163. TRIOSEPHOSPHATE DEHYDROGENASE OF *BACTERIUM COLI*

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THE Booth-Green wet-crushing mill for bacteria has been used previously to prepare a cell-free juice from *Bacterium coli* in which the components of enzyme systems may be studied. In the present paper a juice has been prepared containing the system which oxidizes 3-phosphoglyceraldehyde.

For yeast it has recently been shown that the actual substrate is 1:3-diphosphoglyceraldehyde [Warburg & Christian, 1939]. The evidence in this paper does not conflict with the view that the same is true of *Bact. coli*. The object of this investigation was the partial purification of the triosephosphate dehydrogenase of *Bact. coli* and its comparison with the oxidizing enzyme of yeast fermentation. It has already been shown that the reducing enzyme, alcohol dehydrogenase, occurs in *Bact. coli* [Still, 1940].

I. Preparation

The organism used was *Bact. coli commune* Escherich (National Collection of Type Cultures No. 86). The organism was grown on agar with tryptic digest of casein and mineral salts. After washing the cells were crushed in the mill [Booth & Green, 1938], centrifuged for 30 min. at 3000 r.p.m., the supernatant heated to 55° and centrifuged for 40 min. at 12,000 r.p.m. This supernatant was dialysed against running water. This procedure is essentially that previously followed [Still, 1940]. It was found that in the case of the triosephosphate dehydrogenase more prolonged dialysis was required to remove coenzyme I, and that it was an advantage to dilute the crushed cells more before centrifuging.

The activity of the enzyme was measured by Thunberg tube technique and manometrically by Warburg manometers. The coenzyme I used was made by a modification of the method of Green & Brosteaux [1936]. The hexosediphosphate was estimated with zymoheaxase from rabbit skeletal muscle [cf. Herbert *et al.* 1940].

II. Zymoheaxase

The enzyme solution prepared as above converted hexosediphosphate into phosphoglyceraldehyde. This was shown as follows: 2.0 ml. enzyme, 1.0 ml. *M*/2 NaCN, 1.0 ml. water and 2.0 ml. *M*/20 hexosediphosphate were added to each of two tubes. To one tube 0.32 ml. 40 % trichloroacetic acid was added immediately. The other tube was incubated at 38° and then 0.32 ml. 40 % trichloroacetic acid was added. Two controls without hexosediphosphate were also set up and treated similarly. After standing at 0°, the contents of the tubes were filtered through Whatman No. 40 paper. Changes in the amount of inorganic and alkali-labile phosphate were determined by the method of Lohmann & Jendrassik [1926]. Phosphate rendered labile by treatment with *N* NaOH for 20 min. at room temperature was considered to be derived from phosphoglyceraldehyde.

The results summarized in Table I indicate the presence of zymohexase and phosphatases in the enzyme solution. In view of the instability of phosphoglyceraldehyde hexosediphosphate was used as the substrate in all the subsequent experiments.

Table 1. *Demonstration of zymohexase in enzyme solution*

	Inorganic P (mg.)	Alkali-labile P (mg.)
Enzyme + cyanide + hexosediphosphate: Initial	0.6	0
Final	1.05	3.88
Enzyme + cyanide + hexosediphosphate: Initial	0.19	0
Final	0.31	0.08

III. Components of the oxidation system

The enzyme solution required the addition of coenzyme I and either phosphate or arsenate in order to reduce methylene blue in the presence of hexosediphosphate (Table 2). By analogy with the yeast enzyme this was taken to mean that 3-phosphoglyceraldehyde is first phosphorylated or arsenylated to 1:3-diphosphoglyceraldehyde or 1-arseno-3-phosphoglyceraldehyde both of which are substrates for the enzyme [Warburg & Christian, 1939]. The relative instability of the resulting arsenophosphoglyceric acid probably accounted for the greater activity in the presence of arsenate.

