BACTERIAL OXIDATION OF ARSENITE

II. THE ACTIVITY OF WASHED SUSPENSIONS

By A. W. TURNER* and J. W. LEGGE†

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Summary

Maximum arsenite-oxidizing activity of a pseudomonad (*Ps. arsenoxydans-quinque*) isolated from "oxidized" arsenical cattle-dipping fluids was found in cells 3-4 days old, when cultivated in a synthetic medium containing arsenite. The system is adaptive, cells cultivated in the absence of arsenite showing no arsenite-oxidizing activity.

The oxidizing system in cells harvested at peak activity was optimally active in air at pH 6·4 and 40°C, when tested in several different buffers. It was inhibited by cyanide and azide. Light-sensitive CO inhibition was demonstrated 6 months after the strain was first isolated, but 22 months later, after much subculturing, this property had been virtually lost. Inhibition was also observed with fluoride and pyrophosphate. Certain dicarboxylic acids produced a temporary inhibition between pH 4 and 5. Iodoacetate, diethyldithiocarbamate, α,α'-dipyridyl, and urethane were without effect.

Cells were unable to oxidize arsenite in presence of air after treatment with toluene, acetone, or after being subjected to desiccation.

Since adapted cells can oxidize arsenite anaerobically in the presence of suitable acceptors, it is concluded that the complete system includes an arsenite dehydrogenase and cytochromes.

I. INTRODUCTION

In a previous communication a description was given of 15 strains of arsenite-oxidizing bacteria, comprising five types, isolated from "oxidized" cattle-dipping fluids in Queensland (Turner 1954). Since tervalent arsenite is known to inactivate widely distributed enzymes whose activity depends on $-SH$ groups (Stocken and Thompson 1949), study of the arsenite-oxidizing mechanism of these bacteria, most of which are pseudomonads, may not only be of theoretical biochemical interest, but may throw light on the mechanism by which other microorganisms develop resistance to arsenite and its derivatives.

This paper is concerned with the conditions for obtaining cells with maximum arsenite-oxidizing activity, and with some of the properties of the system in intact cells.

II. METHODS

The experiments were carried out with strain 17, which has been allotted to the species *Pseudomonas arsenoxydans-quinque* (Turner 1954); it grows well,

* Division of Animal Health and Production, C.S.I.R.O., Animal Health Research Laboratory, Parkville, Vic.

† Department of Biochemistry, University of Melbourne.

and provides a high yield of very active cells. Usually these were grown in liquid cultures in KA3 medium (Turner 1954), i.e. Koser citrate medium containing 0·02M $NaAsO_2$. Centrifuged cells could be stored at 4°C for some weeks with very little loss of activity. For use, they were washed, usually once, in the original volume of 0·067M phosphate buffer (pH 6·4), and finally were resuspended in the appropriate buffer. The turbidity was measured in a photoelectric absorptiometer, and in most experiments the volume was adjusted with buffer to a cell concentration containing 0·13 mg dry weight per ml.

The velocity of arsenite oxidation was followed by means of iodometric titration; aeration was obtained by shaking the suspensions in rubber-stoppered 50-ml flasks with a Microid shaker, samples being withdrawn at intervals for titration. Arsenite oxidation was also followed by conventional manometric methods, and by dye reduction in Thunberg tubes.

Strain 17, which was isolated in December 1947, had been maintained by irregular subculturing in KA3 medium. Most of the work reported in the preliminary publication (Turner 1949), including the CO inhibitions, was carried out by June 1948. The investigation of soluble arsenite dehydrogenase was begun in 1949. By October 1949, it was found that cells were giving very low yields of arsenite dehydrogenase, and examination revealed two main colonial types in the culture, of which substrain *L*2 was selected as highly active for this purpose. In March 1950 when the work on CO inhibition was taken up again, it was found that the phenomenon could be obtained only very irregularly with this substrain, which appears to differ from the original isolation.

III. Results

(*a*) *Conditions for Cultivation of Cells with Optimum Arsenite-oxidizing Activity*

(i) *Influence of Age of Culture.*—The arsenite-oxidizing activity per unit mass of cells was found to remain virtually constant for the first 3 days, and then to fall as the incubation period at 25°C increased. In most of the experiments cells were therefore harvested from young cultures, usually not more than 4 days old.

(ii) *Influence of Culture Medium.*—The most satisfactory medium we have used is the simple KA3 medium in which the carbon source is citrate and the nitrogen source is NH_4^+; the yield of cells is not as high as in other media examined, but their arsenite-oxidizing activity is higher. Although meat infusion broth containing 0·02M arsenite produces very heavy yields of cells, the enzymic activity per mg dry weight was only about 10 per cent. of those harvested from KA3 medium. In 1·5 per cent. Difco yeast extract solution containing 0·2 per cent. mixed KH_2PO_4-Na_2HPO_4 buffer salts for pH 7·4, 0·02 per cent. $MgSO_4.7H_2O$, and 0·02M $NaAsO_2$, a high yield of cells was obtained, but with only 40-50 per cent. of the relative activity; this medium, referred to as YA3, corresponds to KA3 in which NH_4^+ is replaced by more complex N sources. The same medium containing in addition 0·15 per cent. $NaNH_4HPO_4$ as in KA3 medium, also gave an excellent yield of cells, but the relative activity was only

30-35 per cent. It appears that the enzyme is formed in greatest amount when the N source is simple, as in NH_4^+, and that it is depressed by "richer" nitrogenous media.

