of bicarbonate buffer is limited from *pH* 6.6 to 8.1. The reaction is most rapid in the alkaline range and is relatively slow on the acid side of neutrality.

Glyceraldehyde can be oxidized by oxaloacetate as readily as by pyruvate (*cf.* Table XI). Manometric analysis showed that all the CO₂ produced arose

Table XI. *Oxidation of glyceraldehyde by oxaloacetate*

Mutase system consisted of 1 ml. enzyme, 1 ml. 0.15% coenzyme and 0.4 ml. *M*/2 NaHCO₃. Other quantities were 0.4 ml. 1% *dl*-glyceraldehyde and 0.1 ml. *M* α -ketonic acid.

	$\mu\text{l. CO}_2$ in 30 min.
Mutase system + glyceraldehyde	91
Mutase system + glyceraldehyde + oxaloacetate	300
Mutase system + glyceraldehyde + pyruvate	312
Mutase system + oxaloacetate	38
Oxaloacetate	0

from bicarbonate as a result of acid formation. The spontaneous decomposition of oxaloacetic acid into pyruvic acid and CO₂ therefore could have played no

Fig. 5.

Fig. 6.

Fig. 5. Effect of the concentration of coenzyme I on the rate of oxidation of triose by pyruvate.

Each manometer contained 1 ml. enzyme, 0.5 ml. 1% *dl*-glyceraldehyde, 0.1 ml. *M* pyruvate and 0.4 ml. *M/2* NaHCO₃. The total volume was 4 ml.

Fig. 6. Effect of the concentration of *dl*-glyceraldehyde on the rate of reaction with pyruvate.

Each manometer contained 1 ml. enzyme, 1 ml. coenzyme, 0.1 ml. *M* pyruvate and 0.4 ml. *M/2* NaHCO₃. The total volume was 4 ml.

Fig. 7.

Fig. 8.

Fig. 7. Effect of the concentration of pyruvate on the reaction with glyceraldehyde.

Each manometer contained 1 ml. enzyme, 1 ml. coenzyme, 0.4 ml. 1% glyceraldehyde and 0.4 ml. *M/2* NaHCO₃. The total volume was 4 ml.

Fig. 8. The effect of pH on the rate of oxidation of triose by pyruvate.

Each manometer contained 1 ml. enzyme, 0.5 ml. coenzyme, 0.4 ml. 1% *dl*-glyceraldehyde and 0.1 ml. *M* pyruvate. For details of bicarbonate concentrations necessary to attain the different pH values in contact with a gas space containing 5% CO₂, cf. Woods [1936].

part in the CO_2 production. It is interesting to note the extraordinary stability of oxaloacetic acid in presence of bicarbonate and CO_2 .

We have been unable thus far to decide with certainty whether the slow production of CO_2 from glyceraldehyde in absence of α -ketonic acid was due to a dismutation or to oxidation by traces of ketonic acids which have defied dialysis. By analogy with other mutases, it is possible that the dismutation is not rapid but does in fact take place.

The triose mutase attacks glycollic aldehyde though not as rapidly as triose. The aldehyde was prepared crystalline from dihydroxymalic acid by the method of Fischer & Taube [1927]. Table XII shows the dependence of the oxidation

Table XII. *Oxidation of glycollic aldehyde*

The quantities used were 1 ml. mutase, 0.5 ml. coenzyme, 0.1 ml. *M* glycollic aldehyde and 0.1 ml. *M* pyruvate.

	$\mu\text{l. CO}_2$ in 15 min.
Mutase + coenzyme	0
Mutase + coenzyme + glycollic aldehyde	33
Mutase + coenzyme + glycollic aldehyde + pyruvate	160
As above without coenzyme	3
Mutase + coenzyme + glycollic aldehyde + oxaloacetate	170

upon the presence of the mutase, the coenzyme and pyruvate or oxaloacetate. There is an appreciable production of CO_2 in absence of α -ketonic acid which again is probably due to a dismutation. By analogy with the oxidation of triose, glycollic aldehyde is probably oxidized to the corresponding acid whereas the α -ketonic acids are reduced to the corresponding hydroxy-acids.

VIII. *The non-identity of the three mutases*

A study of the distribution of the three mutases in various animal tissues indicates that they are distinct enzymes (cf. Table XIII). The enzymes were

Table XIII. *Presence of mutases in acetone preparations from different tissues*

All the tissues except heart and brain were obtained from rabbit. The other two were obtained from ox. The dried acetone powders were ground with water in the proportions of 1 : 10; the mixture was dialysed overnight and centrifuged.

	Lung	Heart	Skeletal muscle	Intestine	Kidney	Liver	Brain
α -Glycerophosphate	0	++	++++	0	0	0	0
Triose	++++	+	++++	+	++++	++++	0
Triosephosphate	++	++++	++++	++++	++	+	++++

prepared from rabbit tissues by the same procedure as for the skeletal muscle enzymes. The results have bearing not so much on the presence or absence of the various mutases in the tissue as on the presence or absence of the mutases in the enzyme preparations. The α -glycerophosphate mutase was found only in the preparations from skeletal and cardiac muscle. The brain preparation showed considerable triosephosphate activity but no activity with glyceraldehyde. Similarly, the heart and intestine preparations were relatively weak in glyceraldehyde activity compared with triosephosphate activity. Aldehyde mutase has been shown by Dixon & Lutwak-Mann [1937] to dismute glyceraldehyde. The high activity found for glyceraldehyde in the kidney and liver preparations is probably due to the aldehyde mutase and not to the triose mutase. These two

mutases can both attack glyceraldehyde. They differ in their action on aldehydes. The aldehyde mutase attacks all aliphatic aldehydes, whereas the triose mutase attacks only glyceraldehyde, glycollic aldehyde and possibly erythrose. There is no activity shown towards aliphatic aldehydes by the mutase preparation of skeletal muscle.