Table 2. *Demonstration of anaerobic oxidation of phosphoglyceraldehyde*

	Additions to tubes (ml.)					
	1	2	3	4	5	6
Enzyme	1.0	1.0	1.0	1.0	1.0	1.0
Phosphate <i>M</i> /15 pH 7.4	—	1.0	—	1.0	1.0	1.0
Arsenate <i>M</i> /10 pH 7.4	—	—	0.7	—	—	0.7
Adenylic acid 1.0 mg./ml.	—	—	—	0.5	—	—
Coenzyme I 5 mg./ml. 6.8% pure	0.1	0.1	0.1	0.1	0.1	—
Methylene blue 0.5%	0.2	0.2	0.2	0.2	0.2	0.2
Hexosediphosphate <i>M</i> /20	0.2	0.2	0.2	0.2	—	0.2
Water	1.6	0.6	0.9	—	0.8	—
Reduction time in min.	>120	17	2½	18	>120	>120

The enzyme was not destroyed when precipitated with acetone in the cold. An acetone preparation relatively free from coenzyme dehydrogenase was made as previously described [Still, 1940] and its activity tested with and without added coenzyme dehydrogenase (diaphorase), the latter being provided in the form of milk flavoprotein (which rapidly catalyses the oxidation of reduced coenzyme I by methylene blue). The results in Table 3 indicate that coenzyme dehydrogenase is a component of the system in which methylene blue is reduced by phosphoglyceraldehyde.

Table 3. *Effect of flavoprotein on acetone preparation*

	Q_{MB}
Enzyme solution	75
Acetone powder	7.2
Acetone powder + milk flavoprotein	36.2

$Q_{MB} = \mu\text{l. H}_2$ transferred to methylene blue per hr. per mg. dry weight of enzyme.

IV. Reaction with molecular oxygen

In the presence of methylene blue the system reacts with molecular O_2 . Table 4 shows that for this reaction also coenzyme I and either phosphate or arsenate are necessary. Fig. 1 shows the extent of the reaction in the presence of phosphate and arsenate.

Fig. 1. Effect of arsenate (*A*) and phosphate (*B*) on the oxidation of phosphoglyceraldehyde. Warburg manometers containing 1.0 ml. enzyme, 0.2 ml. *M*/22 hexosediphosphate, 0.5 ml. 0.5% methylene blue, 1 mg. coenzyme I (14% pure) and 0.1 ml. *M* NaF. Total volume 3.2 ml. *A* contained 0.3 ml. *M*/5 arsenate, *B* contained 1.0 ml. *M*/15 phosphate, and *C* no additions.

Table 4. Aerobic oxidation of phosphoglyceraldehyde.
Additions to Warburg manometers (ml.)

Enzyme	1.0	1.0	1.0	1.0	1.0	1.0
Phosphate <i>M</i> /5 pH 7.7	—	0.5	—	—	—	—
Arsenate <i>M</i> /5 pH 7.7	—	—	0.5	0.5	0.5	0.5
Coenzyme I 5 mg./ml. 27% pure	0.2	0.2	0.2	—	0.2	0.2
Methylene blue 0.5%	0.5	0.5	0.5	0.5	0.5	—
Hexosediphosphate <i>M</i> /20	0.4	0.4	0.4	0.4	—	0.4
Water	1.0	0.5	0.5	0.7	0.9	1.0
Sodium fluoride <i>M</i>	0.1	0.1	0.1	0.1	0.1	0.1
μl. O_2 taken up in 10 min.	4.0	26	154	0	7	23
20 "	8.5	44.5	284	0	10	44
40 "	18.0	77.5	416	5	19	85

The enzyme preparation also oxidized glyceraldehyde under the same conditions, but only to a small extent (Table 5). This may mean that either the glyceraldehyde dehydrogenase or the necessary phosphorylating enzymes have been inactivated by the treatment in preparing the extract. From the work of

Table 5. Aerobic oxidation of glyceraldehyde.
Additions to Warburg manometers (ml.)