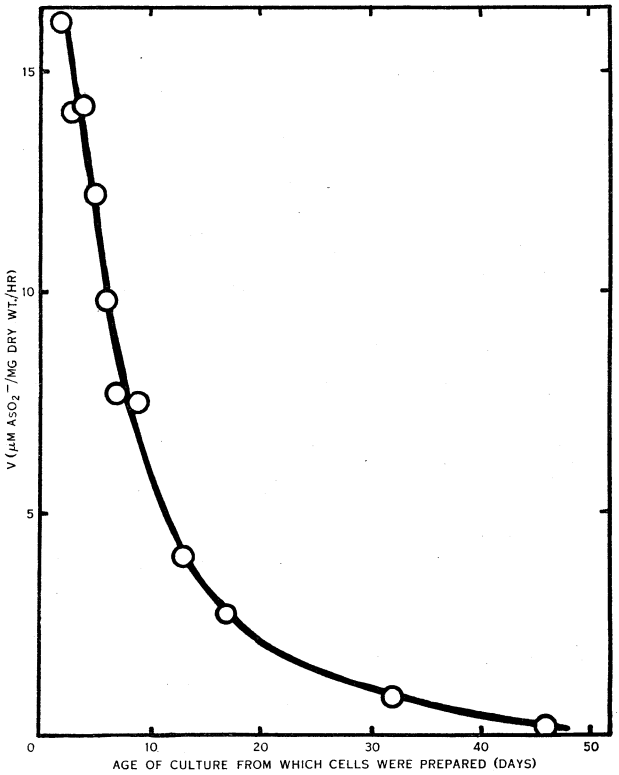

Fig. 1.—Variation of arsenite-oxidizing activity with age of the culture from which the cells were harvested.

(iii) *Adaptive Nature of the Enzyme.*—Cells grown in Koser medium without arsenite, or with 0·02M arsenate instead of arsenite, had no detectable initial arsenite-oxidizing activity, whether they were descended from cells routinely subcultured in the presence of arsenite or in its absence.

In some experiments it was observed that cells grown in the absence of arsenite developed slight activity after a lag period of 60 min or more. Although the post-lag development of activity was slight, it was about twice as great if the cultures yielding the cells had been sown with adapted cells.

These observations suggest the adaptive formation of enzyme, without cell multiplication, from reserve cell materials in the presence of substrate. When a small amount (10 per cent.) of Koser medium was added to the cell-buffer-arsenite system, the optical density doubled after 7 hr shaking at 20°C, indicating a twofold multiplication of cells, but the arsenite-oxidizing activity increased about 28 times compared with that of cells shaken in a system without added nutrient. This suggests adaptive enzyme formation during cell growth.

Cells grown in KA3 medium were equally active, whether the medium had been sown with strains continuously subcultured in medium containing arsenite, or not containing arsenite. This indicates the rapid adaption to arsenite oxidation.

(b) Conditions for Optimum Activity of Washed Cells

(i) *Suspending Buffer.*—The velocity of oxidation by cells washed repeatedly with distilled water was the same, whether they were suspended in 0·05M collidine buffer (Gomori 1946) or in 0·067M phosphate buffer at the same pH (6·4). Evidently an external source of phosphate is not required for arsenite oxidation. In the universal buffer of Teorell and Stenhagen as modified by Östling and Virtama (1946), which contains borate and citrate in addition to phosphate, the initial velocity was likewise the same; after prolonged contact, however, the velocity increased, probably through utilization of citrate followed by consequent cell multiplication, with increased enzyme formation in the presence of arsenite.

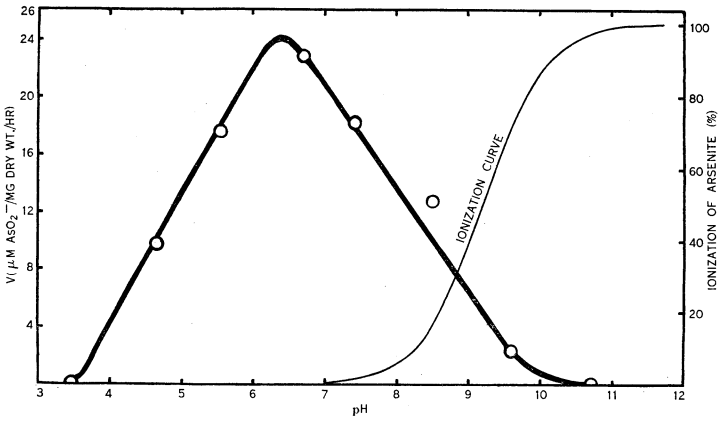

Fig. 2.—Variation of arsenite-oxidizing activity with pH, compared with the dissociation curve of $HAsO_2$.

(ii) *pH Optimum.*—This was determined from the initial rates of oxidation of 0·02M arsenite by aerated washed suspensions in Teorell and Stenhagen's universal buffer, at virtually constant ionic strength, over the pH range 3-11 at 20°C. The reaction was followed by iodometric titration. The values obtained when velocity was plotted against the pH are illustrated in Figure 2, from which it is evident that under these conditions there is a fairly sharp optimum at pH 6·2-6·6. The suspensions held at extreme pH's in these experiments were not tested for viability; but the organism is able to grow in the presence of arsenite between pH 9·5 and 4·5, the growth being very slow at the acid pH. The pH optimum was not significantly altered when the cells tested were harvested from arsenite-containing media in which heavy buffering maintained different pH's during growth.

On the same graph is plotted also the percentage of ionized molecules of sodium arsenite, calculated from the Henderson equation with the pK_a of arsen-

ite as 9·22. It is seen that at pH values up to optimum, virtually all the arsenite exists as undissociated molecules. However, since the activity falls off beyond the optimum pH more rapidly than can be accounted for by the ionization of arsenious acid, the drop of activity is therefore primarily due to effects on the enzyme system.

The same pH optimum was found when the oxygen uptake during arsenite oxidation by washed cell suspensions was measured manometrically.