In addition to the distribution study some experiments on the resolutions of the various mutases have been carried out. The activities of the three mutases were tested after the enzyme solution was subjected to a variety of treatments such as adsorption on kaolin, kieselguhr, charcoal etc. The activity ratios changed very considerably. Thus there can be little doubt that the three mutases are distinct. Similar experiments were carried out to ascertain whether the same enzyme catalyses the dismutation and oxidation of triosephosphate. The results were consistent with the view that only one enzyme is involved.

IX. Specificity of substrates

The three mutase systems were all able to utilize the following α -ketonic acids as acceptors: pyruvic, oxaloacetic, α -ketobutyric, *p*-hydroxyphenylpyruvic, α -ketoisohexanoic and β -indolepyruvic acids (cf. Table XIV). The triosephosphate mutase, however, was negative with *p*-hydroxyphenylpyruvate and

Table XIV. α -Ketonic acids as acceptors

μ l. CO₂ in 15 min.

The various ketonic acids produced no CO₂ in absence of the reductant.

	No addition	Mes- oxalate	<i>p</i> -Hydroxy- phenyl- pyruvate	α -Keto- butyrate	α -Keto- iso- hexanoate	β -In- dole- pyruvate
Glyceraldehyde	31	40	93	140	93	95
Glycollic aldehyde	31	—	90	124	80	90
α -Glycerophosphate	0	0	80	138	106	80
Triosephosphate (+ NaF)	160	230	145	200	—	—

was in fact inhibited. Oxaloacetic acid was prepared by the method of Wohl & Oesterlin [1901], and α -ketobutyric by the method of Tschelinzeff & Schmidt [1929]. The amino-acid oxidase was used as a means of converting leucine and valine into the corresponding keto-acids. After the reaction had finished, the keto-acids were extracted from the deproteinized filtrates with ether and the ether extracts evaporated to a syrup. The keto-acids were then characterized by the 2:4-dinitrophenylhydrazones. We are grateful to Dr D. D. Woods for a gift of β -indolepyruvic acid and to Prof. D. Keilin and Dr Hartree for a gift of *p*-hydroxyphenylpyruvic acid.

Dimethylpyruvate, acetylpyruvate and α -ketoglutarate were the only α -ketonic acids tested which were inactive as acceptors. No β -ketonic acid was found to be active, e.g. acetoacetate. Dihydroxyacetone, acetone, fumarate, aldehyde and laevulinic acid were negative. Braunstein & Vyshepan [1937] have already observed that methyl- and ethyl-pyruvate can replace pyruvate in the reaction with triosephosphate. They found, however, that phenylpyruvate was unsuitable. Mesoxalic acid shows some activity as a hydrogen acceptor with the three mutases of the muscle preparation but in no way comparable with the effect on the aldehyde mutase. From the point of view of velocity of oxidation, pyruvate and oxaloacetate come first, followed by α -ketobutyrate and *p*-hydroxyphenylpyruvate. β -Indolepyruvate, ketoisohexanoate and mesoxalate are the slowest.

Glyceraldehyde could be substituted by glycollic aldehyde and to a slight extent by erythrose (prepared by oxidation of erythritol by H_2O_2 and ferrous sulphate following the method of Fenton & Jackson [1899]). Dihydroxyacetone, glycerol, arabinose, glucose, fructose, xylose and galactose were inactive as substrates. Simple aliphatic and aromatic aldehydes were not attacked by the triose mutase.

X. Purification of the mutases

The work on the purification and resolution of the three mutases is still continuing. The following procedures have been found to effect considerable purification.

(1) The mutase solution is kept at 52° for 15 min. A large precipitate forms of inactive protein which is centrifuged off. The activity of the supernatant remains unchanged.

(2) The mutase solution is acidified with acetic acid and allowed to stand for 15 min. The pH is brought back to neutral with addition of bicarbonate. A very bulky precipitate forms of inactive protein which can be centrifuged off. This procedure does not influence the triosephosphate mutase appreciably but partially inactivates the other two mutases.

(3) Shaking the mutase solution with charcoal and centrifuging off the charcoal occasions little loss in activity and a fair amount of purification.

Electrodialysis completely inactivates the mutases within about 5 hr. Re-precipitation with acetone also causes serious inactivation. Filtration through coarse filter-paper or high speed centrifuging has no effect on the activity of any of the mutases.

XI. Inhibitors

Iodoacetic acid in a concentration from 0.005 to 0.0005 *M* inhibits practically completely the reactions of all three mutases (cf. Tables XV and XVI). It is interesting that no other oxidizing enzymes of animal tissues are known to be

Table XV. *Effect of iodoacetic acid on the oxidation of glyceraldehyde by pyruvate*

The mutase system represents the mutase and coenzyme. The usual quantities were added.

