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The Effect of Various Chemical and Physical Agents on the Dehydrogenating Enzymes of *Eberthella typhosa*

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Knowledge of dehydrogenating enzymes has become a useful tool in the study of the nutrition of bacteria. The effect of physical and chemical agents on such dehydrogenation systems has been studied by many workers. Quastel & Wooldridge (1927*a*) found that exposure of *Escherichia coli* to various chemical and physical injuries resulted in considerable and partly selective inhibition of the dehydrogenating mechanisms. Cell destruction produced by repeated freezing and thawing has been studied by Young (1929), Yudkin (1937*b*) and Adler, Hellström, Günther & v. Euler (1938). The selective action of many types of injury forms the basis of the theory of dehydrogenating mechanisms formulated by Quastel and co-workers (Quastel, 1926; Quastel & Wooldridge, 1927*b*), which stresses the importance of the structural integrity of the cell.

The present investigation deals with the dehydrogenating mechanisms of *Eberthella typhosa* and the effect of chemical and physical conditions on these enzymes. Previous experiments on the influence of physical and chemical factors on bacterial dehydrogenases have been made on *Esch. coli*; it seemed, therefore, of interest to study these problems in connexion with a pathogenic micro-organism with different fermentative properties.

GENERAL METHODS

The following strains of *E. typhosa* were used: the motile H901 and Vi2, and the non-motile O901. The bacteria were grown on ordinary nutrient agar at 37°. After 24 hr. incubation the cultures were suspended in saline and washed three times. Longer incubation than 24 hr. decreased the dehydrogenating abilities of the bacteria. For the detection of hydrogen donators the procedure previously described (Guggenheim, 1944) was used, methylene blue serving as H acceptor. In spite of carefully controlled conditions the dehydrogenase content found for individual bacterial suspensions varied. Consequently, each substrate was tested four to five times with each of the strains. The results represent average values.

The velocity of dehydrogenation of the donators by treated bacteria is expressed in arbitrary units related to the velocity of dehydrogenation of the same donators by normal, untreated bacteria, which was taken as 100.

RESULTS

(1) *The dehydrogenating enzymes of* E. typhosa. The bacteria were grown on agar slants of approximately equal size. The cells of each agar tube, after washing, were suspended in 1·5 ml. saline. The results are classified in the following manner:

Very strong donators: Complete dehydrogenation after 0–15 min.

Strong donators: Complete dehydrogenation after 16–60 min.

Weak donators: Complete dehydrogenation after > 60 min.

Not activated: No dehydrogenation.

All experimental series in which the control tube without donator showed dehydrogenation within 6 hr. were rejected; this occurred only rarely. The results obtained with the strain H 901 are given in Table 1.

It is noteworthy that hardly any difference was found between the strains H 901, O 901 and Vi 2, although these strains differed in their serological properties.

(2) *The effect of ultra-violet irradiation.* In the following experiments the influence of ultra-violet irradiation on some dehydrogenating enzymes of the strain H 901 was studied. A thin layer of washed bacteria was irradiated in open Petri dishes at 50 cm. distance from a quartz lamp; the dehydrogenating activities were compared, after irradiation of 1, 2, 4, 6 and 10 min., with the activities of non-irradiated portions of the same suspension. The results (Table 2) show a diminution of the dehydrogenating activity proportional to the time of exposure. The dehydrogenases for acids were more affected than those for alcohols and sugars.

To exclude the possibility that chemical changes occurring as the result of irradiation in the exposed suspensions had indirectly acted on the ferments, the following experiment was performed. A bacterial suspension was divided into four equal portions, of which one remained untreated, the second was irradiated, the third was irradiated and washed

Table 1. *Substrates activated as hydrogen donators by* E. typhosa (H 901)

Very strong donators	Strong donators	Weak donators	Not activated
Formic acid	Lactic acid	Acetic acid	Oxalic acid
Pyruvic acid	Succinic acid	Propionic acid	*l*-Tartaric acid
Mannitol	*d*-Tartaric acid	Malic acid	Maleic acid
Sorbitol	Ethanol	*dl*-Tartaric acid	Citric acid
Glucose	Glycerol	Fumaric acid	Methanol
Fructose	Arabinose	Xylose	Erythritol
Galactose	Melibiose	Sucrose	Adonitol
Mannose	Trehalose	Melezitose	Dulcitol
Maltose	*d*-Alanine	Raffinose	Inositol
	dl-Alanine	Arginine	Rhamnose
	Serine	Tryptophan	Sorbose
	Aspartic acid	Proline	α-Methyl glucoside
	Glutamic acid		Lactose
	Lysine		Glycine
	Asparagine		*d*-Leucine
	Histidine		*l*-Cystine
			Phenylalanine
			l-Tyrosine
			Urea
			Creatine
			Creatinine
			Guanidine