Enzyme	1.0	1.0	1.0	1.0	1.0	1.0
Phosphate <i>M</i> /5 pH 7.7	—	0.5	—	—	—	—
Arsenate <i>M</i> /2 pH 7.7	—	—	0.2	0.2	0.2	0.2
Coenzyme F 5 mg./ml. 27% pure	0.2	0.2	0.2	—	0.2	0.2
Methylene blue 0.5%	0.5	0.5	0.5	0.5	—	0.5
Glyceraldehyde 1.5%	0.2	0.2	0.2	0.2	0.2	—
Sodium fluoride <i>M</i>	0.1	0.1	0.1	0.1	0.1	0.1
Water	1.0	0.7	1.0	1.2	1.5	1.2
Oxygen uptake μl. in 20 min.	15	22.5	32	16	10.5	8.6

Warburg and Christian, however, it would seem that glyceraldehyde is slowly oxidized by the phosphoglyceraldehyde enzyme, and these authors do not attach much physiological significance to the oxidation of glyceraldehyde.

The aerobic oxidation of 3-phosphoglyceraldehyde to 3-phosphoglyceric acid by the enzyme system in the presence of arsenate is complete (Fig. 1). NaF was added to inhibit the conversion of phosphoglyceric acid into phosphopyruvic acid.

V. Mechanism of reaction

The spectrophotometric method was used to show the oxidation and reduction of coenzyme I in the system Green & Dewan [1937]. The reduction of coenzyme I could be demonstrated only in the presence of arsenate (Table 6).

Reduction of coenzyme I. Five Thunberg tubes were set up as shown in Table 6. The contents were incubated at 38° for 20 min., brought to the boil, left in the ice box for 1 hr. and centrifuged at 12,000 r.p.m. for 20 min. 3.0 ml. of supernatant were made up to 4.5 ml. Samples from tubes 1, 2, 3 and 4 were examined in the "Spekker" spectrophotometer using a sample from tube 5 as control.

Table 6. *Reduction of coenzyme I*

	Additions to tubes (ml.)				
	1	2	3	4	5
Enzyme	1.0	1.0	1.0	—	1.0
Enzyme boiled	—	—	—	1.0	—
Phosphate <i>M</i> /15 pH 7.4	—	1.0	1.0	1.0	1.0
Coenzyme I 5 mg./ml. 14% pure	1.0	1.0	1.0	1.0	—
Hexosediphosphate <i>M</i> /20	1.0	1.0	1.0	1.0	1.0
Arsenate <i>M</i> /10	—	—	0.5	0.5	0.5
Water	2.0	1.0	0.5	0.5	1.5
Log I_0/I at 345 m μ .	0.0	0.1	1.1	0.0	—

Oxidation of reduced coenzyme I. Reduced coenzyme I was prepared using lactic dehydrogenase of pig's heart. The heart was freed from fat, ground in a Latapie mincer and left on ice with 3 vol. of water for 30 min. The suspension was strained through muslin and centrifuged. The supernatant was made half saturated with ammonium sulphate and the resulting precipitate washed with half saturated ammonium sulphate. This precipitate was dissolved in water and dialysed for 16 hr. against running water. Insoluble material was removed by centrifuging.

1.0 ml. lactic enzyme, 5.0 mg. coenzyme I (14% pure), 0.3 ml. 2 *M* cyanide, 0.3 ml. 2 *M* sodium lactate and 1.4 ml. *M*/10 phosphate buffer pH 7.2 were incubated in a Thunberg tube for 30 min. Contents were boiled, 2.0 ml. water added and the mixture filtered through Whatman No. 40 paper. Filtrate was diluted 1 in 1 and examined spectrophotometrically. Log I_0/I at 345 m μ . = 1.3.

