BACTERIAL OXIDATION OF ARSENITE

III. CELL-FREE ARSENITE DEHYDROGENASE

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Summary

A soluble, cell-free arsenite dehydrogenase is liberated from cells of strain 17 of the arsenite-oxidizing pseudomonad (*Pseudomonas arsenoxydans-quinque*) after grinding with powdered glass. The enzyme is moderately stable, retaining its activity for considerable periods on storage at low temperatures. It may be dialysed without loss of activity, and withstands salting out.

Its activity may be measured in the presence of suitable electron acceptors.

In the course of a prolonged series of enzyme preparations, the yield dropped considerably. This was traced to variation in the strain, and substrains were isolated from which original yields of enzyme were obtained.

No evidence was found that the activity of the enzyme was dependent on accessible $-SH$ groups.

I. INTRODUCTION

Previous papers (Turner 1949, 1954; Turner and Legge 1954) gave a detailed description of arsenite-oxidizing bacteria, defined the conditions for obtaining active adapted cells, described their arsenite-oxidizing characteristics, and referred briefly to arsenite dehydrogenase activity in extracts of disrupted cells. The present paper continues the study of the dehydrogenase obtained by disrupting cells with powdered glass. A later paper (Legge 1954) describes the reconstruction of oxidizing systems from disrupted cells.

II. METHODS

(*a*) *Routine Preparation of Enzyme*

Cells for preparing arsenite dehydrogenase were usually harvested from 3-day cultures at 25°C in YA3 medium (Turner 1954), the mean yield being 6·3 g packed cells per l. For special purposes, 6-day cultures in KA3 medium (Turner 1954) were used, the mean yield being 0·93 g packed cells per l.

Cells were harvested in a Sharples supercentrifuge; they were resuspended, and were finally centrifuged to provide 12-25 g of bacterial paste. After storing at 4°C, usually for 5-8 days, the cells were ground in a chilled Utter-Werkman mill with twice their weight of powdered Pyrex glass and twice their weight of M/15 pH 7·2 phosphate buffer. The effluent was then spun at 3500 r.p.m.

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in a Wifug angle centrifuge for 30 min, thus separating the glass and cell debris (P), and the few undamaged cells, from the supernatant solution (S_1). Fraction S_1 contained half to two-thirds of the total arsenite dehydrogenase which could be extracted by further washing of fraction P, and was generally kept separate from the subsequent washings (S_2, S_3). After two washings, fraction P contained only negligible arsenite dehydrogenase activity, although it contained the greater part of the bacterial cytochromes.

S_1, generally a dirty yellowish turbid solution, was then subjected to a further fractionation in the high-speed angle head of the I.E.C. Model 2 refrigerated centrifuge, which was operated to maintain the same temperature as the room. The average gravitational field to which the solutions were subjected was calculated to be 19,000g. After 60 min spinning, a small translucent pink pellet was thrown down (S_1P), leaving a relatively clear supernatant, which was then stored at $-5°$C with a few drops of toluene as preservative.

(*b*) *Measurement of Enzyme Activity*

Dehydrogenase activity was estimated from the rate of reduction of 2,6-dichlorophenol indophenol in Thunberg tubes, or manometrically, from the rate of evolution of CO_2 when ferricyanide was used as the electron acceptor in bicarbonate buffer. Nitrogen was determined by micro-Kjeldahl, a Markham still being used.

The unit of dehydrogenase activity $= 1/t \times 10^5$, when $t =$ the time in minutes for 90 per cent. reduction, at 25°C, of 0·3448mM 2,6-dichlorophenol indophenol by 0·1 ml enzyme in 0·004M arsenite, the volume being made up to 5 ml with buffer. For example, a sample which took 18·2 min to bring about reduction of the dye under those conditions was said to contain 5500 units per ml; this is equivalent to an oxidation of 26·0μM arsenite per ml per hr. The same sample of enzyme, when assayed manometrically with 2×10^{-2}M ferricyanide as acceptor, oxidized arsenite at approximately half this rate.

