A STUDY OF CERTAIN DEHYDROGENASE ACTIVITIES IN A SULFANILAMIDE-RESISTANT STRAIN OF *BACILLUS SUBTILIS*

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There are several reports on the interference of oxidative mechanism in bacteria by drugs (Henry, 1943; Sevag, 1946). Sulfonamides are known to affect the utilisation of glucose (Clifton and Loeuringer, 1942; Dorfman and Koser, 1942; Sevag and Shelburne, 1942; Sevag, Henry and Richardson, 1945), and of essential amino acids (Sevag and Green, 1944). More specifically, their effect has been shown on the metabolism of pyruvate (Sevag *et al.*, 1942, 1945), glycerol and lactate (Macleod, 1939) and succinate and glutamate (Frei, 1942). Sevag *et al.* (1944) suggested a correlation between flavoprotein activity and drug resistance from analysis of various observations.

Macleod (1939) and Macleod and Dadi (1939) reported that sulfapyridine-resistant *Pneumococci* have decreased dehydrogenase activity and a reduced ability to form hydrogen peroxide. Gots and Sevag (1948) also showed that resistant strains of *Pneumococci* have lowered dehydrogenase activity. A difference in the interrelationship of amino acid requirements and glucose metabolism by the susceptible and resistant strains of *Staphylococcus aureus* was also noted by Sevag and coworkers (1949, 1950). The metabolism of glucose provides the necessary energy for the utilisation of amino acids in endergonic reactions. Sevag, Gots and Steers (1950) have reviewed the effects of sulfonamides on various other enzymes.

A comparative study of certain dehydrogenase activities of susceptible and sulfanilamide-resistant strains of *Bacillus subtilis* with different substrates is reported in this communication.

Experimental

Culturing and harvesting.—The studies were carried out with the susceptible and 0·5 per cent. sulfanilamide-resistant strains of *B. subtilis*, a local isolate employed in the earlier studies (Joshi and Sreenivasan, 1958). The culture media used were (i) peptone medium (Stearn's peptone, 1 per cent.; glucose 1 per cent.; and sodium chloride 0·5 per cent. and (ii) the medium of Muir *et al.* (1942) with 1 per cent. casein hydrolysate.

Inocula of susceptible and resistant strains of the organism were grown in 10 ml. lots of the medium, the latter in presence of 0·5 per cent. sulfanilamide, for 18 hours at 37° and washed by centrifugation twice with 0·9 per cent. ice-cold sodium chloride. The packed cells were then transferred aseptically to 100 ml. sterile medium in Roux bottles and incubated at 37° for 18 hours. The cells were centrifuged, washed twice with M/20 phosphate buffer (pH 7·4) and suspended in 25 ml. of the buffer. The volume of cell suspensions was adjusted such that 1 ml. was equivalent to 1·5–2·0 mg. dry weight of cells.

Determination of dehydrogenase activities.—The dehydrogenase activities were measured by the time required to reduce methylene blue anaerobically. Thunberg tubes were used initially but Schnabel technique (1920) was followed later using ordinary rimless narrow diameter tubes. The system consisted of 2 ml. of M/20 phosphate buffer (pH 7·4), appropriate concentrations, as indicated in the text, of methylene blue, substrate, and cell suspension in a final volume of 5 ml.; the surface was covered with 2 ml. petroleum jelly. The tubes were incubated at 37° and the time was noted immediately after adding cell suspension and petroleum jelly. Time of 100 per cent. decolorisation of the methylene blue was recorded.

Results

Preliminary experiments were carried out using the susceptible strain grown in peptone medium and with glucose in a final concentration of $2·7 \times 10^{-4}$ M to ascertain the optimum concentrations of methylene blue and cell suspension for following dehydrogenase activities.

Methylene blue in a concentration of $1·7 \times 10^{-4}$ M was inhibitory as it was not decolorised during 3 hours in the presence or absence of glucose with 1·0 ml. or 2·0 ml. of cell suspension. With $6·8 \times 10^{-6}$ M to $17·0 \times 10^{-6}$ M concentrations of methylene blue, dehydrogenation with glucose as substrate occurred in about 15–40 minutes. At the same time, endogenous activity was low, decolorisation time being more than 180 minutes. A few typical results are given in Table I.

