BACTERIAL OXIDATION OF ARSENITE

IV. SOME PROPERTIES OF THE BACTERIAL CYTOCHROMES

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Summary

Cytochromes *b* and *c* were observed in all substrains of *Pseudomonas arsenoxydans-quinque* examined; in some substrains, however, cytochrome *a* was not visible. Such strains poor in cytochrome *a* were less efficient arsenite oxidizers than those in which this cytochrome component was detectable.

The cytochromes were associated with the insoluble fraction obtained after grinding the packed cells with powdered glass, only traces of cytochrome *c* being found in the soluble fraction.

A significant rate of arsenite oxidation in systems reconstructed from dehydrogenase and oxidase was observed either when both fractions were present in relatively high concentration (about one-seventh that within the cell) or when an artificial carrier (2,6-dichlorophenol indophenol) was present.

Mammalian cytochrome *c* proved ineffective as a carrier. No evidence could be obtained that any natural carrier was present, and calculations indicate that diffusion of the arsenite dehydrogenase-arsenite complex could account for the oxygen uptake found in intact cells without any more readily diffusible electron carrier.

I. INTRODUCTION

A previous paper (Legge and Turner 1954) described some of the properties of the crude arsenite dehydrogenase which can be extracted from strains of *Pseudomonas arsenoxydans-quinque* grown in the presence of arsenite. The present paper describes some properties of the bacterial cytochromes in intact cells or in the sedimentable fractions obtained during the purification of the oxidase.

The observations have thrown some light on the extent of the changes observed during the variation in characteristics of the strain, reported in the above paper, but do not explain the altered sensitivity to carbon monoxide referred to in an earlier paper (Turner and Legge 1954).

II. METHODS

General details for cultivation and harvesting of arsenite-oxidizing pseudomonads are given in earlier papers in this series.

The crude cytochrome oxidase fractions used in the present paper were obtained from the same batches of harvested cells used by Legge and Turner

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(1954) for the preparation of crude arsenite dehydrogenase. Fraction P_1 consisted largely of easily sedimentable cell debris (3500 r.p.m. for 30 min in the Wifug angle centrifuge); fraction S_1P, a mitochondria-like fraction, was thrown down as a reddish translucent pellet when the supernatant from P_1 was spun in a high-speed centrifuge (60 min at an average of 19,000g).

Spectroscopic observations were made with a Hartridge (Beck) or a calibrated small dispersion microspectroscope. Rate of reduction of cytochrome *c* was measured in a DU model Beckman spectrophotometer. Oxygen uptakes were measured by conventional manometric methods.

III. Results

(*a*) *Spectroscopic Observations*

Cytochromes *b* and *c* were invariably present when cells were examined after reduction with buffered arsenite. The *c* component was generally much stronger than the *b*, the α band of the *c* lying at 552 mμ.* Direct comparison left no doubt that the α band of bacterial cytochrome *c* lies slightly to the red side of that of cytochrome *c* of bakers' yeast or ox heart. Bacterial cytochrome *b* lay at about 560 mμ, but accurate measurement was not possible. Where cytochrome *a* was present, the α band lay at 600-603 mμ. It was not possible to observe any splitting of this band in the presence of CO and arsenite, even when the specimens were examined after freezing in liquid air, nor was any change observed with cyanide. Cytochrome a_2 was not seen. In the presence of pyridine and $Na_2S_2O_4$, the band of the pyridine haemochrome of cytochrome *a* was observed at 589 mμ.

If small amounts of arsenite were used, the cells could readily be oxidized on shaking with air. In agreement with the earlier deductions from the redox potential of the arsenite-arsenate system (Turner 1949), the addition of arsenite to oxygenated cells led to the appearance of the *c* band, followed by *b* and *a*. In the presence of malate, which is readily oxidized by the organisms, reduced *b* appears before *c*.