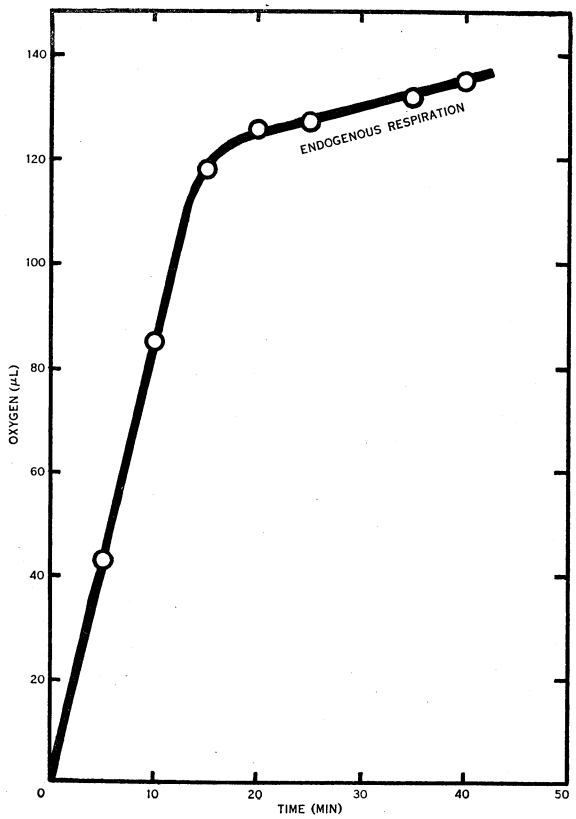

Fig. 3.—Time course of oxidation of 5×10^{-4}M arsenite.

(iii) *Substrate Concentration.*—Figure 3 indicates the course of oxygen uptake when $4{\cdot}3 \times 10^{-3}$M arsenite was oxidized by cells. The velocity of oxidation fell off appreciably only after 90 per cent. of the arsenite had been oxidized.

Figure 4 indicates the rate of oxygen uptake measured immediately after tipping various quantities of arsenite into the vessels. Up to a concentration of 9×10^{-4}M arsenite the rate was virtually a linear function of concentration and at that molarity it appeared to reach a limiting velocity. Half-maximum rate was reached when the concentration of arsenite equalled $4{\cdot}5 \times 10^{-4}$M. Even at the lowest substrate concentrations, however, rates of oxidation were

linear almost to completion, a fact which is unexpected if the relation between rate and substrate concentration were to be interpreted according to the Michaelis-Menten equation. Examination of the data by the methods of Lineweaver and Burk also indicated that they could not be interpreted in this way. This situation, and the above-mentioned linearity of oxygen uptake almost to completion of arsenite oxidation at all the concentrations tested, suggest that the results are complicated by diffusion processes through the cell wall.

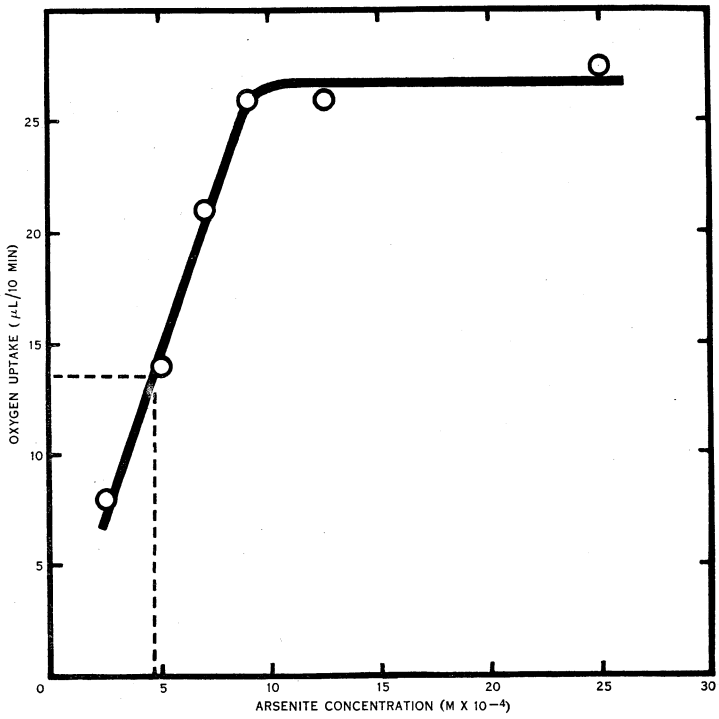

Fig. 4.—Variation in rate of oxygen uptake with the concentration of substrate.

Measurement of the relationship between substrate concentration and rate of arsenite oxidation by iodometric methods gave the same half-maximum rate.

(iv) *Temperature.*—The data in Figure 5 show the dependence of arsenite oxidation rate on temperature. Three suspensions were used, the results being adjusted to the same level of activity (Wilson 1938). The oxidation was followed by titration. For temperatures less than the optimum, the velocity remained constant for 60-80 min, but beyond the optimum it decreased noticeably after 30 min. From Figure 5 it is seen that under these conditions the optimum temperature was near 40°C. The Q_{10} between 20 and 30°C was found from the calculated best-fit line to be 2·4, and between 30 and 40°C, 1·6.*

* In the preliminary note, these Q_{10} values, measured from a visual-fit line, were given as 1·73 and 1·43, respectively.

Of the various equations commonly used to describe the dependence of velocity of biological reactions on temperature, those of Belehrádek (1935) and of Arrhenius were tested. The data are graphed appropriately in Figures 6 and 7 respectively. Statistical analysis shows that the latter method (temperature characteristic $b = 1{\cdot}27 \pm 0{\cdot}08$) gives a better fit of the data than the former (apparent energy of activation = 7750 ± cal/mole).

