XXXVII. THE REDUCING ENZYME OF *BACILLUS COLI COMMUNIS*.

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A reducing enzyme is fairly widespread in the tissues of animals and plants and has of late received the attention of several workers in the province of enzymic chemistry. The results obtained independently by some of the investigators seem to show that the so-called reductase is not a single principle but a system. Thus Bach [1911] working on the reducing enzyme in the calf's liver was able by treating the tissue with a 2 % solution of sodium fluoride and pressing it through a cloth to obtain an inactive filtrate and a residue which when emulsified with water either did not reduce methylene blue at all or did it extremely slowly. On the addition of 1 % of acetaldehyde to the emulsified residue the reducing activity was restored. Harden and Norris too [1914] showed that dried yeast and zymin lost their power of reducing methylene blue and sodium selenite when washed. This loss of the reducing power they found could be restored by the addition of the washings, bouillon and some aldehydes. Recently [1915] they have observed a similar phenomenon with the reducing enzyme of muscle. Schardinger's observation that the addition of acetaldehyde and formaldehyde to milk imparts to it the power of reducing methylene blue to its leuco-base is another illustration of the complexity of reductase.

The authors find that *B. coli communis* (Escherich) when washed will not reduce methylene blue, but that it acquires the power of reduction on the addition to it of various reagents incapable by themselves of effecting the reduction. This property of the organism in question was investigated and the results obtained form the subject of this communication.

The following technique was adopted in the preparation of the bacterial emulsion used in the experiments. Roux bottles containing nutrient agar were inoculated with a 24 hours' broth culture of *B. coli*. The organism was then grown for 48 hours at 37° after which period it was washed off the agar with saline, a glass scraper being used in order to ensure a more thorough removal of the growth. The resulting emulsion was then centrifuged and after removing the supernatant saline fresh salt solution was added, the organisms stirred up, and again centrifuged. This process of washing was repeated two or three times and the residue was finally diluted with saline (the growth from six Roux bottles to 50 cc. of saline) and shaken up with glass beads in order to obtain an even suspension of the bacilli. The emulsion was then ready for use. With very few exceptions a fresh preparation was made daily. If kept in the cold room, however, the emulsion retained its reducing power for some days.

As already mentioned *B. coli* will not reduce methylene blue when washed in saline unless certain substances are added to it. A great number of reagents were tested for this property and a good many of these yielded positive results. The following list comprises the substances tried.

<i>Positive.</i>	<i>Negative.</i>
Washings	Acetaldehyde
Bouillon	Formalin
Sodium lactate	Lactose
Sodium pyruvate	Sorbitol
Sodium glycerate	Dulcitol
Glucose	Raffinose
Galactose	Erythrol
Laevulose	Rochelle salt
Mannose	Glycocoll
Maltose	Potassium oxalate
Arabinose	Anisaldehyde
Mannitol	Oenanthal
Sodium succinate	Phloroglucinol
Sodium formate	Propaldehyde
Alanine	Isovaleraldehyde
Asparagine	Cinnamyl aldehyde
Xylose	Salicylaldehyde
Inulin	Hydroxybutyric acid
Sodium acetate	Butyl alcohol
Isobutyric acid	Linolenic acid
Globulin	Sodium citrate
Albumin	Hydantoin
Horse serum	Creatine
Peptone (Witte)	Guanine hydrochloride
Liebig's Extract	Pyridine
	Hypoxanthine
	Adenine
	Xanthine
	} In suspension

In carrying out the above experiments the following quantities were used: 1 cc. of the bacterial emulsion, 1 cc. of the reagent, 0.2 cc. methylene blue (5 cc. saturated solution diluted to 200 cc. with water). The mixtures were made up to 5 cc. with saline. The salts were employed in N/10 solution, while the other substances, with the exception of the washings, broth, and horse serum, were in 1 % solutions or suspension. All the reductions were carried out at 35–37°. The time taken to reduce the methylene blue varied with the reagent used. The results were regarded as negative when no reduction was observed within an hour. The addition of broth produced one of the highest rates of reduction; an addition of a very small quantity sufficed to impart the reducing power to the emulsion. This is shown by the following experiment:

Emulsion cc.	Broth cc.	Saline cc.	Methylene blue cc.	Time taken for reduction mins.
1	—	4	0.2	(no reduction)
1	0.1	3.9	0.2	20
1	0.2	3.8	0.2	14.5
1	0.3	3.7	0.2	12
1	0.4	3.6	0.2	11.5

It has been suggested that in the process of reduction water is split into H and O and in order that the reduction should occur, the presence of an acceptor for the oxygen is necessary. The great variety in the chemical character of the reagents in the list which gave positive results does not afford definite information as to the precise chemical properties requisite for an acceptor. Bach [1912] in connection with his work on the reductase in calf's liver, discussing the chemical nature of the acceptor or coferment, as he calls it, suggested the possible presence in all solutions capable of acting as acceptors of aldehydes formed from such degradation products of the metabolism of the proteins as α -amino acids. In support of his suggestion he refers to Strecker's observations that α -amino acids are oxidised at ordinary temperature by alloxan forming aldehydes of a series containing fewer carbon atoms and giving off at the same time CO_2 and NH_3 , a process which might possibly occur in nature. The experiments quoted above however show that in the case of *B. coli* all the aldehydes tried with the exception of some of the sugars are incapable of acting as acceptors under the conditions employed. From the same experiments we can also conclude that the reducing enzyme in the organism in question differs to some extent from those present in the liver of the calf and in yeast, since in the former case according to Bach acetaldehyde can act as acceptor and

in the latter case according to Harden and Norris salicylaldehyde, benzaldehyde, anisaldehyde, and isovaleraldehyde are able to restore the reducing power to washed yeast.