Spectroscopic examination of fractions *P* and S_1P, after fragmentation of the cells, added little to the above, although in some cases the S_1P fraction was richer in cytochromes than the *P* fraction. $Na_2S_2O_4$ produced immediate reduction, arsenite and its dehydrogenase a somewhat slower reduction. Attempts to observe preferential reduction of cytochrome *a* by leuco-indophenol failed.

The crude dehydrogenase solution clarified by high-speed centrifugation was greyish brown in colour and contained traces of cytochrome *c*.

The most interesting feature of the spectroscopic examination lay in the difference between the cytochromes in cells from strains with high or low arsenite-oxidizing capacity. The observations are summarized in Table 1.

* We have referred throughout this series of papers to the cytochrome component absorbing maximally at 552 mμ as cytochrome *c*. Chin (1952) and Ryley (1952) refer to a component in the cytochromes of *Aerobacter* and *Tetrahymena pyriformis*, respectively, absorbing at 551-553 mμ, as cytochrome *e*, and restrict cytochrome *c* to the component absorbing at 550 mμ.

Stored cells (*R* to *X* inclusive), which were cultivated during the period of lowered arsenite dehydrogenase activity, were pink in colour, and contained only traces of cytochrome *a*. When substrain 17*L*2 was isolated from the cultures at this stage, it showed the original arsenite dehydrogenase activity, was brown in colour, and contained considerable amounts of cytochrome *a*.

TABLE 1

VARIATIONS OF CYTOCHROMES IN DIFFERENT BATCHES OF *PSEUDOMONAS ARSENOXIDANS-QUINQUE*

Source	Arsenite-oxidizing Activity	Cytochromes*	Colour of Preparation
Stored cells			
F	7700†	*c*, *b*, *a*	Brown
R	2200†	*c*, *b*, *a* absent	Extremely pink
S	2200†	*c*, *b*, trace of *a*	
T	3500†	*c*, *b*, trace of *a*	
V	2800†	*c*, *b*, *a* absent	
X	3900†	*c*, *b*, trace of *a*	
Z (substrain *L*2)	14,100†	*c*, *b*, *a*	Brown
AA	16,600†	*c*, *b*, *a*	
AB	14,200†	*c*, *b*, *a*	
AD	—	*c*, *b*, *a*	
Fresh cultures, May 1950			
Substrain *L*2, KA3	Moderately active	*c*, *b*, *a*	Brown
Substrain *L*2, YA3	Moderately active		
Substrain *L*2, K (no arsenite)	Inactive		
Substrain *X*, KA3	Poorly active	*c*, *b*, trace of *a*‡	Very pink
Substrain *X*, YA3	Poorly active	*c*, *b*, *a* absent‡	Very pink
Substrain *FB*, YA3§	Poorly active	*c*, *b*, *a* absent	
Fresh cultures, June 1950			
Substrain *L*2, KA3	180 μM As/mg N/hr‖	*c*, *b*, *a*	Brown
Substrain *X*, KA3	166 μM As/mg N/hr‖	*c*, *b*, *a*	Brown

* Cells or oxidase fraction observed in 1-2 mm deep paste in reduced state at room temperature, and immediately after being cooled to the temperature of liquid air.

† Measured anaerobically (cf. Legge and Turner 1954).

‡ These cells oxidized only slowly on shaking with air.

§ A large-celled variant isolated at the same time as substrains *L*2 and *S*.

‖ Manometric measurement in air.

The other substrain, 17*X*, isolated at the same time and thought to be the main organism, gave harvests of cells low in cytochrome *a*, and with low arsenite-oxidizing activity, in May 1950; but after further subculturing it regained its high activity, and became indistinguishable spectroscopically from 17*L*2, although still distinguishable from this substrain by colonial habit.

(*b*) *Specificity of Bacterial Cytochromes*

The work of Keilin and Hartree (1930) and of Slater (1949) provides the basis for tests of the donor specificity of cytochromes. The bacterial oxidases, however, differed from the mammalian in a number of ways.

