LXXVII. ON LACTIC DEHYDROGENASE¹.

A CELL-FREE ENZYME PREPARATION OBTAINED FROM BACTERIA.

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Preliminary observations.

DURING an attempt to correlate the dehydrogenating activity of washed bacterial suspensions of *B. coli* with the number of living bacteria present, the rates of reduction of methylene blue by four hydrogen donors (glucose, succinate, formate and lactate) in the presence of the bacterial suspension were plotted at intervals against the viable count. It was soon found that, though the rates of reduction by the first three donors fell off continuously with the age of the suspension, that of the lactate first fell off, then became stationary and finally rose. This led to a search in the filtrate for a dehydrogenase which should activate lactate in preference to the other three substances. Strong evidence for the existence of this was forthcoming, but it was found that the dehydrogenase was better prepared by allowing thick bacterial suspensions to autolyse at 37°; finally the following method of preparation was adopted.

Preparation of the enzyme.

Ten Petri dishes containing tryptic broth agar (p_H 7.6) with the addition of 0.5 % sodium lactate were thickly sown with a 20-hour culture of *B. coli* and incubated at 37° for 48 hours. The growth was then washed off with water slightly buffered and the suspension centrifuged and washed twice and finally suspended in 80 cc. of *M*/2 phosphate buffer at p_H 7.6 and incubated in a stoppered bottle for 5 or 6 days. The suspension was then centrifuged and the activating power of the supernatant liquid towards the four hydrogen donors tested. In this and all subsequent experiments the following method was adopted. 1 cc. of phosphate buffer p_H 7.6, 1 cc. of 1 in 5000 methylene blue, 1 cc. of water, 1 cc. of *M*/20 donor and 1 cc. of the enzyme preparation were put in a Thunberg tube, which was evacuated and put in a water-bath at 40°, and the reduction time was noted. In subsequent experiments this procedure will be assumed and the donor and reduction time alone recorded.

	Glucose	Succinate	Formate	Lactate
Reduction time	∞	2 hrs.	6 mins.	2½ hrs.

¹ A short summary of this work appeared in *Chemistry and Industry*, Nov. 11, 1927.

In order to compare the reducing powers of the cell debris and the supernatant liquid respectively, the preparation was again centrifuged and the deposit made up to the original volume with phosphate buffer, and the dehydrogenating power compared with the supernatant liquid.

			Lactate
Reduction time	deposit		2 hrs.
„ „	supernatant liquid		8 mins.

This observation makes it highly improbable that the reducing power of the preparation is vested in the solid phases of the cell. Further experiments showed that the addition of 1 % sodium fluoride to the phosphate buffer in which the cell suspension was autolysed was useful in preventing the deterioration of the preparation through putrefaction, and was found not to alter the properties of the enzyme in any of the particulars described.

Although by repeated centrifuging it is possible to obtain a preparation of lactic dehydrogenase which is, as far as other common donors are concerned, specific for lactate, this is more rapidly effected by filtering through kieselguhr after centrifuging once or twice; by this means, traces of other dehydrogenases, particularly that acting on formate, are held back. For example, an autolysed cell suspension was centrifuged and the supernatant fluid passed twice through kieselguhr on a Büchner funnel; the preparation was almost water-clear. The comparison of the dehydrogenating power of this preparation (IX liquid) with that of the solid material centrifuged from it and diluted till it was of the same activity towards lactate (IX solid) and also with that of a suspension of washed bacteria illustrates the result of the treatment.

						Reduction time of			
						Glucose	Succinate	Formate	Lactate
						mins.	mins.	mins.	mins.
Cell suspension	...	...	...	...	...	15	62	24	8
Prep. IX solid	...	...	...	...	...	∞	35	7	6
Prep. IX liquid	(after 1st filtration through kieselguhr)					∞	∞	∞	6
„ „	(after 2nd filtration through kieselguhr)					∞	∞	∞	8½

The appearance of the preparation at this stage is either very faintly opalescent or even water-clear. Seen under the microscope, using dark ground illumination, the preparation is found to be quite cell-free though very fine remnants of cell debris may still be visible; although these are reduced by centrifuging, it has not been found possible to eliminate the last traces; as stated earlier, however, the activity of the preparation does not seem to be associated with their presence. Passage through a porcelain filter, however, completely inactivates the enzyme. The enzyme may be concentrated by saturation with ammonium sulphate, being totally carried down with the protein precipitate; the total activity is, however, thereby somewhat decreased.

Exp. 30 cc. of the enzyme preparation were saturated with ammonium sulphate at p_H 7.0 and the whole was centrifuged; the supernatant liquid was poured off and the precipitate redissolved in 10 cc. of buffer p_H 7.6.

				Reduction time with lactate
Original preparation	...	...	...	13 mins.
Supernatant liquid	...	...	...	∞
Redissolved precipitate (original conc. 3)				7 mins.