3.0 ml. reduced coenzyme I prepared as above, 1.0 ml. bacterial enzyme and 0.5 ml. *M*/10 phosphoglycerate were incubated anaerobically for 30 min. Contents were boiled, diluted with 1.5 ml. water and centrifuged. The supernatant examined spectrophotometrically gave log I_0/I at 345 m μ . 0.2. Control with boiled enzyme, log I_0/I 1.2.

Thus if 1:3-diphosphoglyceric acid is the first product of the oxidation, then the juice contains the system which phosphorylates 3-phosphoglyceric acid. According to Warburg & Christian [1939] neither 3-phosphoglyceric acid, 2:3-diphosphoglyceric acid nor phosphopyruvic acid affects the equilibrium between oxidized and reduced coenzyme I in the case of the purified yeast enzyme.

VI. *Kinetics*

Figs. 2, 3, 4 and 5 indicate how the reaction with molecular O_2 varies with the concentrations of four of the components of the system: coenzyme I (Fig. 2),

Fig. 2.

Fig. 3.

Fig. 2. Effect of concentration of coenzyme I. Manometers containing 1.0 ml. enzyme, 0.32 ml. $M/10$ arsenate, 0.1 ml. M NaF, 0.5 ml. 0.5 % methylene blue, 0.4 % ml. $M/22$ hexosediphosphate. Total volume 3.2 ml.

Fig. 3. Effect of concentration of substrate. Manometers containing 1.0 ml. enzyme, 0.2 ml. $M/2$ arsenate, 1 mg. coenzyme I, 0.5 ml. 0.5 % methylene blue, 0.1 ml. M NaF. Total volume 3.2 ml.

Fig. 4. Effect of concentration of carrier. Manometers containing 1.0 ml. enzyme, 0.32 ml. $M/10$ arsenate, 0.4 ml. $M/22$ hexosediphosphate, 0.1 ml. M NaF, and 1 mg. coenzyme I. Total volume 3.2 ml.

substrate (Fig. 3), carrier (Fig. 4) and arsenate (Fig. 5). Over relatively small ranges of concentration the rate of oxidation was proportional to the concentration. In the case of arsenate larger concentrations were found to be inhibitory.

For the anaerobic reaction in Thunberg tubes pH 7.4 was found to be optimum reaction. The behaviour of the uptake of molecular O_2 within the phosphate buffer range is shown in Fig. 6.

Fig. 5.

Fig. 6.

Fig. 5. Effect of concentration of arsenate. Manometers containing 1.0 ml. enzyme, 1 mg. coenzyme I, 0.5 ml. 0.5 % methylene blue, 0.4 ml. *M*/22 hexosediphosphate and 0.1 ml. *M* NaF. Total volume 3.2 ml.

Fig. 6. Effect of pH . Manometers containing 0.5 ml. enzyme, 1.2 ml. *M*/5 phosphate buffer, 0.4 ml. *M*/22 hexosediphosphate, 0.5 ml. 0.5 % methylene blue, 1 mg. coenzyme I and NaF. Total volume 3.2 ml.

Effect of iodoacetate. Inhibition of oxidation of phosphoglyceraldehyde by iodoacetate has been observed in yeast [Dixon, 1937] and in muscle [Green *et al.* 1937]. As the inhibitor is competing with the substrate the effect can be enhanced if the enzyme and inhibitor are incubated together first. The bacterial enzyme also behaves in this way (Table 7).

Table 7. *Effect of iodoacetate*

Figures refer to % inhibition

	<i>M</i> /10,000	<i>M</i> /2000	<i>M</i> /1000	<i>M</i> /100
No incubation	0	27	57	100
Incubation for 10 min.	0	69	75	100

VII. *Oxidation of phosphoglyceraldehyde by oxaloacetate*

The enzyme preparation contained also malic dehydrogenase, so that a coenzyme-linked reaction between phosphoglyceraldehyde and oxaloacetate was possible:

The reaction was followed by the liberation of CO_2 from $NaHCO_3$ - CO_2 buffer, an acid being produced from an aldehyde. Table 8 and Fig. 7 demonstrate the occurrence of this reaction and its components.