Although cytochrome *c* in intact, adapted cells is readily reduced by arsenite, only traces appear to be liberated into solution when cells are disrupted. Nor could it be prepared from the crude bacterial oxidase; various treatments, including trichloracetic acid, strong salt solutions, and bile salts, were invariably unsuccessful in bringing any of the cytochrome *c* into solution.

TABLE 2

SUBSTRATE SPECIFICITY OF DIFFERENT SAMPLES OF STORED OXIDASE

Oxidase	Age (days)	Oxygen Uptake (μl O_2/mg N/hr†) on the Following Substrates*				Cytochrome *a*
		aa+*c*	*ppd*	*ppd*+*c*	*aa*+*ip*	
Fresh, partially purified ox heart	2	3150	2620	2820	3020	Present
AC‡	30	42	910	1060	1740	Not observed
AD	25	160	1080	—	3060	Present
AE	25	304	330	—	4060	Absent
R§	120	100	320	—	3500	Absent
S	112	220	560	—	4250	Trace

* *aa* (ascorbic acid 17×10^{-3}M), *c* (cytochrome *c* $0{\cdot}9\times10^{-4}$M), *ppd* (*p*-phenylene diamine 50×10^{-3}M), *ip* (2, 6-dichlorophenol indophenol $0{\cdot}6\times10^{-3}$M).

† Measurements in phosphate buffer, pH 6·8, 0·07M, 38°C, the gas phase was air.

‡ Fifth to seventh cultures respectively after isolation of substrain *L*2.

§ Oxidase at lowest point of arsenite dehydrogenase production.

Since preparations of cytochrome *c* from such widely different sources as wheat germ and mammalian heart have not been shown to differ markedly in specificity, attempts were made to use ox and horse-heart cytochrome *c* with the bacterial oxidase. The added carrier was found to have little or no effect on the rate of oxidation of *p*-phenylene diamine (cf. Table 2). The oxidase, however, was unsuccessful in bringing about any oxidation of ascorbic acid without added mammalian cytochrome *c*. In regard to the accessibility of the bound cytochrome *c* to those two donors, the bacterial oxidase resembles that of mammalian tissues. In investigations of the variation of the oxidase fraction in the different substrains, mammalian cytochrome *c* was therefore added when ascorbic acid was used, whereas *p*-phenylene diamine was used without added carrier.

In view of the ability of arsenite dehydrogenase to reduce 2,6-dichlorophenol indophenol, and of cytochrome oxidase to oxidize indophenols (Keilin 1925), a

donor system comprising ascorbic acid and 2,6-dichlorophenol indophenol was also tested on mammalian and bacterial oxidases. Table 2 assembles results with these systems obtained in the course of analysis of stored oxidase preparations.

Comparison between the mammalian oxidase and the bacterial oxidases on the basis of their ability to oxidize leuco-2,6-dichlorophenol indophenol is of some interest. While the fresh mammalian specimen gives approximately the same activity whichever hydrogen donor system is used, the bacterial oxidase shows a far greater activity with the leuco dye as substrate, independent of the presence or absence of cytochrome *a*. Since the high redox potential of the indophenol-leuco-indophenol system makes it extremely unlikely that it is oxidized by enzymes other than those with redox potentials of cytochrome *a* level or above, such an oxidase, as yet undetected spectroscopically in these preparations, may be present.

Data obtained with oxidase from freshly harvested cells, prepared by grinding once-washed cells with powdered glass, are set out in Table 3.

TABLE 3

SUBSTRATE SPECIFICITY OF OXIDASE FROM FRESHLY HARVESTED CELLS

Oxidase from Substrain‡	Medium	Oxygen Uptake (μl O_2/mg N/hr†) on Substrates*		Intact Cells Plus Arsenite
		aa+*c*	*ppd*	
F_B	YA3	260	620	130
17*X*	K(no As^{--})	880	1480	59
17*X*	KA3	970	890	234
17*L*2	K(no As^{--})	880	2340	0
17*L*2	KA3	450	1980	370
17*L*2	YA3	170	1680	590

* *aa* (ascorbic acid 17×10^{-3}M), *c* (cytochrome *c* $0{\cdot}9\times10^{-4}$M), *ppd* (*p*-phenylene diamine 50×10^{-3}M).

