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Studies on Relation between Dehydrogenase Activity and Viable Count of Bacterial Cells, with Special Reference to the Action of Antibiotics

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For counting the number of viable bacterial cells in the culture solution or the cell suspension, we have several methods to be applied. By the nephelometric method we cannot tell dead cells from living cells. The plate culture method in which we count colonies, each colony being derived from a single living cell, is appropriate for this purpose from the theoretical point of view. But it requires a considerably long time to accomplish a single experiment, for it is necessary to spend 24—48 hours after incubation to recognize the colony formation and, to more disadvantage, it might need a great amount of skill to attain accurate results. The so-called capillary tube method, adopted by us in the present work, gives a more practicable method. But it takes likewise one or two days and is inapplicable to the purpose when we deal with the strict aerobic bacteria.

It is an important theme to attain accurate count of viable cells of the B.C.G. vaccine in a short time, which takes in the usual way 3—4 weeks, because colony formation of tubercle bacilli is extremely slow. Ôbayashi and co-workers found that dehydrogenating power and viable count of B.C.G. vaccine were closely related to each other and pointed out the possibility of knowing the activity of B.C.G. vaccine through determination of the decoloration time of the dye, for it required only a short time of less than 1 hour to finish a single operation. They used 2·6-dichlorphenol-indophenol as hydrogen acceptor.[1)]

From their study, it will be conceivable that bactericidal as well as bacteriostatic action of antibiotics may also be tested through estimation of dehydrogenating activity. In the present paper we shall report results of experiments on dehydrogenating action of three strains of bacteria which are generally used for tests of antibiotics, *Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli*, and also their viable counts.

Methods

A considerable amount of bacterial cells had to be collected to test the dehydrogenase reaction. At first, bacteria were cultivated in liquid media and after 2 or 3 days' incubation turbid solution was centrifuged

down. Before long we came to perceive that this method is inadequate, for the amount of bacteria thus obtainable is insufficient for this purpose. The most proliferous method was that of culturing them on the surface of potato slice. This may be, however, accompanied with the danger of collecting the bacteria cells mixed with the potato tissues. We used finally bouillon agar in Petridishes, on which bacteria are cultured to form colonies.

15 cc of bouillon agar prepared in the usual way, was poured into a Petridish of 10 cm diameter while hot. When cooled and solidified, test organisms were streaked with a platin loop all over the surface of the agar medium. After 1—2 days' incubation at 38°C the colonies in the 5 plates were collected with a spatula, brought into a mortar and was mashed with a pestle adding a small amount (ca 1 cc) of glycerin in order to make homogeneous suspension. 10 cc of the physiological saline solution was added and shaked for 5 minutes with a vibrator, and then the bacterial cells were centrifuged down. The sediment was diluted to about 5 cc solution to be used. This dilution was regulated in each case so that it would take about 5 minutes for the suspension to decolorize the dye.

The tube of Thunberg was used for the measurement of dehydrogenase, with 0.5 cc of bacterial suspension and 0.5 cc of M/5 phosphate buffer (pH 6.9) placed in the main tube, and 0.5 cc M/5 pottasium lactate as H-donor and 0.5 cc M/500 2·6-dichlorphenol-indophenol as H-acceptor in the curled tube. The whole apparatus was held for 10 minutes in water of a thermostat of temperature 30°C, until it became constant temperature. Then contents of the both tubes were brought together, this being made the starting point for measurement of decolorization.

At first, the plate culture method was tried for the counting of living bacterial cells. But preliminary experiments showed that colonies formed by this method do not always correspond to dilution numbers of the suspension. A new method by Yanagita was then tested, and it was found to be applicable for our purpose. A capillary glass tube of about 1 dm length and 0.2 cc capacity was filled with semi-colloidal bouillon agar (1% agar) mixed with bacterial suspension diluted by an arbitrary amount and laid horizontally in an incubator at 37°C. After 24—48 hours' incubation the number of colonies developed was counted through a magnifying glass, by sliding the capillary tube slowly between two marks, which indicate the boundary lines of 0.2 cc. If it gives appropriate number of bacteria, each colony will lay in a single row and the number can be accurately counted.

Original suspension of each test organism was diluted by adding sterilized water to 10^{-8}—10^{-9}, each divided into a small test tube, to which bouillon agar, cooled to 50°C, was added and well shaked to

be sucked up into the capillary tube.

When kept still, the test organisms were gradually sedimented on the bottom of the tube, causing the reaction to delay. In order to eliminate this defect, we kept them being shaken mechanically during the operation in water of the thermostat.

Experiment 1. Dilution of bacterial suspension and dehydrogenase activity.

Bacillus subtilis, *Bacillus Natto*, *Staphylococcus aureus*, and *Escherichia coli* were used as test organisms. *Bac. Natto* consists of viscous mass and is so hardly to be broken up that the experiment with it is apt to accompany some incorrectness.

