139. ALCOHOL ENZYME OF *BACT. COLI*

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CELL-free extracts of *Bact. coli* were found to contain a highly active alcohol enzyme requiring coenzyme I. This investigation deals with the properties of this enzyme. These are very similar to those of the alcohol enzyme isolated by Negelein & Wulff [1937] from yeast and would suggest the probable identity of the two enzymes.

I. Preparation of the enzyme

The organism used was *Bact. coli commune* Escherich (National Collection of Type Cultures No. 86). It was grown and maintained on agar to which was added tryptic digest of casein and inorganic salts. The cells from 30 hr. growth on 30 Roux bottles were washed off the medium with distilled water and centrifuged. The thick suspension of cells was ground for 2 hr. in the wet-crushing mill [Booth & Green, 1938]. The suspension of crushed cells, volume about 40 ml., was diluted with 60 ml. water and centrifuged for 40 min. at 3000 r.p.m. The supernatant was heated to 55°; cooled immediately and centrifuged for 45 min. at 12,000 r.p.m. The clear yellow supernatant fluid was then dialysed overnight against running water. Such preparations had a dry weight of 8–10 mg. per ml., and when kept on ice retained activity for several weeks.

The activity of the enzyme was measured anaerobically by the Thunberg tube technique, and manometrically with Barcroft and Warburg manometers.

II. Components of the system

The enzyme did not reduce methylene blue in the presence of ethyl alcohol. On addition of coenzyme I reduction took place (Table 1).

Table 1. *Demonstration of alcohol dehydrogenase in extract of Bact. coli*

	Additions to tubes (ml.)			
	1	2	3	4
Enzyme extract	0.5	0.5	0.5	0.5
Phosphate buffer <i>M</i> /15 pH 7.4	1.0	1.0	1.0	1.0
Methylene blue 0.5%	0.1	0.1	0.1	0.1
Ethyl alcohol 9.7%	0.1	0.1	0.1	—
Coenzyme I (14% pure) 5 mg./ml.	0.2	—	0.2	0.2
Semicarbazide 2 <i>M</i>	0.2	0.2	—	0.2
Water	0.5	0.7	0.7	0.6
Reduction time in min.	1½	120	2½	65

Treatment of the enzyme solution in such a way as to destroy coenzyme dehydrogenase diminished the rate of reduction of methylene blue by the enzyme. Addition of milk flavoprotein partially restored this loss in activity (Table 2). The enzyme was precipitated with 5 vol. of acetone and filtered. The precipitate, after washing with acetone and ether, was ground in water and the suspension dialysed overnight against running water. It was then heated at

Table 2. *Effect of coenzyme dehydrogenase on acetone preparation*

	Q_{MB}
Enzyme solution	63.3
Acetone powder	5.3
Acetone powder + milk flavoprotein	25.3

Q_{MB} = μ l. hydrogen transferred to methylene blue per hr. per mg. dry weight of enzyme preparation.

52° for 10 min. and finally centrifuged. The supernatant was then tested under the standard conditions. As a source of coenzyme dehydrogenase milk flavoprotein was used [Corran & Green, 1938].

III. Reaction with molecular oxygen

The system alcohol enzyme, alcohol, coenzyme I and coenzyme dehydrogenase can react with molecular oxygen in the presence of a suitable carrier such as methylene blue. In this reaction a ketone fixative is also required (Table 3). In experiments designed to show the complete oxidation of a known amount of ethyl alcohol to acetaldehyde, the O_2 uptake was never theoretical. There was always a small O_2 uptake in the absence of the substrate. If this blank reaction is assumed to be suppressed in the presence of ethyl alcohol, the observed O_2 uptake would agree with theory (Fig. 1).

Table 3. *Aerobic oxidation of ethyl alcohol by the enzyme*

	Additions (ml.) in Barcroft manometers				
	1	2	3	4	5
Enzyme	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	1.0
Methylene blue 0.5%	0.5	0.5	0.5	—	0.5
Coenzyme I (14% pure) 5 mg./ml.	0.2	—	0.2	0.2	0.2
Ethyl alcohol 10%	0.3	0.3	0.3	0.3	—
Semicarbazide 2 <i>M</i>	0.2	0.2	—	0.2	0.2
Water	—	0.2	0.2	0.5	0.3
O_2 uptake in μ l./10 min.	168	0	54	11	0

Fig. 1. Oxidation of ethyl alcohol to acetaldehyde by enzyme. Manometer *A* contained 1.0 ml. enzyme, 1.0 ml. *M*/15 phosphate buffer pH 7.4, 140 μ g. coenzyme I, 0.2 ml. 2 *M* semicarbazide, 0.4 ml. 0.5% methylene blue and 0.1 ml. 1% ethyl alcohol. Total volume 3.2 ml. Manometer *B* contained no alcohol. Temperature 38°.