† Measurements in phosphate buffer pH 6·8, 0·07M, 38°C, the gas phase was air. Uptake corrected for autoxidation of hydrogen donors.

‡ Cultivated May 1950, same samples as are referred to in Table 1.

The results in Table 3 indicate that the $Q_{O_2 ppd}/Q_{O_2 aa+c}$ ranged from 2·7 to 9·7 in the active 17*L*2 substrain, and from 0·9 to 2·4 in substrains 17*X* and 17*FB*, which were less effective arsenite oxidizers. The values of the arsenite oxidation by intact cells, however, given in the last column of the table show little correlation with the oxidase assays.

The activities with *p*-phenylene diamine and ascorbic acid-cytochrome *c* as substrates seem somewhat higher than those recorded with aged oxidase in Table 2, suggesting that there is an increasing impairment of function with age.

Ranking the $Q_{O_2 ppd}$ (independently of substrain or culture medium) of those oxidase fractions for which spectroscopic observations on the presence of cytochrome *a* are available gives the arrangement in Table 4.

TABLE 4

ASSOCIATION BETWEEN CYTOCHROME *a* AND RATE OF OXIDATION OF *p*-PHENYLENE DIAMINE

μl O_2/mg N/hr	Cytochrome *a*
320	−
330	−
560	±
620	−
890	±
1080	+
1680	+
1980	+
2340	+

−, Not visible; ±, visible with difficulty in thickest parts; +, readily detectable.

This is in accord with the hypothesis that the activity against *p*-phenylene diamine is proportional to the cytochrome *a* content. The $Q_{O_2 aa+c}$ shows no such correlation with the spectroscopic appearance of the preparations.

(*c*) *Reconstructed Oxidase Systems with Carriers*

No oxygen uptake was observed in preliminary experiments in which arsenite, arsenite dehydrogenase, and bacterial oxidase were shaken with air.

Replacement of phosphate by veronal buffer, or inclusion of calcium or magnesium, or both, did not lead to any increase in activity of the reconstructed system. No activation was found when extracts from boiled pseudomonads, yeast, guinea pig heart, or liver were used, or when mammalian cytochrome *c* was added. Since the bacterial oxidase is able to oxidize mammalian cytochrome *c*, and since arsenite is able to reduce the bacterial cytochrome *c* in the intact cell, the above result must be due to the slow rate of reduction of mammalian cytochrome *c* by the dehydrogenase.

This is borne out by a comparison between the rate at which the dehydrogenase reduces ferricyanide, measured manometrically in bicarbonate buffer, and the rate at which it reduces ferricytochrome at the same pH, measured spectrophotometrically. Ferricyanide (2×10^{-2}M) is reduced 10 times faster than $0{\cdot}6 \times 10^{-3}$M cytochrome *c*, and about 100 times faster than $0{\cdot}9 \times 10^{-5}$M cytochrome *c*.

Since arsenite readily reduces 2,6-dichlorophenol indophenol in the presence of the dehydrogenase, and since the oxidase fraction is able to oxidize an

indophenol-ascorbic acid hydrogen donor system extremely rapidly, the dye may be used as a carrier between the arsenite dehydrogenase and the oxidase. Such an experiment is set out in Table 5.

With indophenol it is thus possible to use mammalian cytochrome oxidase to oxidize arsenite in the presence of the bacterial dehydrogenase.

Since the data in Table 5 show that oxidation proceeds rapidly in a dilute system with an artificial carrier, further attempts were made to prepare cell-free suspensions under conditions which would favour the preservation of any labile natural carrier, or which would reduce to a minimum changes in the cytochromes which might render them inaccessible to the dehydrogenase systems.