Endogenous activity was 20–25 per cent. of the dehydrogenase activity. Both endogenous and dehydrogenase activities were proportional to the amount of cell suspension and were independent of methylene blue concentration over the given range. There were variations in the absolute values for dehydrogenase activity with different preparations of cell suspensions and hence comparisons were made only with the same preparation each time.

Preservation of cells at 0° for 48 hours resulted in about 50 per cent. loss in activity. Only freshly harvested cells were therefore used in all experiments.

TABLE I

Dehydrogenase activities of susceptible strain with varying concentrations of methylene blue and cell suspension

Methylene blue ($\times 10^{-6}$ M)	Cell suspension (ml.)	Methylene blue reduction time (minutes)	
		Without substrate	With glucose ($2{\cdot}7 \times 10^{-4}$ M)
170·0	2·0	>180	>180
34·0	1·0	>180	95
13·6	2·0	72	15
6·8	2·0	62	16
13·6	1·0	200	37
6·8	1·0	135	33

Dehydrogenase activities of susceptible and resistant strains.—Dehydrogenase activities of susceptible and resistant strains of *B. subtilis* using as substrates glucose, glycerol, sodium pyruvate, calcium lactate and ethyl alcohol are given in Table II.

The results show that dehydrogenase activities of resistant strain are lowered with all the substrates studied. The susceptible strain has low activity with ethyl alcohol, sodium pyruvate and calcium lactate as substrates. Gots and Sevag (1948) found that glycerol dehydrogenase activities with sulfanilamide-resistant strains of *Pneumococci* grown in peptone medium are impaired. They found no alteration in glucose dehydrogenase activity.

When peptone medium was replaced by the medium of Muir *et al.* (1942) with 1 per cent. casein hydrolysate, somewhat different results were obtained (Table III).

The endogenous activities of both susceptible and resistant strains were rather high and were not also proportional to the amount of cell suspension taken. With the lower concentration of cell suspension, the dehydrogenase

TABLE II

Dehydrogenase activities of susceptible and resistant strains (Peptone medium)

Substrate	Methylene blue ($13\cdot6\times10^{-6}$ M) reduction time (minutes)	
	Susceptible strain (2 ml.)	Resistant strain (2 ml.)
None ..	27	203
Glucose ($2\cdot7\times10^{-4}$ M) ..	7	115
Glycerol (100×10^{-4} M) ..	13	180
Calcium lactate ($4\cdot5\times10^{-4}$ M) ..	19	168
Sodium pyruvate ($9\cdot0\times10^{-4}$ M) ..	18	185
Ethyl alcohol ($8\cdot0\times10^{-4}$ M) ..	22	175

TABLE III

Dehydrogenase activities of susceptible and resistant strain (Synthetic medium)

Substrate	Cell suspension (ml.)	Methylene blue ($13\cdot6\times10^{-6}$ M) reduction time (minutes)	
		Susceptible strain	Resistant strain
None ..	1·0	60	135
None ..	2·0	18	20
Glucose ($2\cdot7\times10^{-4}$ M)	1·0	32	120
Glucose ($2\cdot7\times10^{-4}$ M)	2·0	14	14
Glycerol (1×10^{-2} M)	1·0	32	36
Glycerol (1×10^{-2} M)	2·0	16	14

activity with glucose as substrate is lowered in the resistant strain but the activity with glycerol is not much altered.

With an excess of cell suspension, however, the activities with susceptible and resistant strains are more or less similar. The high endogenous activities and the anomalous behaviour of the cell suspension on dilution preclude quantitative conclusion. Sevag and Gots (1948) also observed somewhat similar behaviour on dilution of cells. The dehydrogenase activity of susceptible cells of *Pneumococci* was not impaired on dilution but resistant cells lost most of the activity on dilution. Higher concentrations of glucose appeared inhibitory.

Dehydrogenase activities of cell-free extract.—A decrease in permeability towards a drug as well as towards substrates during development of resistance might explain the decreased dehydrogenase activities as well as drug resistance. Alteration of the enzymic make-up of a cell as a result of sulfonamide resistance need not therefore preclude permeability changes or development of polysaccharide sheaths (Umbriet, 1944). The difference between the lactose fermenting cells of *B. coli mutabile* and the non-lactose fermenters is chiefly a difference in permeability to lactose (Deere, 1939). This possibility in the case of *B. subtilis* was therefore examined using glucose as substrate with cell-free extracts.