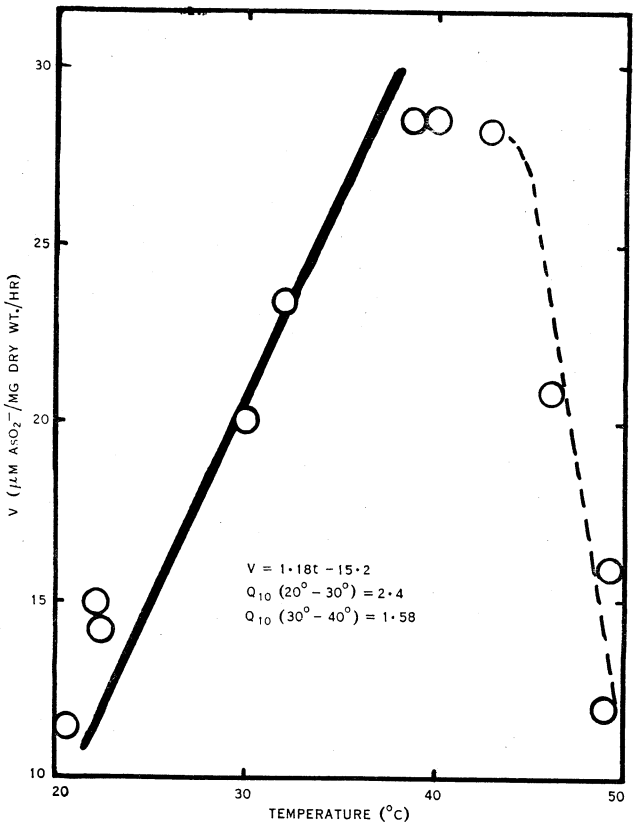

Fig. 5.—Variation of velocity of arsenite oxidation with temperature.

(*c*) *Other Acceptors*

In addition to reducing oxygen in the presence of arsenite, washed adapted cells are able to reduce suitable dyes under anaerobic conditions.

It is necessary to use, as electron acceptors, redox indicators with E_0 values appropriately higher than that of the arsenite-arsenate system itself (E_0' at pH $7{\cdot}0 = +0{\cdot}077$ to $+0{\cdot}167$ V, depending upon which of the several values for E_0 recorded in the literature is accepted). Suitable acceptors are indophenol-2,6-dichlorophenol, phenol blue, and *m*-carboxyphenol-indo-2,6-dibromophenol (E_0' at pH $7{\cdot}0 = +0{\cdot}217$, $+0{\cdot}224$, and $+0{\cdot}250$ V, respectively). With indo-

phenol-2,6-dichlorophenol, arsenite dehydrogenase has an optimum around pH 4·5.

Ferricyanide is also able to oxidize arsenite anaerobically in the presence of washed cells. The reaction was followed in Warburg manometers by measuring CO_2 evolution from bicarbonate buffer, as a result both of the arsenate and of ferrocyanide formed in the reaction. Anaerobiosis was obtained with a small stick of yellow phosphorus in the centre well. Under these conditions, arsenite was oxidized at only one-third the rate at which it is oxidized by air.

These results, which show the existence of an arsenite dehydrogenase able to react with a number of acceptors, are considered further in Paper III of this series.

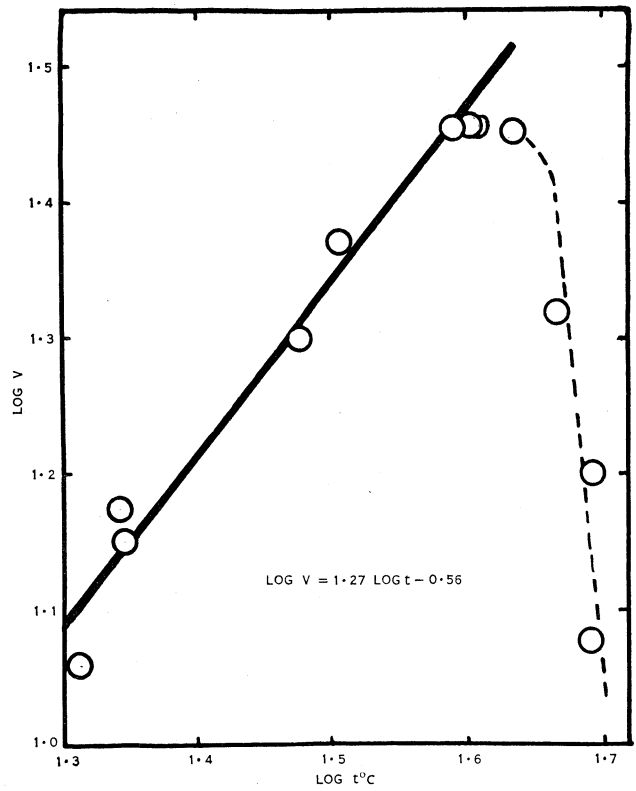

Fig. 6.—Variation of log velocity of arsenite oxidation with log temperature (°C).

(*d*) *Effect of Inhibitors on Arsenite Oxidation by Intact Cells*

(i) *Cyanide and Azide.*—In view of the high redox potential of the arsenite-arsenate system, these inhibitors were used to test for the presence of iron or copper oxidases.

When the rate of arsenite oxidation was measured by iodometric titration, any cyanide or azide present was first removed by acidifying and boiling the suspension.

When cyanide was added at the same time as the arsenite, it was observed that the extent of inhibition was greater in the second half hour than the first. Complete inhibition was produced by 10^{-3}M cyanide after this time. This was not observed when the cell suspension was left in contact with cyanide for 30 min, and then diluted with an equal volume of cyanide-free arsenite.