TABLE 5

RECONSTRUCTED ARSENITE-OXIDIZING SYSTEM WITH 2,6-DICHLOROPHENOL INDOPHENOL AS HYDROGEN CARRIER

Ox	*d*	As^{---}	*ip*	*ppd*	$Fe(CN)_6^{---}$	Oxidation (μ-equiv./hr)
−	+	+	−	−	+	2·5
+	−	−	−	+	−	7·3
+	+	+	−	−	−	0
+	+	+	+	−	−	5·9

Ox = oxidase Zp, 0·2 ml; *d* = arsenite dehydrogenase Zs, 0·025 ml; $As^{---} = 0{\cdot}05M$ arsenite; *ip* = 2,6-dichlorophenol indophenol, $1 \times 10^{-3}M$; *ppd* = *p*-phenylene diamine, $5 \times 10^{-2}M$; phosphate 0·07M; pH 6·8; 38°C; air in gas phase.

$K_3Fe(CN)_6 = 2 \times 10^{-2}M$; HCO_3^- buffer; N_2/CO_2 gas phase; 2·3 ml final volume.

The preparation of oxidase and dehydrogenase fractions from the bacteria generally involved some hours of manipulation at room temperature, although they were ground and subsequently stored at 0°C. Since these conditions might possibly have lead to enzymic destruction of part of the oxidase, bacteria were hand-ground with glass at 0°C; the debris was suspended and immediately transferred to manometric vessels, and arsenite oxidation was measured as soon as temperature equilibration had been reached. The rate of oxidation was found to be only 1/600 that of the intact cells. The results were the same when the cells were ground in the presence of 0·02M nicotinamide.

(*d*) *Reconstruction of Concentrated Systems Without Carriers*

While not providing unequivocal grounds for rejecting the hypothesis of exceptionally labile carriers, or uncontrolled alteration of the cytochromes, the above results suggest consideration of alternative hypotheses, such as the possibility that oxidation in the intact cell involves the diffusion of dehydrogenase-arsenite complex to the cytochromes, and that the failure to find oxidation in disrupted cell suspensions is due primarily to dilution effects.

This could be tested by measuring the oxygen uptake in extremely concentrated systems. Oxidase and dehydrogenase fractions from cultivation *AC* of

substrain 17*L*2 were used after 14 days storage at 0°C. Both fractions were dialysed for 24 hr against water at 0°C in order to remove traces of metabolites or diffusible carriers. The activity per mg N of the oxidase was unaltered by this treatment, that of the dehydrogenase going up somewhat due to the loss of some low-molecular-weight nitrogenous compounds. After dialysis the dehydrogenase fraction represented a dilution about five times the volume of the original cells, whereas the oxidase fraction was diluted after dialysis to a pipettable consistency. A mixture of the two, in the volume ratio of 0·4 oxidase to 0·6 dehydrogenase, was then introduced undiluted, and diluted 2-, 4-, 8-, and 16-fold into manometric vessels. Strong phosphate buffer to give a final concentration of 0·07M, pH 6·8, was added, and arsenite was placed in the side-arms. To the vessel containing the 16-fold dilutions was added 10^{-3}M 2,6-dichlorophenol indophenol. Additional samples of 16-fold diluted solutions were assayed for dehydrogenase activity with ferricyanide, and for oxidase activity with indophenol and ascorbic acid. After 30 min equilibration to estimate blank respiration, arsenite was tipped in, and the measurements were continued for 10 hr, in the 4- and 8-fold dilutions. The uptakes remained perfectly linear over this period, indicating that growth of any organisms left after the crushing could be neglected. The results are set out in Table 6.