	$\mu\text{l. CO}_2$ in 10 min.
Mutase system + glyceraldehyde + pyruvate	248
Mutase system + <i>M</i> /100 iodoacetate	11
Mutase system + <i>M</i> /1000 iodoacetate	16
Mutase system + <i>M</i> /4000 iodoacetate	25

Table XVI. *Effect of iodoacetic acid on oxidation of α -glycerophosphate and hexosediphosphate by pyruvate*

	$\mu\text{l. CO}_2$ in 10 min.
Mutase system + glycollic aldehyde + pyruvate	401
Mutase system + <i>M</i> /200 iodoacetate	44
Mutase system + triosephosphate + pyruvate	198
Mutase system + <i>M</i> /200 iodoacetate	16
Mutase system + α -glycerophosphate + pyruvate	101
Mutase system + <i>M</i> /200 iodoacetate	12

inhibited by low concentrations of iodoacetic acid except the aldehyde mutase of liver. None of the known dehydrogenase systems of animal tissues is appreciably influenced by this inhibitor. This sensitivity of mutases must involve some

common grouping in the various enzymes. The effect of iodoacetic acid on the glyceraldehyde-pyruvate reaction is not instantaneous (cf. Fig. 9). If the enzyme is first mixed with glyceraldehyde and then with iodoacetic acid, the inhibition is considerably reduced. That shows that the substrate competes with iodoacetic acid for the active group of enzyme. When, however, iodoacetic acid is added some minutes before the substrate, inhibition is complete. A complete analysis of the iodoacetic acid effect has been given by Dixon [1937].

Fig. 9. The time factor in the iodoacetic acid effect.

Each manometer contained 1.5 ml. enzyme, 0.5 ml. 15% coenzyme I, 0.1 ml. *M* pyruvate and 0.4 ml. *M*/2 NaHCO_3 . The strength of the iodoacetic acid was *M*/200 final concentration.

Iodoacetic acid inhibits only the oxidation of triosephosphate, α -glycerophosphate and triose respectively but not the reduction of the ketonic acids. The evidence will be considered in a later section. In other words, the oxidizing and reducing parts of the mutase do not show identical reactions to inhibitors.

Table XVII is a summary of the effects of various reagents and inhibitors on the triosephosphate and triose mutases. The effect of salicylaldehyde is probably

Table XVII. *Effect of reagents*

0 indicates no effect. A number refers to the % inhibition.

	Triosephosphate + pyruvate %	Triose + pyruvate %
Insulin	0	0
Glutathione	0	0
<i>M</i> /40 arsenite	—	36
<i>M</i> /40 pyrophosphate	24	69
Salicylaldehyde (1/4 saturated)	—	89
Adrenaline	0	0

one of competitive inhibition. It is interesting that the mutase solution in presence of both salicylaldehyde and glyceraldehyde produces only a fraction of the CO_2 produced by either separately.

XII. *Mutases as dehydrogenases*¹

The reaction between, e.g., triosephosphate and pyruvate may be considered as a coenzyme-linked reaction between two dehydrogenase systems, viz. the triosephosphate and lactic dehydrogenases respectively [cf. Dewan & Green, 1937]:

When, however, the appropriate tests for a triosephosphate and lactic dehydrogenase are carried out, the results are either negative or so weakly positive as to be negligible. The apparent distinction between mutase and dehydrogenase activities may be schematized as follows:

The arrows indicate the direction of transfer of hydrogen. In mutase systems, the coenzyme is reduced and oxidized only by the substrates of the enzyme. In dehydrogenase systems, reduced coenzyme can also react with carriers such as flavin, flavoprotein, adrenochrome, methylene blue etc. At first we considered this distinction to be fundamental, but subsequent experimentation disclosed that dehydrogenases like mutases cannot catalyse the oxidation of reduced coenzyme by means of carriers. The method of preparation of mutases involves the complete destruction of the necessary catalytic factor whereas this factor is present in the usual preparations of the lactic, malic and β -hydroxybutyric dehydrogenases—the best known examples of animal coenzyme dehydrogenases. By suitable treatment it is possible to separate the factor completely from these dehydrogenases. Hence both mutases and dehydrogenases are concerned only in the reaction between the substrate and coenzyme.

Assuming that the inability of mutases to act as dehydrogenases is due to a block in the reaction between reduced coenzyme and the oxygen carrier, we tested the effect of adding extracts of washed muscle prepared as follows. Minced skeletal muscle was washed thoroughly with tap water, ground with sand and phosphate buffer (*M*/50) and filtered through muslin. The filtrate was centrifuged and the supernatant brought to pH 4.6. The precipitate was centrifuged hard and resuspended in *M*/10 phosphate buffer pH 7.2. This extract was rich in dehydrogenases. Table XVIII shows the result of a typical experiment.

Table XVIII. *Effect of factor on the oxidation of triosephosphate*

The quantities used were 1.5 ml. mutase, 1.0 ml. coenzyme, 0.3 ml. of 0.5% methylene blue 0.3 ml. *M*/10 hexosediphosphate and 0.1 ml. 6 *N* KOH in the potash cup.