Hay bacillus and Natto bacillus form endospores in the later stage of their culture and go into resting. The enzyme activity decreases even when the viable count increases, because the spores will show little enzymic activity. It was ascertained microscopically that spore formation did not take place by the time when this experiment was carried out. When we are not ready to undertake the experiment immediately after the culture of bacilli, it must be kept in the refrigerator to avoid the spore formation. Since such preservation had some decreasing effect on the dehydrogenase activity[7], we used cells as far as possible without preservation. Other non-sporulating bacteria were also used while fresh.

This original concentrated suspension of bacteria was weighed with a pipette of 1 cc capacity. The weighing should be cautiously done to avoid incorrectness. We tested the correctness of the weighing as

Table 1. Variability in enzymic activity, depending upon dilution of suspension.

testorganism	amount of cells	enzymic activity					note
		original suspension	1/2 dilution	1/3 dilution	1/5 dilution	1/10 dilution	
Bac. subtilis	0.4 g per 1 cc	1.5 min.	5 min.	11 min.	>30 min.		cultured on potato slices
〃	〃	0.7 〃	3 〃	10 〃			
〃	〃	1.3 〃	5 〃	20 〃	>30 〃		
〃	〃	2.5 〃	6 〃	15 〃	>30 〃		
〃	〃	5 sec.				60 sec.	
Bac. Natto	0.3 g per 1 cc	2 min.	4 min.		13 min.		cultured on potato slices
〃	〃	15 〃	55 〃				
Staph. aureus	one plate per 1 cc	2 min.	25 min.				cultured on Bouillon agar
〃	〃	1 〃	15 〃	>30 min.			
〃	〃	1.5 〃	16 〃	30 〃			
Escher. coli	one plate per 1 cc	3 min.	20 min.	>60 min.			cultured on Bouillon agar
〃	〃	2 〃	21 〃	>60 〃			

follows. 1 cc of the same suspension was weighed 3 times in each weighglass and dried. Dry substance amounted to 0.6 mg, 0.58 mg, 0.6 mg respectively. From these results this weighing method was thought to be practically sufficient in this case. The results obtained are shown in table 1.

Although dehydrogenase activity must be theoretically proportional to the number of the bacterial cells, such was not the case with the above mentioned results. These might have resulted from the incompleteness of the technique, for instance, insufficient breaking up of bacterial colonies, especially of *Bac. subtilis* and *Bac. Natto*. But discrepancies between theory and experimental values are more than those which are to be expected if there is deficit in the techniques. It is difficult to make the standard curve experimentally. Accordingly, number of bacterial cells can not be counted by the decolorization time. Except those bacteria which can be treated under extraordinally defined conditions, for instance, B.C.G.-vaccine, this method is not applicable.

Experiment 2. Changes of dehydrogenase activity by adding antibiotics.

The suspension of bacteria was prepared as mentioned above and the antibiotics were added in a sufficient amount to cause complete inhibition. Four measurements were carried out within 54 hours after mixing two solutions. A large number of Thunberg tubes were set in one shaking apparatus and the measurement was carried out one by

Table 2. Variability in enzymic activity, depending upon the action of antibiotics.

testorganism	antibiotic time of reaction	3hrs	6hrs	12hrs	24hrs
Bac. subtilis	penicillin 500γ per 1 cc	4 min.	4 min.	5 min.	5 min.
	control (no penicillin)	3 〃	3 〃	3 〃	4 〃
Staph. aureus	penicillin 500γ per 1 cc	6 min.	6 min.		7 min.
	control	4 〃	4 〃		4 〃
	penicillin 500γ per 1 cc	1.2 〃	2 〃		2 〃
	control	1 〃	2 〃		2 〃
Escher. coli	penicillin 500γ per 1 cc	3 min.	3 min.		3 min.
	control	1.5 〃	2 〃		2 〃
	penicillin 500γ per 1 cc	2 〃	3 〃		3 〃
	control	2 〃	3 〃		2 〃
Bac. subtilis	streptomycin 500γ per 1 cc	6 min.	6 min.	6 min.	8 min.
	control (no streptomycin)	3 〃	3 〃	3 〃	5 〃
Staph. aureus	streptomycin 500γ per 1 cc	6 min.	9 min.		20 min.
	control	4 〃	4 〃		4 〃
	streptomycin 500γ per 1 cc	1.4 〃	2 〃		2.5 〃
	control	1 〃	1 〃		2 〃
Escher. coli	streptomycin 500γ per 1 cc	3 min.	3 min.		3 min.
	control	3 〃	3 〃		3 〃

one. As antibiotics, streptomycin (Merck) and penicillin (G crystal) were given in excessive dosis.

There are some remarks in the literature whether antibiotics act on the enzyme dehydrogenase.[2] Our experiment concerns also this point.