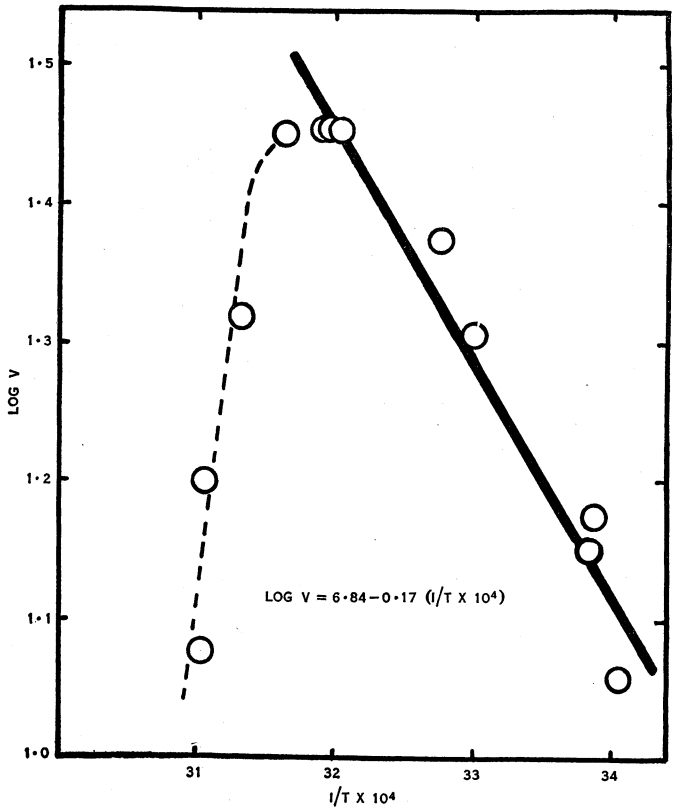

Fig. 7.—Variation of log velocity of arsenite oxidation with $1/T$.

TABLE 1

INHIBITION OF ARSENITE OXIDATION BY CYANIDE AT 23°C

Strain 17, original

Cyanide (final molarity)	Arsenite Oxidized (μM/mg dry wt./hr)	Inhibition (%)
0	31·0	—
10^{-4}	7·50	75·8
10^{-5}	17·8	42·6
10^{-6}	27·8	10·3

Fifty per cent. inhibition was thus produced by between 10^{-4} and 10^{-5}M cyanide under these conditions.

Sodium azide, tested at a concentration of 0·01M, completely inhibited oxidation.

(ii) *Carbon Monoxide Inhibition.*—Carbon monoxide is known to combine with, and to inhibit, cytochrome oxidases as well as the copper oxidases. In the first case, the CO compounds are typically dissociated by light, whereas in the second they are not. Although a typical light-reversible inhibition was observed 6 months after the strain was isolated, suspensions prepared 22 months later reacted differently. The experiments are therefore reported in some detail.

In the earlier experiments, suspensions of strain 17 were equilibrated with relevant gas mixtures, and were placed in rubber-stoppered 50-ml standard flasks, of plain or ruby glass, which had been flushed out with the gas mixture. When aliquots were removed for iodometric titration, the flushing was repeated. The light reversal of the inhibition was obtained either by diffuse daylight or by exposure of the flasks to the concentrated illumination of a battery of flood lights, the beam reaching the flasks in a glass constant-temperature bath after passing through a cooling cell.

TABLE 2

INHIBITION OF ARSENITE OXIDATION BY CO, AND ITS REVERSAL BY LIGHT

Strain 17, original

Experiment	Cell Concentration (mg dry wt./ml)	Temperature (°C)	Light	CO/O_2 or N_2/O_2	Velocity of Oxidation (μM AsO_2^- /mg dry wt./hr)	Inhibition (%)	Light Reversal (%)
1	0·13	23	Diffuse daylight	4	L 20·2 D 18·7 C 24·3	16·8 23·0 —	27
2	0·13	22	960 f.c.	19	L 18·0 D 14·7 C 24·4	26·6 39·7 —	34
3	0·35	1	960 f.c.	19	L 3·47 D 2·41 C 5·00	31·6 51·8	39

L = exposed to light; D = protected from light by ruby flask; C = control. L and D in presence of $CO+O_2$; C in presence of N_2+O_2.

In view of the finding of Kubowitz and Haas (1932) that the light sensitivity of carbon monoxide inhibition of respiration of vinegar bacteria and of yeasts was 4½ times greater at 0°C than at 10°C, some experiments were carried out in water-baths containing an excess of shaved ice. Because of the greatly reduced velocity of oxidation at this temperature, much thicker cell suspensions were then required. Rates of arsenite oxidation were measured over time intervals such that the amount of dissolved oxygen was not significantly lessened during the experiment.

Typical experiments are summarized in Table 2 which demonstrate (*a*) that carbon monoxide inhibited arsenite oxidation, the inhibition being greater in a mixture of 95 per cent. CO and 5 per cent. O_2 than in a mixture of 80 per cent. CO and 20 per cent. O_2; (*b*), that although inhibition was greater at 1°C than at 22°, light reversal of inhibition at 1°C was only slightly, if at all, greater than at 22°C.