	$\mu\text{l. O}_2$ in 20 min.
Mutase + coenzyme + triosephosphate + methylene blue	0
Factor + coenzyme + triosephosphate + methylene blue	0
Mutase + factor + coenzyme + triosephosphate + methylene blue	186
Complete system without triosephosphate	20
Complete system without coenzyme	0
Complete system without methylene blue	75

Neither the mutase nor the extract alone was able to oxidize triosephosphate via methylene blue and oxygen. Mutase and extract together behaved as a typical dehydrogenase system. The objection might be raised that the extract

¹ The discussion of the experiments in Sections XII and XIII was partially rewritten with some additions on 11 December.

contained an active triosephosphate dehydrogenase but no zymohexase. The mutase was therefore merely supplying the enzyme for converting hexosediphosphate into triosephosphate. This is ruled out by the fact that the extract, like the mutase, contains active zymohexase. The reaction with oxygen required the presence of all the components: mutase, coenzyme, extract, hexosediphosphate and carrier. In absence of carrier, a somewhat slower reaction took place. This is due to the coenzyme oxidase in the extract which catalyses the direct reaction between reduced coenzyme and molecular oxygen [cf. Green *et al.* 1937]. We have been unable as yet to find any substance to replace the extract. A highly active factor has also been prepared from pig heart by the same method as for the rabbit muscle extract. Flavoprotein, vitamin B₁ insulin, cytochrome and glutathione are all inactive. Furthermore, the factor responsible for the conversion, as it were, of the mutase into a typical dehydrogenase system is non-dialysable, destroyed by boiling and sedimented by high speed centrifuging. Thus it is clear that some labile component of the extract not replaceable by known carriers can collaborate with the triosephosphate mutase in catalysing the oxidation of triosephosphate by molecular oxygen.

By means of this extract it was possible to demonstrate a whole series of dehydrogenases which reflect either the reducing or oxidizing phases of mutase action, e.g. the lactic, malic, α -hydroxybutyric, α -glycerophosphoric and glyceraldehyde dehydrogenases (cf. Table XIX). The activating effects of the extract were not uniformly as high as with triosephosphate but the effects were always positive. There is a slight complication in testing for some of the dehydrogenases since the extract itself shows some activity towards these substrates. But the effect produced is usually out of all proportion to the activity of the extract.

Table XIX. *Effect of factor on the oxidation of lactate, malate, α -ketobutyrate, α -glycerophosphate and glyceraldehyde*

The system contained coenzyme, methylene blue and cyanide except in the case of glyceraldehyde, in which no cyanide was used and pyocyanine was used instead of methylene blue.

	$\mu\text{l. O}_2$ in 20 min.		
	Mutase + system	Factor + system	Mutase + factor + system
No substrate	0	0	10
Malate	39	40	212
Lactate	30	62	274
α -Hydroxybutyrate	24	0	51
Glyceraldehyde	10	0	39
α -Glycerophosphate	0	29	63

Recently, Banga [1937] described an "activator" of the respiration of muscle extracts, the properties of which agree completely with those of the coenzyme factor. Banga considers that the "activator" makes possible the interaction of triosephosphate and pyruvate.

The coenzyme factor has proved to be an invaluable tool for analysing separately the oxidizing and reducing functions of the mutase. Table XX shows that iodoacetic acid inhibits the oxidation of triosephosphate and not the reduction of pyruvate or oxaloacetate to the corresponding hydroxy-acids (actually the test is made for the reverse reactions). Also we have found that the ratio triosephosphate/lactic activity is not constant and can be changed in the course of purification. As yet there is no evidence for a decision one way or the other on the question whether the mutase is two separate enzymes working together or two enzymes fused into one.

Table XX. *Effect of iodoacetic acid on the oxidation of triosephosphate, lactate and malate*

The mutase system contained mutase, coenzyme and methylene blue. Details as for Table XVIII.

	$\mu\text{l. O}_2$ in 10 min.	% inhibition
Mutase system + factor + hexosediphosphate	110	—
As above with <i>M</i> /200 iodoacetate	17	84
Mutase system + factor + malate + cyanide	133	—
As above with <i>M</i> /200 iodoacetate	112	17
Mutase system + factor + lactate + cyanide	161	—
As above with <i>M</i> /200 iodoacetate	137	15

XIII. *Reaction of mutases with coenzyme dehydrogenase systems*

From the point of view of the theory of mutase action, it was of considerable interest to know whether the mutase could be used as a dehydrogenase in a coenzyme-linked reaction between two systems. We attempted the following coenzyme-linked reactions:

- (16) β -Hydroxybutyrate + α -ketobutyrate $\longrightarrow$ acetoacetate + α -hydroxybutyrate,
 (17) β -Hydroxybutyrate + *p*-hydroxyphenylpyruvate $\longrightarrow$ acetoacetate + *p*-hydroxyphenyl-lactate,
 (18) β -Hydroxybutyrate + mesoxalate $\longrightarrow$ acetoacetate + tartronate.