III. Results

(*a*) *Partition of Arsenite-Dehydrogenase Activity between Cell Juice and Cell Particles*

The extent to which high-molecular-weight particles were removed from the S_1 fractions by this treatment varied in different batches of cells, and could not be correlated with the other changes discussed below. In some cases the supernatant from the first centrifugation of the disrupted cells showed only a faint Tyndall effect, and little or no further clarification after high-speed spinning. In other cases, the supernatant after 60 min at high speed showed the presence of a high-molecular-weight component which was concentrated in the lower part of the centrifuge tubes. In nine out of 12 preparations in which the enzyme activity of both upper and lower fractions was measured, the lower showed 7-17 per cent. greater activity. In one instance, the lower fraction was significantly (12 per cent.) less active than the upper.

Since all the enzyme remains in solution after low-speed centrifugation, it is concluded that 17 per cent. represents the maximum amount of enzyme remaining bound to relatively high-molecular-weight particles, and that most exists as a true protein solution. No correlation could be observed between the amount of arsenite dehydrogenase bound to high-molecular-weight particles, and the enzyme activity of the S_1 fractions. The relationship, if any, between the slowly sedimenting, high-molecular-weight particles carrying bound arsenite dehydrogenase, and the pink or buff cytochrome-rich pellets sedimenting more rapidly in the high-speed head, was not investigated in detail. The finding that the cytochrome-rich pellet may be washed free of arsenite dehydrogenase suggests that the particles may be different.

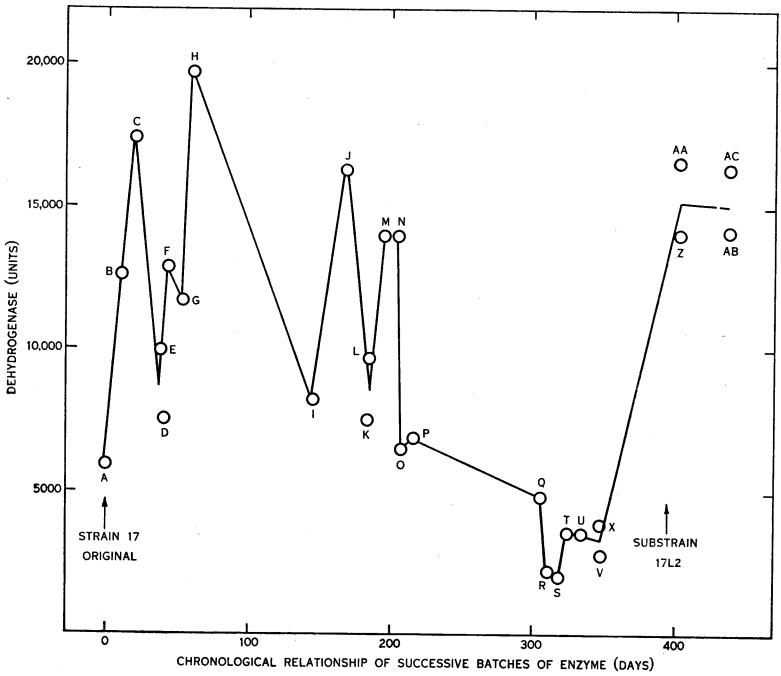

Fig. 1.—Secular variation in activity of 27 successive batches of arsenite dehydrogenase.

(*b*) *Secular Changes in Enzyme Yield*

When the procedure for fractionating the enzymes had been put on a routine basis, fresh preparations were made at intervals in order that stocks of enzyme could be accumulated.

Storage of packed washed cells at 4°C outside the limits of 5-8 days was rare, and appeared unrelated to the gross changes in enzyme yield found to occur. These are set out in Figure 1, and represent the arsenite dehydrogenase activity in the first extract (S_1) from the crushed cells. Essentially the same picture is obtained when the enzyme content of the second and subsequent extractions of the crushed cells is taken into account. The data must only be

considered as semi-quantitative, since it was not always possible to assay the enzyme activities of the extracts immediately after preparation, but deterioration at 4°C is usually slow (cf. Fig. 2).