The harvested cells (peptone medium) were crushed with the aid of glass powder in a glass mortar and pestle and extracted twice with 10 ml. of M/20 phosphate buffer (pH 7·4), centrifuged to remove glass powder and made upto 25 ml. with buffer. The opalescent extract was used for determination of dehydrogenase activity (Table IV).

TABLE IV

Dehydrogenase activities of cell-free extracts

Cell-free extract (ml.)	Methylene blue reduction time (minutes)			
	Susceptible strain		Resistant strain	
	No substrate	With glucose	No substrate	With glucose
0·5	>1260	>180	>1260	>120
1·0	1275	180	220	110
1·5	220	110	105	60
2·0	105	61	60	40

Methylene blue, $13 \cdot 6 \times 10^{-6}$ M; Glucose, $2 \cdot 7 \times 10^{-4}$ M.

The resistant strain has more endogenous activity which diminished proportionately with the extract taken. An accelerated endogenous activity would obscure and weaken dehydrogenase activity. When dehydrogenase activities are compared with endogenous activities as the basal line of reference, it is seen that the resistant strain is far less active than the susceptible strain. The observations show that the behaviour of the cell-free extract of either susceptible or resistant strain is comparable to that of the corresponding intact cells. It seems therefore that the development of resistance and decreased dehydrogenase activity of the resistant strain may not be due to alteration in cell-wall permeability. Yegian and Budd (1945) also found that sulfonamide-resistant *Mycobacteria* were not less permeable than the parent non-resistant strains. Sevag and Gots (1948) reported that the drug resistant cells of *Pneumococci* did not undergo decrease in permeability to drugs, substrates or riboflavin. In fact the data suggested that resistant cells might manifest greater permeability to these agents.

Discussion

The foregoing observations show that dehydrogenase activities of the resistant strain of *B. subtilis* are impaired as compared to those of the susceptible strain. Gots and Sevag (1948) also observed an impairment in dehydrogenase activities in *Pneumococci* rendered resistant to atabrine, propamidine, optochin, acriflavine and sulfathiazole when grown in peptone medium. The alteration in the enzyme content is dependent upon the initial activity of the organism and the number of organisms present in the system and, in certain cases, also on the nature of the organism, substrate and substrate concentration.

Sevag and Gots (1948) studied the effect of atabrine, acriflavine and related drugs structurally similar to riboflavin and found that reduction in dehydrogenase activity could be restored by riboflavin. In sulfathiazole-resistant strains of *Pneumococci*, they found that only glycerol dehydrogenase activity was lowered and that this retarding effect was not reversed by riboflavin. Catalase was used to guard against the toxic action of hydrogen peroxide which may be produced. The observations of Sevag and Gots, however, were not in complete agreement with those of Macleod (1939) who reported sulfapyridine inhibition of glycerol, lactate, and pyruvate dehydrogenase activities with type 1 *Pneumococcus*. The dehydrogenase activities in all substrates examined by Sevag and Gots (1948) were completely unaffected by high concentrations of sulfonamides. As suggested by them, the variable activity may be a reflection of varied metabolic capacities of different organisms. The findings emphasise the difficulties involved in such studies.

As the synthesis of most amino acids depends on glucose metabolism and since this is susceptible to sulfathiazole, the enzymes involved in the metabolism of glucose and the tricarboxylic acid cycle supplying α-Keto acids for amino acid synthesis must represent a critical site of sulfonamide action.

The blocking of glucose and amino acid metabolism by sulfonamides would constitute the primary site of drug action and unhindered functioning of the physiological activities of the resistant organisms must depend on metabolically active sites not susceptible to the action of the drug.

The present studies, although essentially preliminary, would point out that cell permeability is not a factor in itself contributing to changes in enzymic make-up of susceptible and resistant strains of *B. subtilis*.

SUMMARY

The dehydrogenase activities of susceptible and sulfanilamide-resistant strains of *B. subtilis* grown in peptone or semi-synthetic media have been studied with glucose, glycerol, calcium lactate, sodium pyruvate and ethyl alcohol as substrates. The resistant strain showed a marked decrease in dehydrogenase activities with all the substrates.

The behaviour of the cell-free extracts of susceptible or resistant strains was comparable to those of the corresponding intact cells, indicating that decreased dehydrogenase activities of the resistant strain may not be due to alteration in cell-wall permeability.

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