Table 2. *The effect of ultra-violet irradiation on certain dehydrogenases of* E. typhosa

		Irradiation time (min.)					
		0	1	2	4	6	10
		Relative dehydrogenating velocities (normal untreated bacteria = 100)					
Donator							
Acids (formic, pyruvic, glutamic, *d*-alanine)	Range	100	68–80	63–70	35–62	24–53	12–29
	Mean	100	75	66	52	43	21
Alcohols and sugars (mannitol, sorbitol, glucose, fructose)	Range	100	78–100	70–89	64–80	58–67	37–44
	Mean	100	88	84	71	60	41
Total average of donators tested		100	82	75	62	51	31

by centrifugation; to the fourth the supernatant fluid of the third portion was added. Equal reduction rates were noted in the first and fourth specimens on the one hand, and in the second and third on the other. From these results it may be concluded that ultra-violet irradiation acts directly on the dehydrogenating mechanisms of the cells.

(3) *The effect of salt solutions.* The influence of NaCl solutions of varying concentration was tested on eight dehydrogenases (formic and pyruvic acids, mannitol, sorbitol, glucose, fructose, *d*-alanine, glutamic acid) of the strain H 901. Equal amounts of washed bacteria were suspended in NaCl solutions of varying concentrations, and after 4 hr. their dehydrogenating properties were tested. From the results (Table 3) it follows that: (*a*) dehydrogenation in distilled water is slightly faster than that in 0·9 % NaCl; (*b*) hypertonic NaCl solutions suppress the dehydrogenating mechanisms, the inhibition increasing with increasing salt concentration; (*c*) the dehydrogenase for formic acid showed exceptional resistance and was influenced only by the highest NaCl concentration.

Table 3. *The effect of various* NaCl *concentrations on certain dehydrogenases of* E. typhosa

Donator		NaCl concentration (%) 0	0·9	3·0	5·0	7·0	10·0	15·0	30·0
		Relative dehydrogenating velocities							
Formic acid		100	100	100	100	100	100	100	67
Other donators (pyruvic acid, mannitol, sorbitol, glucose, fructose, glutamic acid, *d*-alanine)	Range	99–141	100	70–92	61–92	27–75	5–35	6–17	0·5–3
	Mean	112	100	83	78	53	21	9	2

(4) *The effect of freezing and thawing.* Repeated freezing and thawing considerably alter cell integrity. In our experiments a washed suspension of *E. typhosa* was frozen in an ice + salt mixture and thawed in a water-bath at 40°. This procedure was repeated ten times, the activity of the suspension being tested, for 14 donators (see Table 4), after every second freezing and compared with that of an untreated portion of the same suspension. In other experiments the number of surviving bacteria was determined. The examination carried out on the strains H 901 and O 901 yielded, essentially, the same results for both types of bacteria and, therefore, only the results obtained with H 901 are given here. A gradual decrease in the activity of almost all dehydrogenases was observed, which increased with each freezing. It is noteworthy that, out of 14 dehydrogenating mechanisms tested, only one, that for formic acid, remained completely uninfluenced by ten freezings. A comparison of the death-rate of the bacteria with loss in dehydrogenating capacity showed that the former always outweighed the latter. A possible explanation is that in estimating the number of 'living' bacteria we actually determine only those which are able to multiply, and this ability involves many fermentative processes which, as a whole, are more sensitive to freezing than the mechanisms of dehydrogenation.