Table 8. *Oxidation of phosphoglyceraldehyde by oxaloacetate.*
Addition to manometers (ml.)

Enzyme	1.0	1.0	1.0	1.0	1.0	1.0
NaHCO ₃ <i>M</i> /10	0.8	0.8	0.8	0.8	0.8	0.8
NaF <i>M</i>	0.1	0.1	0.1	0.1	0.1	0.1
Hexosediphosphate <i>M</i> /20	0.4	0.4	0.4	—	0.4	0.4
Oxaloacetate 12.5 mg./ml.	0.4	0.4	0.4	0.4	0.4	—
Coenzyme I 5 mg./ml. 27% pure	0.2	0.2	0.2	0.2	—	0.2
Arsenate <i>M</i> /10	—	—	0.32	0.32	0.32	0.32
Phosphate <i>M</i> /10	—	0.32	—	—	—	—
Water	0.3	0.02	0.02	0.38	0.12	0.32
μl. CO ₂ in 10 min.	33.5	38.5	421	16	19.5	45.5

Fig. 7. Effect of arsenate (*A*) and phosphate (*B*) on the oxidation of phosphoglyceraldehyde by oxaloacetic acid. Manometers contained 1.0 ml. enzyme 0.8 ml. *M*/10 NaHCO₃, 0.2 ml. *M*/22 hexosediphosphate, 1 mg. coenzyme I, 0.1 ml. *M* NaF and 2.5 mg. oxaloacetic acid. Total volume 3.2 ml. Gas space N₂ 5% CO₂.

This oxido-reduction is also inhibited by iodoacetate as shown in Table 9. The enzyme used in this experiment had not been dialysed free of phosphate, and arsenate was not added.

Table 9. *Effect of iodoacetate on oxido-reduction*

Manometers contained: 1.0 ml. enzyme, 0.3 ml. hexosediphosphate 0.057 *M*, 0.8 ml. *M*/10 NaHCO₃, 1 mg. coenzyme I 6.8% pure, 2.2 mg. oxaloacetic acid. Total volume 3.2 ml. in Barcroft manometers. Iodoacetate was added from Keilin cups.

Complete system	μl. CO ₂ /16 min.
" " - oxaloacetate	208
" " + <i>M</i> /100 iodoacetate	21
" " " " - oxaloacetate	15
" " " " " "	20

VIII. *Dismutation of phosphoglyceraldehyde*

The enzyme preparation dismuted phosphoglyceraldehyde (Table 10). Presumably the reaction is:

Comparison of columns 3 and 6 in Table 8 shows that under identical conditions the rate of dismutation is about one-tenth the rate of oxido-reduction when oxaloacetic acid is the acceptor [Green *et al.* 1937].

Table 10. *Dismutation of phosphoglyceraldehyde by enzyme.*
Additions to manometers (ml.)

Enzyme	1.0	1.0	1.0	1.0
NaHCO ₃ M/10	0.8	0.8	0.8	0.8
NaF M	0.1	0.1	0.1	0.1
Hexosediphosphate M/20	0.4	0.4	0.4	—
Coenzyme I 5 mg./ml. 27% pure	0.2	0.2	—	0.2
Arsenate M/10	0.32	—	0.32	0.32
Phosphate M/10	—	0.32	—	—
Water	0.38	0.38	0.58	0.78
μ l. CO ₂ in 30 min.	95	27	2	10

IX. *Product of oxidation*

In § IV it was shown that the manometric data corresponded to an uptake of one atom of oxygen per molecule of phosphoglyceraldehyde, i.e. an oxidation to phosphoglyceric acid. In an experiment similar to that in Table 8 it was shown that difficultly hydrolysable P accumulated in the presence of NaF. Phosphate not hydrolysed by 3 hr. boiling with *N* HCl was considered as phosphoglyceric acid. As arsenate was present the method of Pett [1933] was used in the estimation of phosphate. Of the acid formed 85% could be accounted for as phosphoglyceric acid. In the absence of fluoride no difficultly hydrolysable phosphate accumulated.