It was intended to use the β -hydroxybutyric dehydrogenase preparation of heart in combination with the muscle mutase to catalyse the above reactions. However, the lactic dehydrogenase present in the heart preparation was found to catalyse the reduction of all the ketonic acids without added mutase. The effect of mutase was therefore greatly obscured. But it is significant that in presence of the mutase, reaction (17) proceeded almost twice as fast as in its absence (cf. Table XXI). Thus it appears that the mutase can link up anaerobically with dehydrogenases. The formation of acetoacetic acid was used as

Table XXI. *Linking of the β -hydroxybutyric dehydrogenase system (pig heart preparation) with the mutase of skeletal muscle (rabbit)*

The dehydrogenase system contained the heart enzyme and coenzyme I. For details of similar experiments and methods of estimation, cf. Dewan & Green [1937].

	$\mu\text{l. acetoacetic}$ CO_2
Dehydrogenase system + β -hydroxybutyrate	15
Dehydrogenase system + β -hydroxybutyrate + α -ketobutyrate	338
As above with mutase	352
As above without α -ketobutyrate	15
Dehydrogenase system + β -hydroxybutyrate + <i>p</i> -hydroxyphenyl-pyruvate	196
As above with mutase	354
Dehydrogenase system + β -hydroxybutyrate + mesoxalate	284
As above with mutase	434

the measure of reaction. The ketonic acid was estimated manometrically as CO_2 by the aniline method of Ostern [1933]. Dewan & Green [1937] have already demonstrated that the aldehyde mutase could be coupled with the β -hydroxybutyric dehydrogenase via coenzyme I.

The specificity of the typical lactic dehydrogenase as distinguished from the mutase one, towards the higher members of the series of α -hydroxy-acids has not been studied. It appears that the specificity is wider than has hitherto been suspected. A knowledge of the specificities of the two lactic enzymes might permit a decision as to their identity.

The similarities between the typical lactic dehydrogenase and that of the mutase are many, e.g. dependence upon a coenzyme, specificity for *l*(+)-lactate, reaction with compounds related to pyruvate such as α -ketobutyrate and *p*-hydroxyphenylpyruvate, insensitivity to iodoacetic acid etc. But there are also some differences. The mutase is soluble and is unaffected by high speed centrifuging. The typical lactic dehydrogenase cannot be washed out of minced tissue—being extracted only after complete grinding and disintegration of cell organization. In contrast the mutase is easily washed out of coarsely minced tissue without any grinding being necessary. However, these differences are not fundamental. We have recently been able to obtain the dehydrogenase in the form of a water-clear solution. The tentative hypothesis is that the lactic-pyruvic end of the mutase is identical with the typical dehydrogenase.

Another apparent difference between the lactic enzyme of the mutase and the ordinary lactic dehydrogenase lies in the power of reacting with molecular oxygen via carriers. The former is unable to do so except in presence of an additional factor. One may argue that possibly the lactic dehydrogenase on further purification would similarly fail to react with carriers. We have been able by a procedure to be described in a later paper to separate the lactic dehydrogenase from the coenzyme factor. Such a preparation of the lactic enzyme, like the mutase, is unable to react with carriers except in presence of the factor. The ability to react with molecular oxygen via carriers is therefore not characteristic either of coenzyme dehydrogenases or mutases.

Our experiments thus far on the coenzyme factor have been confined to dehydrogenases of animal source. Flavoprotein fulfils the same role in the case of yeast coenzyme systems as coenzyme factor in the case of animal systems. Flavoprotein however is not identical with coenzyme factor.

XIV. *Oxidation and reduction of coenzyme*

The spectrophotometric method employed for studying the reduction and oxidation of the coenzyme has been previously described by Green & Dewan [1937]. Tables XXII and XXIII summarize the experiments. Glyceraldehyde, α -glycerophosphate, lactate, malate and glycollic aldehyde all rapidly reduce coenzyme I. It is necessary to use a fixative such as cyanide when testing reduction by lactate, malate and α -glycerophosphate in order to shift the equilibrium in favour of reduced coenzyme. In experiments on the reduction of coenzyme by lactate, it was found that the reaction was practically instantaneous. No reduction of coenzyme by hexosediphosphate (i.e. triosephosphate)

Table XXII. *Reduction of coenzyme*

For details of procedure, cf. Green & Dewan [1937]. For each estimation, 0.1 ml. mutase and 2.5 ml. 0.2% crude coenzyme I were used. The controls for reduction of the coenzyme in absence of the enzyme were all negative. The time of these experiments was 5 min.

	$\log I_0/I$ at 340 m μ
Mutase + coenzyme	0.2
Mutase + coenzyme + glyceraldehyde	1.5
Mutase + coenzyme + glycollic aldehyde	1.3
Mutase + coenzyme + lactate + cyanide	2.0
As above—no cyanide	0.3
Mutase + coenzyme + malate + cyanide	2.0
Mutase + coenzyme + α -glycerophosphate + cyanide	1.3
As above with <i>M</i> /200 iodoacetate	0.2
Mutase + coenzyme + hexosediphosphate	0.2

Table XXIII. *Oxidation of reduced coenzyme*

	log I_0/I at 340 m μ
Mutase + reduced coenzyme	2.0
Mutase + reduced coenzyme + pyruvate	0.3
As above with boiled enzyme	2.0
Mutase + reduced coenzyme + oxaloacetate	0.4
As above with boiled enzyme	2.0
Mutase + reduced coenzyme + α -ketobutyrate	0.7
Mutase + reduced coenzyme + hexosediphosphate	0.3

could be demonstrated either in presence or in absence of fluoride. This negative result does not mean that the coenzyme is not being reduced. Hexosediphosphate instantaneously gives rise to α -glycerophosphate and phosphoglycerate—the latter of which can oxidize reduced coenzyme. In fact, triosephosphate acts not only as a hydrogen donator but also as an acceptor. The kinetic balance is therefore wholly in favour of the oxidation of the coenzyme. Consistent with that hypothesis is the fact that reduced coenzyme is oxidized completely in presence of the mutase and hexosediphosphate. Pyruvate, oxaloacetate and α -ketobutyrate all rapidly oxidize reduced coenzyme.