(5) *The effect of autolysis.* The formic dehydrogenase is apparently not affected by the destruction of the cell on repeated exposure to freezing, i.e. it is not bound to the intact cell structure. Cell-free extracts of bacteria were therefore tested for their dehydrogenating properties. The extracts were prepared by autolysis. A heavy suspension of *E. typhosa* (strain H 901) in phosphate-buffer solution at pH 7·2 was kept at 37° for 5 days; it was then strongly centrifuged and the supernatant liquid was used for dehydrogenating experiments. Out of 12 substances which were found to be activated as strong donators in the intact cells (formic, lactic and pyruvic acids, mannitol, sorbitol, mannose, glucose, fructose, galactose, maltose, *d*-alanine and glutamic acid), lactic acid was the only one to be dehydrogenated by the autolysate.

Table 4. *The effect of freezing and thawing on the survival and on certain dehydrogenases of* E. typhosa

Donators		No. of freezings 0	2	4	6	8	10
		Death-rate (%) 0	52	80	84	89	93
		Relative dehydrogenating velocities					
Formic acid		100	100	100	100	100	100
Other donators (lactic, pyruvic, succinic acids, sorbitol, mannitol, fructose, galactose, mannose, glucose, maltose, *d*-alanine, glutamic acid, asparagine)	Range	100	60–89	55–84	43–79	38–63	31–60
	Mean	100	78	68	55	47	40

The different methods of cell lysis used have, therefore, different effects. Although most of the dehydrogenases are affected, formic dehydrogenase remains uninfluenced by freezing and thawing, and lactic dehydrogenase is not destroyed by autolysis at 37°.

DISCUSSION

Dehydrogenating enzymes of *E. typhosa* have already been studied by v. Vasarhelyi (1935). His strains activated essentially the same substances as did ours, but there are a number of differences between the two sets of results: acetic, propionic, malic and tartaric acids, glycerol, sucrose, alanine and lysine were not dehydrogenated in v. Vasarhelyi's experiments, while rhamnose was dehydrogenated. Dehydrogenation of formic, lactic and succinic acids by *E. typhosa* was also demonstrated by Stickland (1929). It is interesting to compare our results with those obtained by other authors with other bacteria of the coli-typhoid group. Quastel & Whetham (1925) noted strong activating properties in *Esch. coli* for formic and succinic acids, glucose, fructose, galactose and mannitol. Aaron (1933), working with *Salmonella schottmuelleri* and using the same technique as ours, found a rapid dehydrogenation of lactic acid, mannitol, sorbitol, xylose, glucose, fructose and maltose; and Braun & Woerdehoff (1933) noted powerful activation of formic, lactic and pyruvic acids, glucose, fructose and maltose by *Shigella paradysenteriae* (Flexner). It is, therefore, evident that practically the same substances are dehydrogenated by the various bacteria of this family.

It is noteworthy that no significant difference could be detected in the dehydrogenating abilities of the *E. typhosa* strains O 901, H 901 and Vi 2. Their different antigenic structures are not correlated with any distinct dehydrogenating mechanism. Braun & v. Vasarhelyi (1940) have shown that different strains of *Proteus vulgaris* also activate the same substrates.

Hypertonic NaCl solutions retard and hypotonic solutions accelerate the dehydrogenating velocity of most enzymes: the former reduce by shrinkage the active surface whilst hypotonic solutions, by swelling the cells, enlarge the cell surface and thus accelerate the dehydrogenating activities. Formic dehydrogenase, however, is little affected by these changes.

Young (1929) found that freezing and thawing strongly inhibited the lactic and glucose dehydrogenases of *E. coli* and left almost unaffected the formic and succinic dehydrogenases. In Yudkin's (1937*b*) experiments glucose dehydrogenase was eliminated, while that for lactic acid remained intact. Young's statement concerning formic dehydrogenase of *Esch. coli* agrees with our results obtained on *E. typhosa*. In the experiments of this author too, the ability of bacteria to multiply was affected more than their dehydrogenating properties. Adler *et al.* (1938) prepared by repeated freezing and thawing a cell-free extract of *Esch. coli* which contained the dehydrogenases for lactic and glutamic acids. The reduced activity, caused by freezing and thawing, of the dehydrogenases other than formic dehydrogenase may be due to an injury either to the cell structure or to the mechanism of hydrogen transport. The latter possibility is suggested by Yudkin's (1933, 1937*a*) observations: with glucose and lactic dehydrogenases of *Esch. coli* the loss of activity on dilution of the bacterial suspension was very great owing to dilution of a coenzyme. The activity of the enzyme could be restored by the addition of suspensions of heated bacteria. Lysis of a cell is invariably accompanied by a dilution of its content, but in experiments to test the effect of this it was found that neither the addition of heated bacteria nor of yeast cozymase was able to restore the activity, after ten freezings, of the enzymes tested (those for pyruvic acid, mannitol, sorbitol, glucose, fructose, *d*-alanine and glutamic acid). In these instances, therefore, the enzymes appeared to be linked in some way to the structure of the cell. In fact, when a bacterial suspension was frozen ten times and afterwards centrifuged, all the dehydrogenases remained in the sediment, the supernatant fluid being completely inactive. Slightly different results, however, were recorded in our experiments with cell-free extracts obtained by autolysis. It is interesting to note that among 12 substances tested, which were found to be strong donators when activated by living bacteria, only lactic acid was dehydrogenated by our extract. It follows, therefore, that lactic dehydrogenase is in some way independent of the cell structure.