Specificity.

This enzyme reduces lactate; a number of hydrogen donators activated by suspensions of *B. coli* [Quastel and Whetham, 1925, 1, 2] have been tested and found negative; the following are among those tried: glucose, succinate, formate, acetate, glycerol, malate, alanine, ethyl alcohol and acetaldehyde. In their work on associated systems of enzymes in bacterial suspensions, obtained by treatment of the suspensions by various cell poisons, it was found by Quastel and Wooldridge [1927] that the dehydrogenation of lactate was usually associated with that of α -hydroxybutyrate; on their suggestion, this substance was tested with the lactic dehydrogenase and was found to be slowly dehydrogenated. As compared with lactate the process was much slower and it was found necessary to use the α -hydroxybutyrate in ten times the concentration of the lactate, and even then the reduction took about four times as long.

Exp.

Donator	Conc. in Thunberg tube	Reduction time mins.
Lactate	<i>M</i> /100	11
Hydroxybutyrate	<i>M</i> /5	42
"	<i>M</i> /10	48
"	<i>M</i> /20	68
"	<i>M</i> /40	192

These slow rates suggested that the reduction might be due to traces of lactic acid present as impurity; the α -hydroxybutyric acid was therefore distilled at 1 mm. pressure¹. The first fraction distilling at about 120° was collected and the distillation stopped when the temperature reached 125°. The distillate and residue were then dissolved and neutralised, and the reduction times of a series of dilutions compared.

Exp.

Hydroxybutyrate. Concentration	Reduction time		
	<i>N</i> /5	<i>N</i> /10	<i>N</i> /20
	mins.	mins.	mins.
Distillate	29	42	63
Residue	33	48	68

These inconsiderable differences do not suggest that the reduction is due to lactate which—if present—should be concentrated in the distillate.

Behaviour towards Clark's indicators.

In order to determine, if possible, the position of the reducing system on the oxidation reduction potential scale, solutions of Clark's series of indicators

¹ For suggesting this procedure and for help in carrying it out I must thank Mrs Needham.

(kindly supplied to me by Dr Dixon) were made in concentrations equivalent to 1/5000 methylene blue and the reduction times with the enzyme and lactate compared.

Indicator	No.	Reduction time	Order of reduction		r_H range of indicator
			Lactic de-hydrogenase	Xanthine oxidase	
Indigotindisulphonate	1	About 2 hrs.	7	7	7.2-11.2
Indigotintrisulphonate	2	" 5 "	8	8	8.3-12.3
Indigotintetrasulphonate	3	> 5 "	9	9	10 -14
Methylene blue	3 a	13½ mins.	—	—	13 -17
1-Naphthol-2-sulphonate- dichloro-indophenol	4	106 "	6	6	15.2-19.2
1-Naphthol-2-sulphonate- indophenol	5	75 "	5	5	15.4-19.4
o-Cresol-2 : 6-dichloro-indophenol	6	12 "	3	4	17.3-21.3
o-Cresolindophenol	7	14 "	4	2	17.7-21.7
2 : 6-Dibromophenolindophenol	8	7½ "	2	3	18.3-22.3
o-Chlorophenolindophenol	9	6 "	1	1	19 -23

It is seen that all the indicators were in time completely reduced and that, with the exception of Nos. 1, 2 and 3, the order closely follows that which would be predicted from the position of the indicators on the r_H scale; the behaviour of xanthine oxidase is appended for comparison [Dixon, 1926]; the r_H range therefore lies outside that of any of these indicators, *i.e.* below 7.

Behaviour towards molecular oxygen.

Whereas the enzyme rapidly transfers hydrogen to the reducing dyes, its behaviour towards oxygen is otherwise. Thus enzyme + buffer + lactate (same quantities as used in the Thunberg tubes), if placed in a small wash-bottle through which a stream of air is passed, produces no pyruvate as shown by the nitroprusside test; if, however, methylene blue be added to the system, rapid oxidation of the lactate occurs and pyruvate is formed. This is shown by first removing the methylene blue by shaking with kieselguhr and filtering and then applying the nitroprusside test.

The observation may be confirmed by a study of actual oxygen uptakes. For this purpose the Barcroft differential manometer was employed with the flat-bottomed cups used in tissue oxidation work, with 40 % potash in the vertical tubes.

Exp.

Left-hand cup

1 cc. buffer p_H 7.6
1 cc. enzyme
1 cc. water

Fig. 1. Curve 1.

Right-hand cup

1 cc. buffer
1 cc. enzyme
1 cc. 0.012*M* sodium lactate

Fig. 1. Curve 2.