IV. *Kinetics*

Fixatives. Semicarbazide, cyanide and hydrazine accelerated the reduction of methylene blue anaerobically. Hydroxylamine was inhibitory. However, cyanide and hydrazine accelerated the blank reduction in the absence of substrate. Accordingly semicarbazide was used where a fixative was required.

pH. The optimum pH for the reduction of methylene blue by alcohol in the presence of the enzyme was found to be 7.4 (Table 4). Borate appeared to inhibit the activity of the enzyme.

Table 4. *Effect of pH on activity*

In Thunberg tubes containing enzyme 0.25 ml., coenzyme I (14% pure) 0.5 mg., buffer *M*/15 2.0 ml. and 0.5% methylene blue 0.1 ml.

pH	Reduction time in min.	pH	Reduction time in min.
5.8	75	7.0	9½
6.4	37	7.4	3
6.8	12½	7.75	4½

Concentrations of substrate, coenzyme I and carrier. The relation of the rate of oxidation to the concentrations of substrate, coenzyme I and methylene blue is shown in Table 5 and Figs. 2 and 3.

Table 5. *The effect of concentration of ethyl alcohol*

In Barcroft manometers. 1.0 ml. enzyme, 1.0 ml. phosphate buffer pH 7.4, 0.2 ml. semicarbazide 2*M*, 0.5 ml. methylene blue 0.5%, 1.0 mg. coenzyme I (14% pure coenzyme) and ethyl alcohol. Total volume 3.2 ml. Temperature 38°.

Alcohol concentration (<i>M</i>)	O ₂ uptake in µl./25 min.
0	9
0.007	47
0.022	63
0.074	149
0.22	141

Fig. 2.

Fig. 3.

Fig. 2. Effect of concentration of coenzyme I. Barcroft manometers containing 1.0 ml. enzyme, 1.0 ml. *M*/20 phosphate buffer pH 7.4, 0.2 ml. 2*M* semicarbazide, 0.5 ml. 0.5% methylene blue, and 0.3 ml. 10% ethyl alcohol (in both cups). Total volume 3.2 ml. Temperature 38°.

Fig. 3. Effect of concentration of carrier. Barcroft manometers containing 1.0 ml. enzyme, 1.0 ml. *M*/20 phosphate buffer pH 7.4, 0.2 ml. 2*M* semicarbazide, coenzyme I 141 µg., 0.3 ml. 10% ethyl alcohol (in both cups). Total volume 3.2 ml. Temperature 38°.

V. *Specificity of substrate*

The enzyme oxidizes readily the lower aliphatic alcohols. Methyl alcohol is oxidized only slowly (Table 6).

Table 6. *Various alcohols as substrate*

Using Thunberg tubes containing 1.0 ml. enzyme, 1.0 ml. phosphate buffer *M*/15 pH 7.4, 0.1 ml. methylene blue 0.5%, 0.2 ml. semicarbazide 2 *M*, 1.0 mg. coenzyme I (14% pure coenzyme) and substrate 0.1 ml.

Substrate	Reduction time in min.
—	60
Methyl alcohol	17
Ethyl alcohol	1½
Propyl alcohol	1½
<i>n</i> Butyl alcohol	2
Amyl alcohol	5
Methyl and ethyl alcohols	2
Glycerol	52

VI. *Mechanism of reaction*

Coenzyme I and coenzyme dehydrogenase have been shown to be components of the system. By analogy with the yeast enzyme one would expect the coenzyme I to undergo a cycle of reduction by alcohol and oxidation by acetaldehyde. This was found to be the case with the bacterial enzyme also.

Reduction of coenzyme I. 1.5 ml. enzyme, 0.2 ml. semicarbazide, 1.0 ml. *M*/20 phosphate buffer pH 7.4, 0.1 ml. 97% ethyl alcohol, 2.2 ml. water and 5.0 mg. coenzyme I (14% pure coenzyme) were incubated in a Thunberg tube for 30 min. at 37°. Similar tubes, one without cozymase and one with enzyme previously boiled, were also incubated. The contents of the tubes were brought to boiling and filtered. 3.0 ml. of the filtrate in each instance were made up to 4.5 ml. with water and examined in the Hilger "Spekker" apparatus [cf. Dewan & Green, 1937]. For the complete system $\log I_0/I$ at 345 m μ was 1.7. The control with boiled enzyme gave $\log I_0/I = 0.5$ at 345 m μ .

Reoxidation of reduced coenzyme I. Gale & Stephenson [1939] showed that malic dehydrogenase is extracted from *Bact. coli* by crushing and resides mainly in the liquid fraction of the crushed cells. It survives further treatment and occurs in the enzyme solution used in the present investigation. Reduced coenzyme I was prepared by means of this enzyme. 1.5 ml. of enzyme, 2.0 ml. *M*/20 phosphate buffer pH 7.4, 1.0 ml. water, 0.4 ml. 2 *M* potassium cyanide, 1.1 ml. *M* sodium malate and 15 mg. coenzyme I (14% pure coenzyme) were incubated in a Thunberg tube for 30 min. A similar tube without coenzyme I was also set up. The contents of both tubes were boiled and filtered. The filtrate was diluted 1 in 4 and examined as above. $\log I_0/I = 1.7$ at 345 m μ .