A cell-free lactic acid-dehydrogenating enzyme of *Esch. coli* has already been prepared by Stephenson (1928) and by Adler *et al.* (1938): Stephenson's method of preparation was similar to ours. A peculiar property of formic dehydrogenase, resembling that found by us, was also noted by Stickland (1929); after destruction of the cell structure by tryptic digestion almost all dehydrogenases except that for formic acid were destroyed.

SUMMARY

1. Fifty-nine substances were tested for their ability to be activated as hydrogen donators by *Eberthella typhosa*. The most active donators were formic and pyruvic acids, mannitol, sorbitol, mannose, glucose, fructose, galactose and maltose. No significant difference could be detected between the dehydrogenating abilities of the serologically different strains H 901, O 901 and Vi 2.

2. Ultra-violet irradiation of the cells suppressed the dehydrogenating activity. Dehydrogenases for acids were more affected than those for alcohols and sugars.

3. Hypertonic NaCl solutions inhibited the dehydrogenating mechanisms for almost all donators, hypotonic ones slightly accelerated them. Only formic dehydrogenase was nearly insensitive to osmotic changes.

4. Repeated freezing and thawing inhibited the activity of almost all dehydrogenases tested, but formic dehydrogenase remained uninfluenced by as many as ten freezings. The diminution of the dehydrogenating velocity was not due to the dilution of a coenzyme.

5. Cell-free extracts, prepared by centrifugation of autolyzed bacteria, contained lactic acid dehydrogenase.

6. It is concluded that most dehydrogenating systems are in some way linked to the cell structure. Only formic and lactic dehydrogenases are in some degree independent of the intactness of cell structure.

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The Determination of Starch in Apple Tissue

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In the determination of starch present in apple tissues by the use of an enzyme, the tissue must first be obtained in a finely divided state so that the starch can be easily extracted and hydrolyzed. It is noticeable in the literature on starch determinations in apples that difficulty is experienced in obtaining complete extraction of starch, and that different values may be obtained according to the method of extraction and the enzyme which is used for hydrolysis.

In 1926 Gerhardt was able to extract starch quantitatively with boiling water from the ethanol-insoluble residue of dried apple tissue after removal of the substances soluble in 10% ethanol.

Widdowson (1932) records that she was unable to extract all the starch from apple tissue, unless the ethanol-insoluble residue was previously extracted, with a 1% K-oxalate solution, to remove pectin. Even after this treatment the residue had to be thoroughly ground in a mortar before complete hydrolysis of the starch by takadiastase could be effected.

Denny (1934) found it necessary to extract the ethanol-insoluble residue several times with ethyl ether and then to grind the dried tissue to a powder. After grinding the powder with water in a mortar he was able to determine starch quantitatively with takadiastase. He also describes a method based on the preliminary extraction of the starch in hot concentrated $CaCl_2$ solution, which, he states, gave true values for starch.

In 1936 Hanes determined starch in ethanol-extracted apple tissues by first rendering the fibre soluble by treatment with ethanolic HCl, and then hydrolyzing with β-amylase, which is specific for starch. His starch determinations were made on a set of ten apple residues supplied by Dr Kidd from an experiment with Bramley's apples, for which the authors had already obtained values by hydrolysis with takadiastase. Comparison of the results suggested the presence of a fraction hydrolyzable by takadiastase, but not by β-amylase.

The authors have not experienced difficulty in carrying out complete hydrolysis with takadiastase;