Left-hand cup

1 cc. buffer with 1/5000 meth. blue
1 cc. enzyme
1 cc. water

Right-hand cup

1 cc. buffer with 1/5000 meth. blue
1 cc. enzyme
1 cc. 0.012*M* sodium lactate

Fig. 1 shows the absence of uptake in the system enzyme + lactate and the positive uptake when methylene blue is added; the calculated uptake for the change of lactate to pyruvate is shown by the horizontal dotted line at 137 mm.³

Fig. 1.

----- Oxygen uptake by enzyme + 1 cc. 0.012 *M* sodium lactate.
 ——— Oxygen uptake of same system + 1 cc. 1/5000 methylene blue.

Fig. 2. Effect of cyanide.

————— Oxygen uptake without cyanide. } Enzyme + 1 cc. 0.012 *M* Na lactate + 1 cc.
 - - - - - Oxygen uptake with cyanide. } 1/5000 methylene blue.

It thus appears that this enzyme is a pure dehydrogenase, that is to say, able to catalyse the transfer of hydrogen to an acceptor already activated but powerless to do so to molecular oxygen except by the aid of an intermediary; this property agrees with its behaviour towards cyanide.

<i>Exp.</i>	Left-hand cup (1)	Fig. 2. Curve 1.	Right-hand cup (2)
	1 cc. buffer in 1/5000 meth. blue		1 cc. buffer in meth. blue
	1 cc. enzyme		1 cc. enzyme
	1 cc. water		1 cc. 0.012 <i>M</i> sodium lactate

Fig. 2. Curve 2.

Same as Curve 1 with 0.001*M* KCN in both cups.

From Fig. 2 it is seen that the system enzyme + methylene blue + lactate is not appreciably inhibited by cyanide.

It is interesting to compare the behaviour of the cell-free lactic dehydrogenase with the same system in the cell suspensions¹. For this purpose a suspension of washed bacteria (*B. coli*) was prepared in buffer and diluted so as to reduce methylene blue in the presence of lactate in the same time as an equal volume of cell-free enzyme and the oxygen uptake of the two preparations in the presence of lactate compared.

<i>Exp.</i>	Cup 1	Fig. 3. Curve 1.	Cup 2
	1 cc. buffer		1 cc. buffer
	1 cc. bacterial suspension		1 cc. bacterial suspension
	1 cc. water		1 cc. 0.012 <i>M</i> sodium lactate

	Cup 1	Fig. 3. Curve 2.	Cup 2
	1 cc. buffer in 1/5000 meth. blue		1 cc. buffer in 1/5000 meth. blue
	1 cc. enzyme		1 cc. enzyme
	1 cc. water		1 cc. 0.012 <i>M</i> sodium lactate

	Cup 1	Fig. 3. Curve 3.	Cup 2
	1 cc. buffer in 1/5000 meth. blue		1 cc. buffer in 1/5000 meth. blue
	1 cc. bacterial suspension		1 cc. bacterial suspension
	1 cc. water		1 cc. 0.012 <i>M</i> sodium lactate

It is seen that in the case of the washed cell preparation (Curve 1) the uptake is rather more rapid and much more prolonged than with the enzyme (Curve 2), and that it continues long past the point at which the lactate is oxidised to pyruvate. On adding methylene blue to the cell suspension (Curve 3), no acceleration occurs, but a slight retardation and a stoppage slightly higher than in the case of the enzyme preparation + methylene blue, at a point approximating to the oxidation of lactic to pyruvic acid. The washed cell therefore differs in its behaviour towards lactate from the cell-free enzyme in being able to transfer hydrogen to oxygen direct; the addition of methylene blue decreases instead of, as in the case of the enzyme, conferring this power, and would appear to check the uptake near the pyruvic stage.

The action of cyanide on the washed cell system confirms the fact that a component of the system is here present which is absent from the cell-free enzyme.

¹ Comparable with "resting bacteria" employed by Quastel and Whetham [1925, 1].

Fig. 3.

- Curve 1. —●—●— Cell suspension + lactate.
 „ 2. —x—x—x Enzyme + lactate + methylene blue.
 „ 3. —○—○—○ Cell suspension + lactate + methylene blue.

Fig. 4.

- Curve 1. —●—●— Cell suspension + lactate.
 „ 2. —■—■—■ Cell suspension + lactate + cyanide.
 „ 3. —○—○—○ Cell suspension + lactate + methylene blue.
 „ 4. —x—x—x Cell suspension + lactate + methylene blue + cyanide.

Exp.

Cup 1

1 cc. buffer
1 cc. water
1 cc. bacterial suspension

Fig. 4. Curve 1.

Cup 2

1 cc. buffer
1 cc. bacterial suspension
1 cc. 0.012*M* sodium lactate

Fig. 4. Curve 2.

Same as Curve 1 + 0.001*M* potassium cyanide.

Fig. 4. Curve 3.