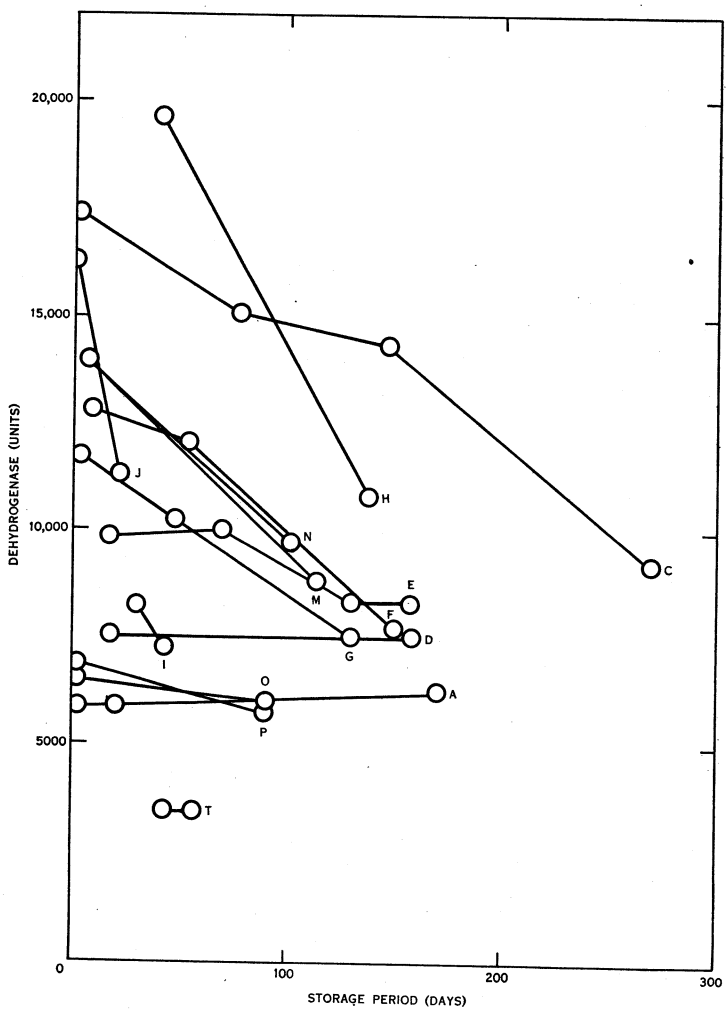

Fig. 2.—Stability of arsenite dehydrogenase on storage between −5°C and + 1°C.

Until mid 1949, the activity of the arsenite dehydrogenase varied between 6000 and 17,000 units in S_1; the succeeding seven preparations, however, gave extracts whose activity varied from 2000 to 5000 units. This was correlated with a drop in arsenite-oxidizing activity of the whole cells: cultures from which high yields of enzyme had been obtained were able to oxidize arsenite at rates varying from 70 to 140μM/mg N/hr, measured manometrically, whereas cells

from which enzymes *Q* to *X* had been prepared oxidized only 13-20μM/mg N/hr. The low yields of enzyme were, therefore, not due to incomplete extraction, but to a lowered production by the cell. The activity remained low not only in YA3, but in KA3, even when this was enriched with iron.

The strain then in use was then plated out, and separate colonies were subcultured in arsenite-Koser medium to which brom-phenol blue had been added (Turner 1954); substrains were then selected according to the speed of production of acid. In this way two substrains were isolated, 17*X* and 17*L*2, the former cells being able to oxidize arsenite at the rate of approx. 30μM/mg N/hr and the latter, approx. 100μM/mg N/hr. The secular change in enzyme yield from crushed cells is explained, therefore, by the transformation of the original strain 17 into the two variants 17*X* and 17*L*2, the former of these showing more rapid growth in the media used.