DISCUSSION

Recently Wiggert *et al.* [1940] have described the preparation of a cell-free juice from certain bacteria by grinding the cells with finely powdered pyrex glass. Their preparation from *Escherichia coli* when shaken with glucose and hexosediphosphate in NaHCO₃-CO₂ buffer produced acid, the amount of which was increased by addition of boiled yeast extract. Acetone preparations of *Bact. coli* carry out this reaction with catalytic amounts of hexosediphosphate and in the presence of NaF phosphoglyceric acid can be demonstrated as a reaction product [Endo, 1938].

It is now established that *Bact. coli* contains two enzymes similar to if not identical with the reducing and the oxidizing enzymes of yeast fermentation, alcohol and triosephosphate dehydrogenases. Endo [1938] concluded that the same coupled reactions and the same phosphorylated intermediate products occurred in *Bact. coli* fermentation as in yeast fermentation. The oxidizing enzyme probably plays the same part in the two processes; i.e. the oxidation of phosphoglyceraldehyde to phosphoglyceric acid. The origin of acetaldehyde to act as substrate for the reducing enzyme is not clear. However, according to Tikka [1935] the alcohol resulting from the fermentation arises from α -glycerophosphate.

Pyruvic acid is the parent substance of alcohol in yeast fermentation. From the results of unpublished work by the author with cell-free preparations of *Bact. coli* it would seem that this organism contains no carboxylase but oxidizes pyruvic acid to acetic acid and CO₂. Inorganic phosphate, cocarboxylase and a heavy metal are involved in the reaction *in vitro*.

SUMMARY

1. A cell-free extract of *Bact. coli commune* containing zymohexase and phosphoglyceraldehyde dehydrogenase has been made.

2. As coenzyme I and either inorganic phosphate or arsenate are required for the oxidation of phosphoglyceraldehyde the following course of reaction is assumed in the presence of fluoride:

3-phosphoglyceraldehyde $\rightarrow$ 1:3-diphosphoglyceraldehyde $\rightarrow$
1:3-diphosphoglyceric acid $\rightarrow$ 3-phosphoglyceric acid.

3. The reaction is shown to be reversible with respect to the reduction and reoxidation of coenzyme I.

4. The kinetics of this reaction where molecular oxygen is the oxidant have been investigated.

5. Phosphoglyceraldehyde dehydrogenase is inhibited by iodoacetate.

6. As malic dehydrogenase is also present in the enzyme preparation, the phosphoglyceraldehyde can be oxidized in a coenzyme-linked reaction with oxaloacetic acid.

7. The enzyme preparation will dismute phosphoglyceraldehyde under suitable conditions.

8. The product of oxidation was not isolated but evidence is given that phosphoglyceric acid is the main or only product.

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REFERENCES

- Booth & Green (1938). *Biochem. J.* **32**, 855.
Dixon (1937). *Nature, Lond.*, **140**, 806.
Endo (1938). *Biochem. Z.* **296**, 56.
Green & Brosteaux (1936). *Biochem. J.* **30**, 1489.
— Needham & Dewan (1937). *Biochem. J.* **31**, 2327.
— & Dewan (1937). *Biochem. J.* **31**, 1069.
Herbert, Gordon, Subrahmanyam & Green (1940). *Biochem. J.* **34**, 1108.
Lohmann & Jendrassik (1926). *Biochem. Z.* **178**, 419.
Pett (1933). *Biochem. J.* **27**, 1672.
Still (1940). *Biochem. J.* **34**, 1177.
Tikka (1935). *Biochem. Z.* **279**, 264.
Warburg & Christian (1939). *Biochem. Z.* **303**, 40.
Wiggert, Silverman, Utter & Werkman (1940). *Iowa State Coll. J. Sci.* **14**, 179.