Cup 1

1 cc. buffer in 1/5000 meth. blue
1 cc. bacterial suspension
1 cc. water

Cup 2

1 cc. buffer in 1/5000 meth. blue
1 cc. bacterial suspension
1 cc. water

Fig. 4. Curve 4.

Same as Curve 3 + 0.001*M* potassium cyanide.

It is seen here that 0.001*M* cyanide inhibits the oxygen uptake of the intact cell in the presence of lactate by 80 %; whilst the oxygen uptake of the cell in the presence of methylene blue is only very slightly inhibited by cyanide (3.3 %), thereby resembling the enzyme system. In short, the intact cell possesses a complete oxidation system for lactate which is strongly inhibited by cyanide; the addition of methylene blue to the intact cell cuts down the oxidative mechanism to a point approximating to that of the cell-free enzyme and renders it almost independent of cyanide.

Behaviour of the solid residue after the extraction of the enzyme.

The solid residue after the removal of the enzyme still retains the power actively to dehydrogenate lactate; this power is, however, not specific, formate, for example, being also powerfully activated. The action of fluoride in modifying the power of these various preparations in taking up oxygen needs mention. The presence of fluoride during autolysis appears to have an inhibitive action on the oxygen uptake of the cell residue and it seemed at first as if the same factor might be held accountable for the failure of the enzyme to dehydrogenate to molecular oxygen. As, however, this inability in the enzyme (though not in the residue) exists also in preparations made in the absence of fluoride, this factor cannot be held accountable for the inactivity of the enzyme towards oxygen. An investigation into the action of fluoride in this respect is being undertaken.

Search for the missing factor in the dehydrogenase system.

Since the washed cell possesses some factor, absent from the cell-free dehydrogenase, which enables the hydrogen to be transferred to molecular oxygen it is of interest to discover its nature. The method adopted was to aerate in small wash bottles:

buffer	...	...	...	...	...	1 cc.
enzyme	...	...	...	...	...	1 cc.
0.05 <i>M</i> lactate	...	...	...	...	...	1 cc.
<i>x</i>	...	...	...	...	...	1 cc.

the experiment being controlled by one in which *x* is represented by 1 cc. of

methylene blue (1/5000). Both tubes were aerated at 40° for 3 hours; at the end of that time both contents were separately shaken with kieselguhr to remove the methylene blue from the control tube, filtered, and pyruvate tested for in the filtrate by the nitroprusside test. The search has not yet been successful; during the investigation x has been represented by the following, all of which gave negative results: boiled yeast suspension, boiled suspension of *B. coli*, yeast suspension heated to 65° for 30 minutes, *i.e.* till the dehydrogenases were inactivated to methylene blue, a suspension of *B. coli* similarly treated, nitrate and finally glutathione both in the oxidised and reduced forms.

Comparison with other systems.

These results confirm and extend the views of Fleisch [1924] with regard to the succinic acid dehydrogenase of muscle. Working in a system where washed muscle took the place of bacterial suspensions and succinate replaced lactate he found the oxygen uptake of the system succinate-enzyme totally inhibited by 0.001*M* cyanide and this completely restored—though not at the initial rate—by methylene blue. This led him to postulate a dual enzyme mechanism for the oxidation of succinic acid by oxygen in muscle: (1) a succino-dehydrogenase in the Wieland-Thunberg sense; (2) a catalyst for the activation of oxygen which “may be an independent oxidising enzyme, or it may be iron in the sense of Warburg [1921].” As has been shown, a precisely analogous state of affairs exists in the behaviour of bacterial suspensions towards lactic acid, but in this case the obtaining of a cell-free dehydrogenase unable to reduce molecular oxygen brings additional confirmation to the view of Fleisch on the dual nature of the mechanism, and incidentally assists towards reconciling the opposing views of Wieland and Warburg.

Lactic dehydrogenase differs from another cell-free oxidase, *viz.* the Schardinger enzyme, which, as Dixon [1927] has shown, reduces not only methylene blue but also molecular oxygen without any intermediate mechanism, the process being independent of cyanide (except at such high concentrations that the whole enzyme is destroyed).

SUMMARY.

1. By autolysing washed bacterial suspensions (*B. coli*) in phosphate buffer p_H 7.6 a dehydrogenase is extracted; by repeated centrifuging and filtration through kieselguhr this may be obtained in clear aqueous solution free from the other dehydrogenases of the cell.

2. This preparation in the presence of methylene blue actively dehydrogenates lactate and also, though much more slowly, α -hydroxybutyrate: the author has not identified any other substrate upon which the enzyme acts.

3. This enzyme differs from the intact cell in its behaviour towards lactate, not only in specificity but in the fact that it cannot reduce molecular oxygen except in the presence of a carrier such as methylene blue.

4. The enzyme is unaffected by cyanide.

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