The reoxidation of reduced coenzyme I by the alcohol enzyme was demonstrated as follows: 1.5 ml. enzyme, 1.25 ml. water, 1.25 ml. reduced coenzyme I and 1.0 ml. *M* acetaldehyde were added to a Thunberg tube. Two other tubes, one without reduced coenzyme I and one with boiled enzyme, were also set up. The tubes were incubated for 30 min. at 38°, the contents boiled and filtered. The filtrate was examined as before. For the complete system $\log I_0/I = 0.2$ at 345 m μ . The control with boiled enzyme gave $\log I_0/I = 1.4$ at 345 m μ .

VII. *The product of oxidation*

The product of oxidation was isolated from a reaction mixture made up of the following: 23 ml. enzyme, 10 ml. 2 *M* semicarbazide, 25 ml. *M*/15 phosphate buffer, 50 mg. coenzyme I (14% pure) and 1.3 ml. 96% ethyl alcohol. This mixture was incubated at 38° for 2 hr. during which it was aerated with a stream of oxygen. Protein was removed with colloidal iron and methylene blue removed by filtration through kieselguhr. 1 g. 2:4-dinitrophenylhydrazine was added to the filtrate together with enough HCl to make the final concentration normal. The solution became opalescent immediately. After 24 hr. standing at room temperature a yellow precipitate was filtered off and recrystallized once from absolute alcohol and twice from ethyl acetate and ligroin. The yellow crystals obtained gave M.P. 161° decomp. The known derivative from acetaldehyde melted at 160° with decomposition. Mixed melting point 160°. *Analysis* (Weiler): C=44.27%; H=3.42%; N=25.40%. Calculated for $C_8H_8N_4O_4$: C=44.44%; H=3.70%; N=25.93%.

VIII. *Inhibition by iodoacetate*

Dixon [1937] found that *M*/1000 iodoacetate inhibits completely the yeast alcohol dehydrogenase. Lutwak-Mann [1938] found that the aerobic oxidation of ethyl alcohol by *Acetobacter suboxydans* was unaffected by prolonged incubation with *M*/100 iodoacetate and the anaerobic oxidation only slightly affected. In the case of the cell-free enzyme of *Bact. coli* incubation with *M*/500 iodoacetate for 5 min. caused 75% inhibition of the activity.

IX. *Oxidation of alcohol by washed suspensions of Bact. coli*

Cook & Stephenson [1928] observed that a strain of *Bact. coli* oxidized acetate, the O_2 consumption being $\frac{2}{3}$ of that required for the complete oxidation to CO_2 . The organism did not oxidize ethyl alcohol or acetaldehyde under the same conditions. Dismutation of acetaldehyde was observed.

Table 7 shows that washed suspensions of the organism used in this investigation oxidized alcohol anaerobically. Aerobically, the washed suspensions took up O_2 in the presence of phosphate and either ethyl alcohol or acetaldehyde. Dismutation of acetaldehyde in bicarbonate- CO_2 buffer at pH 7.4 could not be observed.

Table 7. *Demonstration of alcohol oxidation by washed suspensions of Bact. coli*

	Additions to tubes (ml.)			
	1	2	3	4
Bacterial suspension 9 mg./ml. dry weight	1.0	1.0	1.0	1.0
Phosphate buffer <i>M</i> /15 pH 7.4	1.0	1.0	1.0	1.0
Methylene blue 0.5%	0.2	0.2	0.2	0.2
Ethyl alcohol 97%	0.1	0.1	0.1	—
Coenzyme I (14% pure) 5 mg./ml.	0.2	0.2	—	0.2
Semicarbazide 2 <i>M</i>	0.2	—	0.2	0.2
Water	—	0.2	0.2	0.1
Reduction time in min.	1½	2¼	2	150

The addition of coenzyme I to suspensions of whole cells made no appreciable difference to the rate of reduction of methylene blue in the presence of alcohol. *Bact. coli* has been shown to contain coenzyme I [Gale & Stephenson, 1939].

SUMMARY

1. A cell-free preparation of alcohol dehydrogenase has been made from *Bact. coli commune*.

2. The enzyme oxidizes ethyl alcohol in the following system: ethyl alcohol—alcohol dehydrogenase—coenzyme I—coenzyme dehydrogenase—methylene blue—oxygen.

3. The effect of variation in the concentration of these components, the specificity of substrate and the effect of fixatives have been investigated.

4. The reoxidation of reduced coenzyme I by the enzyme in the presence of acetaldehyde demonstrates that the enzyme is reversible.

5. The product of the oxidation is acetaldehyde which has been isolated as the 2:4-dinitrophenylhydrazone.

6. The enzyme is inhibited by iodoacetate.

7. The whole organism oxidizes both ethyl alcohol and acetaldehyde. The cell-free preparation does not oxidize acetaldehyde.

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