TABLE 6

RECONSTRUCTED ARSENITE-OXIDIZING SYSTEM WITHOUT ADDED HYDROGEN CARRIER

	Arsenite Oxidation		
Dehydrogenase : oxidase = 6/4	Rate (μl O_2/hr)	Enzyme N/Vessel (mg)	μl O_2/mg N/hr
Undiluted	273	6·68	38
Twofold dilution	86	3·54	24
Fourfold dilution	26	1·82	14
Eightfold dilution	12	0·99	11
16-fold dilution (indophenol as carrier)	89	0·50	178
16-fold dilution (ferricyanide as acceptor)	52*	0·50	104
16-fold dilution (indophenol and ascorbate as donors)	350	0·50	700

* Recalculated from CO_2 output.

The above figures are not corrected for the blank oxygen uptake. This presents some difficulty: from a knowledge of the total uptake to be expected from the amount of added arsenite, it can be calculated that the oxygen uptake due to hydrogen donors other than arsenite is decreased from 11 μl O_2/mg N/hr in its presence. The uptake observed in the 16-fold diluted vessel in the presence of indophenol indicates the inefficiency of oxidation in the unsupple-

mented vessels. Owing to an oversight, control measurements of the arsenite-oxidizing capacity of the separate fractions were omitted. However, if the uptake were due to a small amount of "active" dehydrogenase-oxidase complex, dissociable with difficulty, in either fraction, the activity should be independent of the dilution, and should not go up with increasing concentration.

The oxygen uptake with indophenol-ascorbic acid, recorded in the last column of Table 6, indicates that some component of the oxidase capable of oxidation at appropriate redox level is present in great excess at all dilutions.

The nitrogen content of the solutions containing the undiluted enzymes corresponds approximately to a seven-fold dilution of bacterial cell contents. If the reconstructed system differs from that within the intact cell only by being more dilute, one would arrive at a figure of 1860 μl O_2/mg N/hr ($7^2 \times 38$ μl O_2/mg N/hr) for the uptake of intact cells on the assumption of an approximate proportionality between activity and the square of reactant concentration (cf. Table 6). This should be compared with the observed figure of 810 μl O_2/mg N/hr. When the treatment to which the *AC* cells were subjected during fractionation is recalled, as well as the likelihood that much of the oxidase may be enveloped in structural debris, the calculated activity might well have been expected to be less than the observed activity. The fact that the latter is smaller could be due to higher intracellular viscosity or to non-uniform distribution of oxidase. When these considerations, for which adequate quantitative correction cannot be made, are taken into account, the knowledge that the observed figure disagrees with that calculated by a factor of only two is not unsatisfactory.

IV. Discussion

If there is no diffusible electron carrier between arsenite dehydrogenase and oxidase, it is of interest to calculate whether diffusion of the activated arsenite dehydrogenase within the cell is sufficient to account for the observed oxygen uptake in the presence of arsenite.

In view of the simplifying assumptions necessary, such a calculation can only be expected to establish limits within which diffusion between large molecules would not be the limiting factor. Arsenite oxidation at rates of 100μM/mg N/hr in the presence of air may be achieved with present strains. The oxidase may be considered saturated at this oxygen pressure, because little increase in oxidation rate is observed in 100 per cent. O_2, and at 4 per cent. O_2 the rate only drops to 60 per cent. of that in air. Data presented earlier (Turner and Legge 1954) show that arsenite is oxidized to completion without much falling off in rate, even at substrate concentrations far below the optimum. This suggests that the permeability of the cell wall to arsenite is not a limiting factor under conditions in which uptakes of 100μM/mg N/hr were recorded.

Let us assume that the oxidase is evenly distributed throughout the cell, and is saturated with oxygen. Further, let as assume that the limiting factor within the cell is the diffusion of the activated arsenite-dehydrogenase complex to the oxidase.

The appropriate diffusion equations, which set limits to the radii of cylindrical and spherical organisms beyond which a diffusible compound being consumed uniformly throughout the mass of the organism does not reach the central region, are

$$r = \sqrt{4c\ D/A}$$

and

$$r = \sqrt{6c\ D/A},$$

respectively, where r is the radius, c is the concentration of metabolite, A is the rate at which the metabolite is being consumed, and D is the diffusion constant of the metabolite.