Like the original strain (Turner 1949), the arsenite dehydrogenase activity of both the new substrains is adaptive.

(*c*) *Characteristics of Crude Enzyme Solution*

So far, purification of the enzyme present in the crude extracts has not been attempted. The following observations refer, therefore, to enzyme solutions of unknown purity, and, probably, in view of the alterations observed in the strains of organisms used, of unknown homogeneity.

The stability of the enzyme appears maximum at about pH 6. When solutions were acidified, no change occurred down to pH 4·8, but at 4·2 the enzyme was totally precipitated. After standing at 23°C for 24 hr, the enzyme could be redissolved by adjusting the pH, over 70 per cent. of the activity being recovered. Some samples were stable to dialysis, whereas others lost up to 30 per cent. of their activity.

Dilute enzyme solutions have been observed to increase in activity on standing 24 hr at room temperature; some material precipitated out under these conditions.

Solutions of arsenite dehydrogenase stored between -5°C and $+1$°C in presence of toluene were periodically sampled. The data set out in Figure 2 show that the enzyme may be classed as relatively stable, and that the initial level of activity has no significant effect on the keeping quality. The one sample of enzyme (*T*) prepared from variant *X* appears to show stability of the same order as solutions obtained from earlier cultures.

(*d*) *Search for Inhibitors of Arsenite Dehydrogenase*

A number of enzyme inhibitors have been examined for influence on arsenite oxidation by intact cells (Turner 1949; Turner and Legge 1954). The most effective were those whose point of attack was on the oxidase end of the system. The separation of the dehydrogenase from the cells enabled further inhibitors to be examined without regard to the limitations of permeability, inseparable from experiments on intact cells.

Thunberg techniques were used since, in the presence of the dehydrogenase, arsenite reduces 2,6-dichlorophenol indophenol more readily than ferricyanide. A number of enzyme preparations were used. Substances which inhibited arsenite dehydrogenase activity of the clarified cell juice are listed in Table 1, and those which did not inhibit, or brought about an acceleration, are listed in Table 2.

TABLE 1

SUBSTANCES WHICH INHIBITED ARSENITE DEHYDROGENASE ACTIVITY OF CLARIFIED CELL JUICE

Enzyme	Substance	Molarity	Incubation Period	Activity $\frac{t \text{ control}}{t \text{ inhibitor}} \times 100$
19.x.48	Arsenate*	$4{\cdot}4\times10^{-3}$	Nil	93
		$4{\cdot}4\times10^{-2}$	Nil	86
19.x.48	Cu^{++}*	2×10^{-5}	Nil	96
		2×10^{-4}	Nil	75
		2×10^{-3}	Nil	33
N†	Hg^{++}	10^{-3}	—	94
	Cu^{++}	10^{-3}	—	58
V†	Hg^{++}	10^{-3}	—	89
	Cu^{++}	10^{-3}	—	35
T‡	*p*-Chlormercuribenzoate	10^{-3}	1 Hr§	46
M‡	*p*-Chlormercuribenzoate	10^{-3}	5 Hr§	55

* pH 5·9, buffer.

† Arsenite 20μM, indophenol 1μM, volume 2·5 ml, veronal buffer, pH 6·8.

‡ Arsenite 20μM, indophenol 1μM, volume 2·5 ml, phosphate buffer, pH 6·8.

§ Dialysed 16 hr.

No inhibition was observed when *p*-chlormercuribenzoate or *o*-iodobenzoate was added with the substrate, or was incubated for 15-20 min with enzyme before addition of arsenite. With the exception of Cu^{++}, therefore, none of the reagents reacts immediately with groups in the enzyme. The inhibition found with *p*-chlormercuribenzoate is apparently the result of a slower reaction which forms a compound stable to dialysis.