Euler *et al.* [1936] have already shown that the mutase solution in presence of α -glycerophosphate can reduce coenzyme I and that in presence of pyruvate it can oxidize reduced coenzyme I. They attributed these oxidoreductions to typical dehydrogenase systems.

XV. *Synthesis of triosephosphate*

Triosephosphate and pyruvate in presence of the appropriate mutase and coenzyme I react until all the triosephosphate is oxidized. That is to say equilibrium is reached only when the reaction has proceeded practically to completion. In order to reverse the forward reaction, some means must be found of shifting the equilibrium point. Since cyanide combines with both triosephosphate and pyruvate, the use of this reagent should enable the reaction to proceed in the reverse direction. The reduction of phosphoglycerate to triosephosphate involves the disappearance of an acid. Hence, if the experiment is conducted manometrically, the course of the reaction should be indicated by an absorption of CO_2 from the gas space into the bicarbonate solution containing the reactants. Table XXIV is a summary of a typical experiment. The absorption of CO_2 depends upon the presence of the coenzyme, lactate, phosphoglycerate and the mutase. There is an appreciable blank in absence of coenzyme but this is also true of the forward reaction.

Table XXIV. *Reaction of lactate with phosphoglycerate*

The following quantities were used; 1 ml. mutase, 1.0 ml. 0.2% coenzyme I, 0.4 ml. $M/5$ phosphoglycerate (natural) 0.2 ml. $2M$ HCN and 0.1 ml. M *dl*-lactate.

	$\mu\text{l. CO}_2$ absorbed in 10 min.
Mutase + coenzyme + lactate + cyanide + phosphoglycerate	394
Complete system without lactate	60
Complete system without cyanide	0
Complete system without mutase	0
Complete system without phosphoglycerate	0
Complete system without coenzyme	80

There are a few experimental details that are important in connexion with the synthesis of triosephosphate. The cyanide solution ($2M$) should be neutralized immediately before the experiment. The neutralized solution should be just

colourless to phenolphthalein and distinctly blue to bromothymol blue. The neutralized solution should not be kept more than 2 hr.

Table XXV. *Oxidation of malate and α -hydroxybutyrate by phosphoglycerate*

Details as for Table XXIV.		$\mu\text{l. CO}_2$ absorbed in 10 min.
Mutase + coenzyme + cyanide + malate + phosphoglycerate		352
Complete system without phosphoglycerate		0
Complete system without malate		60
Mutase + coenzyme + cyanide + α -hydroxybutyrate + phosphoglycerate		103
As above without phosphoglycerate		0

Not only will lactate react with phosphoglycerate but malate and α -hydroxybutyrate do so as well (cf. Table XXV). The three reactions may be summarized by equation (19):

Only *l*(+)-lactate and *l*(-)-malate are active as reductants for (+)-phosphoglycerate:

	$\mu\text{l. CO}_2$ absorbed in 10 min.
<i>l</i> (+)-Lactate	398
<i>d</i> (-)-Lactate	38
<i>l</i> (-)-Malate	236
<i>d</i> (+)-Malate	19

The production of triosephosphate and pyruvate was also demonstrated chemically. The former was estimated as alkali-labile phosphate by the method of Meyerhof & Lohmann [1934]; the latter was isolated as the 2:4-dinitrophenylhydrazone and identified as such by a mixed m.p. with a known sample. Table XXVI compares the absorption of CO_2 with the production of alkali-labile

Table XXVI. *CO_2 absorption and formation of alkali-labile phosphate*

	$\mu\text{l. CO}_2$ absorbed	mg. alkali labile phosphate produced
Complete system	210	0.70
Without coenzyme	100	0.34
Without cyanide	0	0
Without lactate	0	0
Without phosphoglycerate	0	0

phosphate. The parallelism, although not exact, indicates that the reaction is proceeding according to equation (19).

In addition to the production of alkali-labile P, a considerable liberation of inorganic phosphate is observed coincidently with the oxidoreduction. In absence or in presence of iodoacetic acid, the production of inorganic phosphate as well as of alkali-labile phosphate is suppressed. The mechanism whereby the inorganic phosphate is derived from phosphoglycerate is unknown. But it appears likely that the production of inorganic phosphate is dependent upon the oxidoreduction. This observation recalls the results of Meyerhof *et al.* [1937] who found with

yeast preparations that dephosphorylation of phosphopyruvate was under certain conditions greatly accelerated by the presence of a trace of hexosediphosphate provided that coenzyme I and an active oxidoreduction system were also present. The production of inorganic phosphate has no direct bearing on the synthesis of triosephosphate. It is not due for example to breakdown of the triosephosphatecyanohydrin. Hexosediphosphate in presence of the mutase solution and cyanide gave rise quantitatively to triosephosphatecyanohydrin. No inorganic phosphate was found.