Since the arsenite-dehydrogenase complex exists only within the cell, c refers to the concentration at the outermost region of the cytoplasm. It is assumed that $D = 7 \times 10^{-7}$ cm^2 sec^{-1} for the arsenite-dehydrogenase complex, this being of the order for spherical proteins of molecular weight 40-50,000.

The organism is a rod, 2-3 μ long, and about 1 μ diameter, and its critical radius would probably be better described by an equation

$$r = \sqrt{5c\ D/A},$$

the constant being arbitrarily taken as lying between that for a rod, 4, and for a sphere, 6.

The oxygen uptake is equivalent to 100μM/mg N/hr. Converting this to moles cm^{-3} sec^{-1}, and assuming the nitrogen content to be 2 per cent. of the wet weight, a value is reached for $A = 5{\cdot}5 \times 10^{-7}$ moles cm^{-3} sec^{-1}. Inserting this value for A, 7×10^{-7} cm^2 sec^{-1} for D, and $0{\cdot}5 \times 10^{-4}$ cm for r, in the converted equation

$$c = \frac{r^2 A}{5D},$$

we obtain

$$c = \frac{2{\cdot}5 \times 10^{-9} \times 5{\cdot}5 \times 10^{-7}}{5 \times 7 \times 10^{-7}} \text{ moles cm}^{-3}$$
$$= 3{\cdot}9 \times 10^{-10} \text{ moles cm}^{-3},$$

or a concentration of activated enzyme-substrate complex of $3{\cdot}9 \times 10^{-7}$M.

The wet organism contains about 13 per cent. total protein, and 5 per cent. soluble protein. The assumption of a mean molecular weight of 48,000 for the latter leads to a figure for the molarity of $1{\cdot}0 \times 10^{-3}$, in which case the enzyme would represent a fraction of only $3{\cdot}9 \times 10^{-4}$ of the total soluble protein, or $3{\cdot}9 \times 10^{-2}$ per cent.

Few readily accessible data are available on the enzyme content of microorganisms expressed in units of mass rather than in units of activity. The above estimate that the arsenite dehydrogenase content of *Pseudomonas arsenoxydans-quinque* is $3{\cdot}9 \times 10^{-2}$ per cent. of the soluble protein would appear reasonable. It is certainly far lower than the catalase content of *Micrococcus lysodeicticus* found by Herbert and Pinsent (1948); this was 1-2 per cent. of the dry weight, or of the order of 2-4 per cent. total protein or 4-8 per cent. soluble protein.

Certain of the assumptions made in deriving the estimate may now be reconsidered. It is obvious that it makes little difference whether diffusion

formulae appropriate for cylinder or sphere are taken as the basis of the calculation. If the enzyme is assumed to be very much larger and to have a diffusion constant one-tenth that of a spherical protein of molecular weight 48,000, it would still represent only 0·39 per cent. of the total soluble cell protein.

The oxidase was assumed to be evenly distributed throughout the body of the cell. The fact that little may be separated from the insoluble fraction of crushed cells, a great part of which may be cell wall, suggests that the oxidase may be found in higher concentration at the periphery of the cell. This situation would require a different diffusion equation from the one used, but it is unlikely that this would lead to impossibly high values for the arsenite dehydrogenase content. If the dehydrogenase was loosely attached to oxidase within the cell, a far lower concentration would be needed to give the rates of arsenite oxidation found, irrespective of whether the oxidase is assumed to be distributed peripherally or evenly within the cell.

The calculation has been made on an arsenite oxidation rate of 100μM/mg N/hr found for the strains under present cultivation. The calculated enzyme content is so low that increased amounts could well be present in strains with greater arsenite-oxidizing ability.

The conclusion from diffusion theory therefore does not conflict with that reached from experiments on reconstructed systems, namely, that the observed oxygen uptakes in intact cells may be accounted for without postulating a diffusible carrier between dehydrogenase and oxidase.

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