In a number of instances, the addition of substances examined for possible inhibiting action led to an enhancement of enzyme activity. The substance to be tested was always mixed with the enzyme in the Thunberg tube, and the reaction was started when temperature equilibration had occurred. Control measurements showed that the enhanced reduction of dye was not due to uncatalysed reactions with the added substance.

The possibility that the active centre contains a disulphide grouping was also considered. Five hours' incubation with 0·1M $NaHSO_3$ and subsequent dialysis, however, had no effect on enzyme activity.

IV. Discussion

The ease with which the enzyme may be separated from the crushed cells under conditions in which autolytic changes are minimum strongly suggests that the enzyme is not firmly bound to the cell wall, or to insoluble particles in the cytoplasm. Since crude enzyme exhibits little or no oxygen uptake, arsenite oxidation in the intact cell must involve a loose association between the enzyme and the cytochromes, or, alternatively, some intracellular carrier. Facts presented in a subsequent paper (Legge 1954) make the first hypothesis the more likely.

Table 2

SUBSTANCES WHICH FAILED TO INHIBIT, OR WHICH ACCELERATED, ARSENITE DEHYDROGENASE ACTIVITY OF CLARIFIED CELL JUICE

Enzyme	Substance	Molarity	Activity $\frac{t \text{ control}}{t \text{ addition}} \times 100$
*L**	Lewisite oxide†	1×10^{-3}	100
	Chloroacetophenone†	2×10^{-3}	100
*T**	*o*-Iodobenzoate‡	1×10^{-3}	100
19.x.48	Pyrophosphate§	2×10^{-2}	118
		1×10^{-1}	127
20.x.48	Pyrophosphate§	2×10^{-2}	114
		1×10^{-1}	138
20.x.48	Pyrophosphate‖	2×10^{-2}	95
		1×10^{-1}	87
19.x.48	Phenyl $HgNO_3$§	2×10^{-4}	106
		2×10^{-3}	158
19.x.48	NaF§	1×10^{-1}	116
20.x.48	NaF§	1×10^{-1}	120
20.x.48	Iodoacetate§	1×10^{-2}	189
19.x.48	Thiourea§	2×10^{-2}	195
20.x.48	Thiourea	2×10^{-2}	230
11.xi.48	$(NH_4)_2SO_4$	$0{\cdot}5 \times 10^{-1}$	167
		1×10^{-1}	201

* Arsenite 20μM, indophenol 1μM, volume 2·5 ml, phosphate buffer, pH 6·8.
† Incubated for 1 hr.
‡ Incubated for 1 hr, and dialysed for 16 hr.
§ pH 5·9.
‖ pH 7·0.

Some enzyme appears bound to particles sedimenting more slowly than the cytochrome-rich pellet. These may be the bacterial analogues of the mammalian microsomes (Schachman, Pardee, and Stanier 1952), and the small part of the enzyme attached to such particles may be the fraction which Brachet and Jeener (1944) suggested is still associated with the systems which synthesize it.

The secular variation in enzyme activity observed in the course of many preparations, which was traced to the emergence of a variant, could not be related to any alteration of conditions of culture. It was, in fact, observed that decreased production of arsenite dehydrogenase was associated with decreased production of cytochrome *a* (Legge 1954).

There was no good evidence for our initial hypothesis that – SH groups might be associated with the activity of the enzyme. Considerable inhibition was observed at a 2×10^{-3}M concentration of copper ion, but since none of the more powerful – SH reactants gave substantial inhibition, and some even led to activation, this result may be due to binding at some other site than – SH.

It is difficult to explain the activation observed with some substances. The compounds implicated are dissimilar in nature, including a number of anions as well as thiourea, a neutral substance. With the exception of phenylmercurinitrate, the substances were present in relatively high concentration. Possibly in some instances the activation may have been due to inactivation of inhibiting impurities present in the crude cell juice; that these occur is suggested by the observation that when enzyme solutions were left at room temperature, a precipitate tended to form, and that the activity then increased.

V. Acknowledgment

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