Thus far we have been unable to produce the following reversals: glycerate + lactate, α -glycerophosphate + phosphoglycerate and α -glycerophosphate + glycerate. There are three possibilities: (1) these reactions are irreversible, (2) cyanide is inhibiting the triose and α -glycerophosphate mutases and (3) cyanide does not fix glyceraldehyde efficiently.

A direct and simple method demonstrating the reduction of phosphoglycerate to triosephosphate lies in the use of oxidoreduction indicators. Glyceraldehyde in presence of the mutase, coenzyme I and the factor at pH 7.2 reduced benzylviologen practically to completion (intense blue colour). When the reduction had proceeded to completion, phosphoglycerate was introduced. The blue colour of the reduced form gradually faded and in a few minutes the solution became colourless. Glyceraldehyde is merely a reducing agent for the coenzyme, reduced coenzyme in turn reducing benzylviologen. By addition of phosphoglycerate, the equilibrium is shifted in favour of oxidized coenzyme and hence in favour of the oxidized or colourless form of benzylviologen (for a complete discussion of the theory of the method, cf. Green *et al.* [1937]).

The formation of lactate and inorganic phosphate from hexosediphosphate in muscle is usually regarded as taking place in six stages: (1) formation of triosephosphate, (2) oxidoreduction between triosephosphate and a trace of pyruvate, (3) transformation of 3-phosphoglycerate into 2-phosphoglycerate, (4) transformation of 2-phosphoglycerate into phosphopyruvate, (5) dephosphorylation of phosphopyruvate by reaction with adenylic acid, the pyruvate thus formed taking part in the second reaction and (6) formation of free phosphate and adenylic acid from adenosinetriphosphate. Of these stages, (1), (3) and (4) have been shown by Meyerhof and his collaborators to be reversible. Needham & Pillai [1937] have shown that stage (6) can be reversed by coupling with an energy-yielding reaction. Thus stage (5) is the only one which has not so far been reversed *in vitro*. An investigation of the conditions under which adenylypyrophosphate might phosphorylate pyruvate, or under which pyruvate might react with inorganic phosphate is being undertaken.

These facts lead on to the consideration of a possible path of carbohydrate synthesis from lactate. Given a trace of phosphoglycerate, reaction with lactate would produce triosephosphate and pyruvate. This reaction would proceed to an appreciable degree if the triosephosphate formed were continually and completely removed—as would happen if, for example, the hexosediphosphate formed were being converted into glycogen. As for the pyruvate formed in the oxidoreduction, once it had been phosphorylated (a step at present obscure), its conversion into a further supply of phosphoglycerate to react with the lactate would readily follow. Thus given an energy source for the phosphorylation of pyruvate and for other energy-requiring reactions, it should be possible to convert lactate quantitatively into glycogen.

XVI. *Aldehyde mutase*

The kinetics and quantitative relations of the aldehyde mutase of liver have been thoroughly studied by Dixon & Lutwak-Mann [1937]. We have been interested merely in comparing the aldehyde mutase with the muscle mutases in respect of the reaction with α -ketonic acids. Addition of α -ketonic acids such as pyruvate, oxaloacetate, mesoxalate, α -ketoglutarate and α -ketobutyrate considerably increases the rate of production of CO_2 from a mixture of the

Table XXVII. *Oxidation of acetaldehyde by ketonic acids*

The mutase was prepared as described in § I. The quantities used were 1 ml. enzyme, 1 ml. 0.15% coenzyme I, 0.1 ml. *M* ketonic acid, 0.4 ml. *M*/2 NaHCO_3 and 0.1 ml. *M* acetaldehyde. The aldehyde was placed in both the right and left cups of the Barcroft manometer to compensate for the extra vapour pressure developed.

	$\mu\text{l. CO}_2$ in 10 min.
Mutase + aldehyde	335
Mutase + aldehyde + pyruvate	803
Mutase + aldehyde + oxaloacetate	720
Mutase + aldehyde + α -ketoglutarate	644
Mutase + aldehyde + mesoxalate	900

aldehyde mutase, coenzyme I and acetaldehyde in bicarbonate solution (cf. Table XXVII). In the case of pyruvate as oxidant, the dismutation is suppressed and the only reaction which proceeds is:

The evidence for this is the equivalence between the manometric CO_2 and the production of lactic acid.

	$\mu\text{l. CO}_2$ in 15 min.
Mutase + aldehyde + coenzyme	340
Mutase + aldehyde + coenzyme + pyruvate	480
Lactic acid produced from added pyruvic (expressed as $\mu\text{l. CO}_2$)	410

i.e. 85% of the total CO_2 could be accounted for by the oxidation of aldehyde by pyruvate.