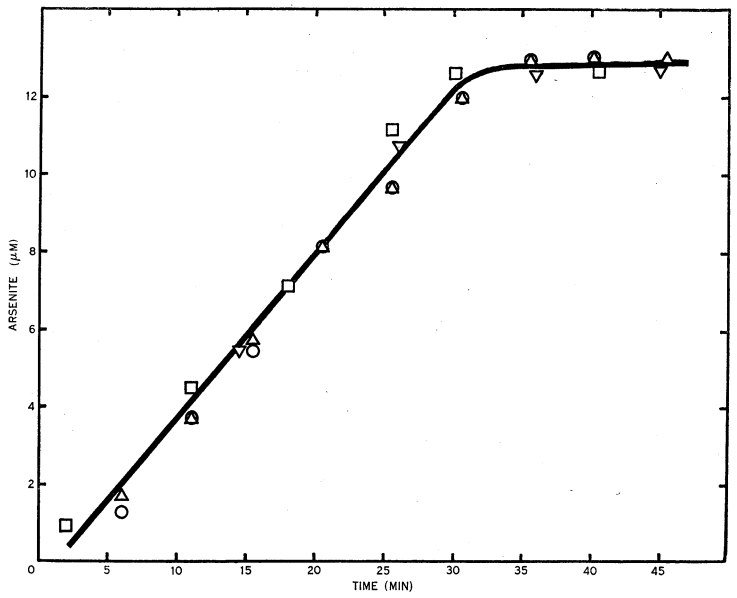

Fig. 8.—Time course of arsenite oxidation in presence and absence of carbon monoxide, measured by oxygen uptake and by iodometry. Substrain *L*2.

The effect of carbon monoxide was re-examined after substrain 17*L*2 had been isolated in the course of later experiments (Legge and Turner 1954). When arsenite oxidation was measured manometrically at 38°C, reproducible inhibition of oxygen uptake by carbon monoxide in the dark with CO/O_2 ratios up to 30 to 1, could not be obtained; in some cases, indeed, there was a slight acceleration. Although it had been previously observed by Fujita and Kodama (1934) that carbon monoxide did not inhibit the respiration of pseudomonads, the failure to confirm our earlier findings was unexpected. Further experiments were therefore performed in order to throw some light on this capricious phenomenon.

Impurities in the carbon monoxide, which was prepared either by dehydrating formic acid with concentrated sulphuric acid or by heating calcium carbonate and zinc, were considered to be most unlikely; indeed, samples of bakers' yeast showed a typical inhibition, with some light reversal, and with complete reversibility when the CO/N_2 mixture was replaced by O_2/N_2 mixtures.

The possibility that carbon monoxide might be oxidized by the organisms, and thus conceal an inhibited oxygen uptake due to arsenite oxidation, was

considered. No evidence was found to support the hypothesis of carbon monoxide oxidation, and an experiment in which arsenite disappearance was followed iodometrically in parallel with oxygen uptake showed that arsenite oxidation by the *L*2 substrain was insensitive to CO inhibition by either method. At given periods the vessels were removed from the manometers, solid sodium carbonate was added, and the remaining arsenite was titrated iodometrically; the titration was completed within 2 min of removing the vessels. The results are set out in Figure 8.

It can be seen that, whether measured by oxygen uptake or by iodometric titration, the disappearance of arsenite was not influenced by the presence of CO, the CO/O_2 ratio being 16·5.

TABLE 3

COMPARISON OF EFFECT OF CO ON ARSENITE OXIDATION BY *L*2 CELLS IN PHOSPHATE BUFFER, AND IN BICARBONATE BUFFER

Gas Mixture	Arsenite Oxidation (μM AsO_2^-/mg N/hr)	
	Phosphate Buffer*	Bicarbonate Buffer†
3·5% O_2/CO	37·8	36·5
3·7% O_2/N_2	42·2	35·8
21% O_2/N_2	58, 60·8	42·1

* $3·5 \times 10^{-3}$M, pH 7·4.

† $3·5 \times 10^{-3}$M HCO_3^- with $3·5 \times 10^{-4}$M CO_2, pH 7·4.

Since Warburg (Warburg, Kubowitz, and Christian 1931) had recorded instances in which the CO inhibition of respiration in animal tissues required the presence of CO_2/bicarbonate, and failed in the presence of NaCl-phosphate buffers, the experiments were repeated in the presence of CO_2. These were rendered difficult because of two opposing requirements: high buffering capacity was required in order to protect the system against the strongly acid oxidation product, arsenic acid, and, at the same time, high partial pressure of CO was required, thus limiting the partial pressure of CO_2. The method of Pardee (1949) was used; the diethylamine-carbonate buffer system was chosen to give a partial pressure of $1·5 \times 10^{-2}$ atm CO_2. With $3·5 \times 10^{-4}$M dissolved CO_2 and $3·5 \times 10^{-3}$M HCO_3^-, it was calculated that the initial pH was 7·3. Since its buffer capacity was low, only the initial rate of arsenite oxidation was considered.

The results with a suspension of KA3 cells previously stored for 60 days in the refrigerator, but still highly active, are shown in Table 3. For comparison, arsenite oxidation was measured, in the presence of KOH papers, in phosphate buffer pH 7·4, of the same molarity as the HCO_3^-. In order to estimate the oxygen affinity of the cell oxidase, the measurements of uptakes in these two buffers were also carried out in air. The temperature was 38°C.

Slight CO inhibition (10 per cent.) was observable in the phosphate buffer, but the results in bicarbonate buffer showed no significant difference. Since the CO/O_2 ratio of 25 in the above experiment exceeded the ratio of 20, which in earlier experiments was found to produce 50 per cent. inhibition of arsenite oxidation, and since 10 per cent. inhibition was, with one exception, the highest found in the present series, it is apparent that a substantial change in the characteristics of the oxidase had taken place. Since a significant decline in oxidation rate at the low partial pressure of oxygen was observed in both buffers, it is apparent that the failure to find CO inhibition was not due to an abnormally high affinity of the oxidase for oxygen.

The insensitivity of the *L*2 substrain to carbon monoxide inhibition was observed with substrates other than arsenite; with formate, for example, which is oxidized readily, one experiment with CO/O_2 ratio of 4 showed 10 per cent. inhibition. An experiment in which the oxidation of *p*-phenylene diamine was measured at 0°C showed no inhibition with 50 per cent. CO in O_2.