As table 2 shows, dehydrogenase activity does not decrease markedly at least within 24 hours, despite the bacteria were damaged by the antibiotics. In the controls, the enzyme activity was also scarcely changed. Here we are reminded of the papers in which it was reported that bovine semen lost its dehydrogenase but was still viable after storage in the presence of penicillin for approximately 3 weeks.[3][4][5] On the contrary, our experiments made it clear that dehydrogenase of the three kinds of bacteria was not markedly attacked by the two antibiotics.

Experiment 3. Viable counts and dehydrogenase in the presence of antibiotics.

Lastly, both the enzymic activity and viable count were measured in the presence of streptomycin or penicillin. The antibiotics were

Table 3. Enzymic activity and viable count in the presence of antibiotics.

testorganism	antibiotic \ incubation time		0hr	3hrs	6hrs	24hrs
Bac. subtilis	streptomycin	viable count	58	5	1	0
		decolor. time	2 min.	2 min.	3 min.	4 min.
	penicillin	viable count	58	3	0	0
		decolor. time	2 min.	2 min.	2 min.	3 min.
	control	viable count	58	58	10	5
		decolor. time	2 min.	2 min.	2 min.	2 min.
Staph. aureus	streptomycin	viable count	131	94	13	0
		decolor. time	1.4 min.	1.4 min.	2 min.	2.5 min.
	penicillin	viable count	131	131	50	27
		decolor. time	1.2 min.	2 min.	2 min.	2 min.
	control	viable count	131	131	50	27
		decolor. time	1 min.	1 min.	1 min.	1 min.
Escher. coli	streptomycin	viable count	∞	∞	142	53
		decolor. time	3 min.	3 min.	3 mim.	5 min.
	penicillin	viable count	∞	∞	235	113
		decolor. time	2 min.	2 min.	2 min.	5 mim.
	control	viable count	∞	∞	∞	∞
		decolor. time	1 min.	1 min.	1 min.	4 min.

given 500 γ per 1 cc. The viable count indicated the number of colonies, visible in 0.2 cc in the glass tube. The results are shown in Table 3.

Similarly to the cases of Table 1 and Table 2, dehydrogenase activity remained almost unchanged, although viable count decreased markedly in the presence of the antibiotics. When left for a longer time, e.g. 24 hours, however, the enzymic activity used to be decayed slightly.

Stephenson has separated lactic dehydrogenase out of coli bacillus[6] and Quastel and Wooldridge (1927) observed that lactic dehydrogenase is still active in cells no longer viable, although glucose and amino acid dehydrogenases are greatly reduced in dead cells.[7] Wooldridge and Glass (1937) reported later that glucose and amino acid dehydrogenases were not also dependent upon viable counts.[8] Our observations show good agreement with these reports.

In conclusion, as it is obvious from the results of the three experiments, there is no definite relation between the viable count and dehydrogenase activity, so that the dehydrogenase method can not be applied to the test of the antibiotic activity.

Summary

An attempt to test inhibitory power of antibiotics by the way of dehydrogenase (lactico-) measurements of test organisms ended in failure. Results of 3 experiments are briefly summarized as follows.

1. Bacterial suspensions were prepared in serial dilutions, while dehydrogenase of each dilution was measured. But no proportionality was recognized between concentration and dehydrogenase.

2. Streptomycin or penicillin was added in dosis of complete inhibition (500 γ per cc), but no conspicuous reduction of the dehydrogenase activity was seen.

3. Both viable count and dehydrogenase measurement in the presence of antibiotics were carried out. In this case, the enzymic activity remained almost constant, although viable count decreased, and the reaction progressed in almost the same way as in the control.

Literature

1) Ôbayashi, Y., Tsugiki, M., and Furuya, S.: Studies on dehydrogenating reaction of B.C.G., with special reference to the test of B.C.G. vaccine. Medicine and Biology, **10** (1947), 311, 314, 317.

2) Prat, R., and Dufrenoy, J.: Recent research on penicillin action. Texas Rep. Biol. & Med., **7** (1947), 180-219.

3) Almquist, J.O., Thorp, W.T.S., and Knodt, C.B.: The effect of penicillin upon the livability, glycolysis, and bacterial content of bovine semen. J. Dairy Sci. **31** (1948), 11-19.

4) Foote, R.H., and Salisbury, G.W.: The effect of pyridium, penicillin, furacin, and phenoxethal upon the livability of spermatozoa and upon the control of bacteria in diluted bull semen. J. Dairy Sci., **31** (1948), 763-768.

5) Hennaux, L., Dimitropoulos, E., and Cordiez, E.: L'action de la penicilline sur la vitalité du sperme de taureau. Compt. rend. Soc. Biol., **142** (1948), 408-410.

6) Stephenson, M.: On lactic dehydrogenase. A cell-free enzyme preparation obtained from bacteria. Bioch. J., **22** (1928), 605-614.

7) Quastel and Wooldridge: The effects of chemical and physical changes in environment on resting bacteria. Bioch. J., **21** (1927).

8) Wooldridge, W.R., and Glass, V.: Variability in the activity of bacterial enzymes II. Factors associated with viability and growth. Bioch. J., **31** (1937), 526-531.

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