With oxaloacetic acid, both reactions take place simultaneously. The malic acid produced is equivalent only to the difference in manometric CO_2 between the value for acetaldehyde + oxaloacetate and that for acetaldehyde alone:

	$\mu\text{l. CO}_2$ in 90 min.
Mutase + aldehyde + coenzyme	373
Mutase + aldehyde + coenzyme + oxaloacetate	811
Increase in CO_2 due to oxaloacetate	438
Malic acid formed (expressed as $\mu\text{l. CO}_2$)	421

Malic acid was estimated as acetaldehyde by the method of Friedemann & Graesser [1933]. The malic acid produced showed no optical activity. Thus the reduction of oxaloacetate by the aldehyde mutase is symmetrical whereas that by the muscle mutases is asymmetric. In the reaction between aldehyde and mesoxalate, disappearance of ketonic acid could be demonstrated by the iodine-bisulphite titration method. Aldehyde was first removed by aeration.

We attempted to produce aldehyde from a mixture of alcohol and acid in presence of cyanide but with no success. It is conceivable that the reaction is reversible but that cyanide inhibits the enzymic activity.

XVII. *Optical specificity*

All the muscle mutases reduced α -ketonic acids to the corresponding optically active α -hydroxy-acids of the *l*-stereochemical series, e.g. *l*(+)-lactate and

l(-)-malate. Conversely *d*(-)-lactate and *d*(+)-malate were inactive in the reaction with the natural *d*(+)-phosphoglycerate. The α -glycerophosphate produced by the dismutation of triosephosphate was completely estimated by the muscle dehydrogenase system which specifically attacks only the *l*(-) component. This agrees with the results of Fischer & Baer [1937], who synthesized *l*(-)- α -glycerophosphate, and found it to be identical with the natural compound. They point out that chemical oxidation of natural α -glycerophosphate gives rise to the non-natural *l*-glyceraldehydephosphate and *l*(-)-phosphoglyceric acid. It can readily be seen from structural formulae that enzymic formation of dihydroxyacetonephosphate and *d*-glyceraldehydephosphate from hexosediphosphate, followed by oxidoreduction between these two, would give rise to *l*(-)-glycerophosphate and *d*(-)-phosphoglyceric acid. When pyruvic acid oxidizes *l*(-)-glycerophosphate under the influence of the mutase, *d*(-)-phosphoglyceric acid must be formed via inactive dihydroxyacetonephosphate. Of the two forms of glyceraldehyde, apparently only the *d*(+)-enantiomorph is attacked.

XVIII. *Theory of mutase action*

We may define mutases as enzymes which catalyse the oxidation of their substrate by α -ketonic acids and which depend upon the collaboration of coenzyme I. Most of them can also catalyse the dismutation of the substrate. But that ability is not characteristic of all the members of the class. The objection may be raised that the term mutase was originally applied to enzymes catalysing dismutations and that the sense in which we are now employing it will introduce confusion. Dr M. Dixon has therefore suggested the term oxidoreductases to cover the exceptional mutases which do not catalyse dismutation. We feel, however, that the problem of nomenclature must await further work on the nature of mutases.

Our provisional conception of mutase action is the following. Each mutase consists of a protein with two active groups—one of which activates the substrate and catalyses the oxidation of the substrate by the coenzyme, the other activates the α -ketonic acids and catalyses the reduction of the α -ketonic acid by reduced coenzyme. The coenzyme is the liaison agent between the two active groups. The dismutation of the substrate involves a dual activation, i.e. the substrate has to be simultaneously reduced and oxidized. It is impossible to say at the moment whether only one active group is sufficient to catalyse the dismutation or whether the dismutation like the oxidoreduction involves the two different active groups.

The difficulties still in the way of a clear understanding of mutase action may be summarized as follows:

(1) In the reaction between e.g. triosephosphate and pyruvate are we dealing with two different enzymes or with a single enzyme unit? By enzyme is implied both the protein bearer and active group together.

(2) Is the mutase enzyme which is involved in the reduction of pyruvate to lactate and of the other α -ketonic acids to their corresponding hydroxy-acids a different enzyme from the lactic dehydrogenase?

The experiments described above supply partial answers to some of these difficulties. But it would be dogmatic to reach a definite decision.

The participation of α -ketonic acids other than pyruvic acid in the reaction with triosephosphate, triose and α -glycerophosphate has some bearing on modern conceptions of muscle glycolysis. It would appear that only three of a very large number of possible oxidoreductions have been given prominence. The artificiality of such schemes is obvious.

SUMMARY

1. The properties and reactions of the triose, triosephosphate and α -glycero-phosphate mutases have been studied. The activities of all these three mutases depend upon coenzyme I.

2. The mutases all catalyse the oxidation of their substrates by α -ketonic acids. Some, like the triosephosphate mutase, also catalyse the dismutation of the substrate.

3. Mutase action is inhibited almost completely by low concentrations of iodoacetic acid.

4. The coenzyme functions in these oxidoreductions by undergoing a cycle of oxidation and reduction.

5. Mutase systems cannot react with oxygen carriers such as flavin, methylene blue etc. in absence of a thermolabile factor found in skeletal and cardiac muscle extracts. The factor is non-dialysable and sedimented by high speed centrifuging. It is not identical with any of the known carriers.

6. The reaction between lactate and phosphoglycerate yielding triosephosphate and pyruvate has been shown to take place in presence of the triosephosphate mutase system and strong cyanide.

7. The theory of mutase action is discussed.

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