The effect of the addition of increasing quantities of broth was next studied and it was found that the rate of reduction increased at first with the increase in the quantity of broth added until a certain optimum was reached, after which any further increase in the quantity of broth added decreased the rate of reduction. This is illustrated by the following experiment.

Emulsion cc.	Broth cc.	Saline cc.	Methylene blue cc.	Time of reduction mins.
1	—	4	0.2	(not reduced)
1	0.1	3.9	0.2	28.5
1	0.2	3.8	0.2	24
1	0.3	3.7	0.2	22.5
1	0.4	3.6	0.2	19
1	0.5	3.5	0.2	14.5
1	1	3	0.2	15
1	1.5	2.5	0.2	15.5
1	2	2	0.2	18
1	2.5	1.5	0.2	20
1	3	1	0.2	20.5
1	3.5	0.5	0.2	24

This decrease in the rate of reduction produced by increased addition of broth after a certain optimum is an observation of some interest. Palladin [1914] found that the addition of glycerol and pyridine to hefanol produced a deterrent effect on the activity of the reductase in it and that the extent of the retardation varied with the amount of the reagent added. He ascribes the fact to the displacement of the water by the glycerol and pyridine. Pyridine does not act as an acceptor when added to *B. coli*, glycerol on the other hand does, but its behaviour was observed to be similar to that of broth, since it has at first an activating influence which is proportional to the amount added, but after a certain point any increase in the amount of the reagent decreases the rate of reduction. This is shown in the following experiment.

Emulsion cc.	Glycerol 50 % cc.	Saline cc.	Methylene blue cc.	Time of reduction mins.
1	—	4	0.2	(no reduction)
1	1	3	0.2	20
1	2	2	0.2	16.75
1	3	1	0.2	20.5
1	4	—	0.2	23

This experiment and also the one carried out with the broth suggest that both reagents possess an activating, as well as a retarding action on the reductase of *B. coli*. The experiments throw no direct light on the cause of the retarding influence, but at the same time they do not exclude Palladin's explanation. It is quite conceivable in this case that glycerol for instance can act chemically as an acceptor, while as it displaces the water owing to its physical condition it can also retard and even stop the action of the reducing system of which it forms a part (i.e. acceptor). It is also plain from the experimental data that initially the rate of reduction is influenced more by the activating than by the retarding factor, but as the quantities of the reagents added are increased the reverse takes place, and this accounts for the decrease in the rate after a certain amount of the reagent has been added.

It was of further interest to investigate how the time of reduction is influenced by varying the amounts of methylene blue and emulsion respectively, and accordingly experiments were instituted with that object. In the first set the amount of emulsion was kept constant and the quantity of methylene blue changed. In the second set the methylene blue was kept constant and the amount of emulsion changed. The following are the results obtained:

	Emulsion cc.	Broth cc.	Saline cc.	Methylene blue cc.	Time of reduction mins.
1.	1	1	2.9	0.1	19
	1	1	2.8	0.2	21.5
	1	1	2.7	0.3	25
	1	1	2.6	0.4	26.5
	1	1	2.5	0.5	29
2.	0.1	1	3.9	0.2	43
	0.3	1	3.7	0.2	20.5
	0.5	1	3.5	0.2	13
	1	1	3	0.2	7.5
	2	1	2	0.2	5

As it is seen from the figures the time taken to reduce increases as the quantity of methylene blue is increased, while in the case of the varying quantities of emulsion the time decreases with the increase in the amount of emulsion.

No essential difference in the results could be observed when some of the experiments were carried out anaerobically, i.e. by boiling the saline previous to using it and by displacing the atmosphere in the tubes and the dissolved oxygen in the emulsion by nitrogen.

SUMMARY.

1. *B. coli* does not reduce methylene blue when washed and suspended in saline, but does so on addition of certain reagents.

2. A wide variation in chemical nature is exhibited by the reagents capable of acting in this way.

3. The effect of the addition of various amounts of broth and glycerol respectively show that there are two factors concerned in the effect of those reagents which influence the process of reduction, i.e. an activating factor and a retarding one. The former is thought to be chemical while the second is possibly physical.

4. The time of reduction of a certain quantity of methylene blue decreases with increase in the amount of emulsion, while as the amount of methylene blue is increased for a constant amount of emulsion, the time of reduction increases.

REFERENCES.

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