TABLE 4

FAILURE OF CO INHIBITION OF ARSENITE OXIDATION BY (*a*) *L*2 CELLS, CONTRASTED WITH INHIBITION OF ASCORBATE OXIDATION BY (*b*) *L*2 CYTOCHROME OXIDASE AT 38°C

Treatment	Oxygen Uptake (μl O_2/hr)		Inhibition (%)	
	(*a*)	(*b*)	(*a*)	(*b*)
N_2/O_2	194	276	—	—
CO/O_2 light	192	194	0	30
CO/O_2 dark	192	134	0	51

In the whole series, only one experiment showed a typical CO inhibition. This was carried out with KA3 cells of the *L*2 substrain, 5 wk old, in phosphate buffer pH 6·8, 38°C, with CO/O_2 or N_2/O_2 ratios of 10. One vessel containing CO was irradiated with a 300 W "Hanovia" u.v. lamp through a window in the bath. In N_2/O_2, arsenite oxidation proceeded at 131 μM/mg N/hr; the rate was inhibited 29 per cent. in CO/O_2 in the dark, and only 15 per cent. when irradiated. When a sample of cells of the 17*X* substrain, cultivated in KA3 medium, was tested under conditions identical with the above, an inhibition of 19 per cent. was observed in the dark, with negligible light reversal.

Although the arsenite-oxidizing activity of the 17*L*2 substrain was almost completely insensitive to carbon monoxide inhibition, a cytochrome oxidase preparation from this substrain (cf. Legge and Turner 1954), as well as from the 17*X* substrain, was more readily inhibited. Table 4 sets out the results with oxidase AA, ascorbic acid, and 2,6-dichlorophenol indophenol, at 37°C, CO/O_2 and N_2/O_2 ratios 11·5. For comparison, 17*L*2 cells oxidizing arsenite were included. Vessels were irradiated from below by light reflected from a 250 W u.v. low-pressure lamp, control vessels being covered with metal foil.

It can be seen that the activity of the cytochrome oxidase was decreased 50 per cent. by CO in the dark, and only 30 per cent. when illuminated. The oxidase recovered its original activity on replacement of the CO/O_2 mixture by N_2/O_2.

(iii) *Other Enzyme Inhibitors.*—Experiments with other enzyme inhibitors were less informative than those with cyanide and carbon monoxide. Thus fluoride, which inhibits Ca^{++} or Mg^{++} activated enzymes, as well as the cytochrome system (cf. Borei 1945; Slater and Bonner 1952), inhibited arsenite oxidation only 21 per cent. after being in contact with cells for 90 min (0·01M NaF, pH 6·4-7·0, 20°C). With 0·1M NaF the inhibition increased with time, reaching 67 per cent. after 100 min. Under the same conditions, 0·02M pyrophosphate produced only 16 per cent. inhibition.

Other inhibitors of mental enzymes, such as 0·001M α,α'-dipyridyl (for Fe^{++}) or diethyldithiocarbamate (for Cu^{++}) were without effect. The sulphide ion, which could not be used in presence of arsenite because of arsenious sulphide formation, had a negligible effect when cells exposed to 0·001M concentration for 15 min were subsequently tested.

Neither urethane (0·1M) nor the well-known — SH enzyme inhibitor iodoacetate (0·01M) inhibited the arsenite oxidation.

In view of the likelihood that the terminal oxidation of arsenite takes place on the cytochrome system, the effect of malonic, succinic, and fumaric acids was tested. Although only the cytochromes of higher redox potential were likely to be involved in the arsenite oxidation, the possibility was recognized that electron transport from arsenite might have to compete with that from other substrates.

At pH 6·4, when malonic acid is present mainly as the malonate ion, no significant inhibition was observed. At pH 4, however, 30-40 per cent. inhibition was observed with 0·05M malonate which was overcome after 40-60 min; 0·05M succinate and fumarate behaved similarly, and the effect of malonate added together with fumarate was simply to prolong the period of inhibition; after 80 min, arsenite oxidation proceeded equally rapidly in the presence of any of these acids.

These experiments were carried out in Universal buffer pH 4·5, which contains citrate. On repeating in 0·05M phosphate buffer, the initial rates in the presence of the above dicarboxylic acids were greater than the control rate. Between 20 and 60 min, the arsenite oxidation rate slackened, and then approximated to the original. Since the opacity of the suspension increased during the experimental period in comparison with the control, it appears probable that interference with arsenite oxidation is complicated by secondary adaptive phenomena related to the contact between the KA3-trained cells and a new, assimilable carbon source (cf. Turner 1954). The initial effects observed may indeed be of a non-specific character owing to the pH regulating mechanism of the cell being overwhelmed by a permeable acid.

(*e*) *Stability of Enzyme System*

The cell suspensions were inactivated by drying or by acetone treatment. Addition of 0·5 per cent. toluene was followed by a distinct clearing of the suspension, and lysis of many cells. Such treated suspensions no longer oxidized arsenite.

IV. Discussion

Experiments on cell-free arsenite dehydrogenase, and on the cytochrome oxidase of the organism, are reported in subsequent papers. This communication is limited to experiments with intact cells; hence restrictions due to permeability must be recognized, particularly when the activity of possible inhibitors is considered.

The significance of the value obtained for the apparent energy of activation ($\mu = 7750$) calls for discussion. Dehydrogenase reactions probably do not limit the reaction at conditions approaching the optimum, since the characteristic values for dehydrogenases are of a higher order; thus for succinic dehydrogenase of bacteria, $\mu = 16{,}700$; for dehydrogenase of *B. coli*, $\mu = 19{,}400$; and for heart dehydrogenase-cytochrome-cytochrome oxidase, $\mu = 16{,}000$ (Sizer 1943). It is equally unlikely that the cytochrome system is responsible for the low value, since, although Hadidian and Hoagland (1940) obtained $\mu = 9500$ for oxidation of phenylene diamine by cytochrome oxidase, the cytochrome-cytochrome oxidase reaction has been found to have a μ value of 16,000; indeed, in the succinic oxidase system, for which $\mu = 11{,}000$, succinic dehydrogenase is the limiting reaction (Stearn 1949). Our finding of 7750 cal/mole for arsenite oxidation is, therefore, less than values which others have found for oxidations; on the other hand, it lies in the range found for diffusion processes (Barrer 1949). This is consistent with the evidence based upon the relationship between arsenite concentration and rate of oxidation.

In view of the rapidity with which many microorganisms are able to modify their protoplasm in response to changes in the environment, the use of washed cells, under conditions which may be made to approach "physiological conditions" closely, may render difficult the interpretation of inhibitor data in simple enzymic terms. This may be so with cyanide, with which the degree of inhibition changed with time. The observations of Chin (1950) and of Ephrussi and Slonimski (1950) on the rapidity with which yeast is able to alter its cytochromes in response to changes in partial pressure of oxygen in a nitrogen-free medium suggest that such an interpretation is worthy of further experimental investigation. The same may be so with the inhibition observed with dicarboxylic acids. Here, however, the process may be complicated by competition between two oxidizable substrates for the same oxidase, and by the possibility that the cells may be adapting to these carbon sources during the course of the experiment.

The variable results obtained when measurements of the carbon monoxide inhibition were made should be noted. Since samples of bacterial oxidase with differing spectroscopic properties (cf. Legge 1954) show carbon-monoxide inhibition of leuco-indophenol oxidation, the failure of the strains from which

these oxidases were derived to show significant carbon monoxide inhibition of arsenite oxidation cannot be due to the lack of a cytochrome component. Since fresh samples of packed cells, as well as samples which had been stored in the refrigerator for periods up to 60 days, showed similar behaviour, pathological changes in the stored cells cannot be implicated. The possible significance of the buffer used, whether bicarbonate or phosphate, has been excluded by experiment, and the agreement between arsenite disappearance measured by oxygen uptake or iodometric titration excludes significant carbon monoxide oxidation. It would appear most unlikely that the insensitive cells differed from the sensitive cells in the permeability of their walls to such a small uncharged molecule as that of carbon monoxide.

We can only conclude that the change in carbon monoxide sensitivity of the arsenite oxidation must be due to altered reactivity of the cytochromes within the cell. The fact that most of the substrains used in the manometric studies had less arsenite-oxidizing activity than the earliest substrains isolated, in which undoubted carbon monoxide inhibition was observed, is confirmatory evidence that additional changes in the organism, which we have been unable to probe experimentally, have taken place during the period of the investigation.

The adaptive character of the arsenite dehydrogenase makes it a particularly interesting enzyme to investigate. So far, our studies of the conditions for the cultivation of maximally active cell suspensions have shown that the enzyme system is less active in richer culture media than in those containing inorganic nitrogen and sulphur, with citrate as the only carbon source. The variation in activity reported in the succeeding paper shows that other factors must be taken into consideration, chief among these being the cultivation of cells with a high concentration of cytochrome components with high redox potential. This is now being reinvestigated.

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VI. References

Barrer, R. M. (1949).—*Trans. Faraday Soc.* **45**: 358.
Belehrádek, J. (1935).—Cited by Wilson (1938).
Borei, H. (1945).—*Ark. Kemi. Min. Geol.* **20** A: 215.
Chin. C. H. (1950).—*Nature* **165**: 926.
Ephrussi, B., and Slonimski, P. P. (1950).—*Biochim. Biophys. Acta* **6**: 256.
Fujita, A., and Kodama, K. (1934).—*Biochem. Z.* **273**: 186.

GOMORI, G. (1946).—*Proc. Soc. Exp. Biol. Med., N.Y.* **62**: 33.
HADIDEAN, Z., and HOAGLAND, H. (1940).—*J. Gen. Physiol.* **23**: 81.
KUBOWITZ, F., and HAAS, E. (1932).—*Biochem. Z.* **255**: 247.
LEGGE, J. W. (1954).—*Aust. J. Biol. Sci.* **7**: 479.
LEGGE, J. W., and TURNER, A. W. (1954).—*Aust. J. Biol. Sci.* **7**: 496.
ÖSTLING, S., and VIRTAMA, P. (1946).—*Acta Physiol. Scand.* **11**: 289.
PARDEE, A. B (1949).—*J. Biol. Chem.* **179**: 1085.
SIZER, I. W. (1943).—*Advanc. Enzymol.* **3**: 35.
SLATER, E. C., and BONNER, W. D. (1952).—*Biochem. J.* **52**: 185.
STEARN, A. E. (1949).—*Advanc. Enzymol.* **9**: 25.
STOCKEN, L. A., and THOMPSON, R. H. S. (1949).—*Physiol. Rev.* **29**: 168.
TURNER, A. W. (1949).—*Nature* **164**: 76.
TURNER, A. W. (1954).—*Aust. J. Biol. Sci.* **7**: 452.
WARBURG, O., KUBOWITZ, F., and CHRISTIAN, W. (1931).—*Biochem. Z.* **242**: 120.
WILSON, P. W. (1938).—Physical-chemical theory of enzyme reactions. *In* "Respiratory Enzymes." Ed. Elvehjem, C. A., and Wilson, P. W. (Burgess Publ. Co.: Minneapolis.)