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ARSENATE UPTAKE AND RELEASE IN RELATION TO THE INHIBITION OF TRANSPORT AND GLYCOLYSIS IN YEAST*

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Abstract—The amount of arsenate in yeast cells exposed to arsenate plus fermentable sugar for varying periods of time is determined by the kinetics of influx and efflux. Influx occurs only in the presence of fermentable substrate via a transport mechanism that can be divided by kinetic properties into two components with Michaelis constants of 4×10^{-6} and 4×10^{-4}. During exposure to arsenate the transport system is gradually reduced in capacity and, with sufficient concentrations of arsenate, may be almost completely inactivated. At the same time, previously absorbed arsenate leaks out of the cell with first-order kinetics. The balance of inward transport, inactivation of the transport system, and outward leakage results in the arsenate content of the cell reaching a maximal value and then decreasing. Arsenate also inhibits the fermentation of the intact cell, but only to a maximal extent of 60 per cent, compared to 100 per cent in broken cells. The data indicate that only a small fraction of the cellular arsenate is involved in the inhibition of metabolism and of transport and that its distribution and metabolic turnover must be complicated. Simple fractionations of cellular arsenate confirm this supposition.

IN CELL-FREE systems[1–3] and in intact cells,[4–9] arsenate presumably inhibits metabolic reactions by competing with inorganic phosphate for esterification reactions. In cell-free systems arsenate has direct access to the enzyme centers, but in the intact cells the cell membrane and possibly other barriers are interposed, so that the movement of arsenate across the membranes may be the primary limiting factor that characterizes the inhibitory effect.

Arsenate enters the yeast cell via a transport system that is normally responsible for phosphate uptake, so the two anions, when present together, compete for entry.[10–14] The transport system has been characterized as a carrier-mediated, active-transport system involving not only competition of ions but also "saturation" kinetics, uptake against large chemical gradients, and dependence on specific metabolic pathways.[14] One unusual feature of the transport of phosphate into the cell is the virtual absence of any phosphate efflux, before, during, or immediately after the period of uptake. Thus, permeability to phosphate, exchanges of phosphate, and backflow of phosphate through the transport system are minimal.[14]

In contrast to phosphate, arsenate continuously inactivates the transport system in an irreversible manner so that uptake eventually ceases.[13] The total amount of arsenate

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delivered to the inside of the cell where it is available to inhibit metabolic reactions is thus determined jointly by the kinetics of transport and by the kinetics of inactivation of the transport system. Although it is obvious that the inhibitory effects of arsenate on cellular metabolism depend on the delivery of the arsenate into the cell by the transport system, the converse may also be true. Because the operation of the transport system is itself dependent on energy derived from glycolysis,[14] any inhibitory effects of arsenate on metabolism may reflect themselves in a reduction of the rate of transport. In the present study an attempt has been made to sort out the relationships among arsenate transport, net arsenate uptake, inhibition of transport, and inhibition of metabolism.

METHODS

Fresh bakers yeast (Standard Brands, Inc.) was thoroughly washed, starved overnight by aeration in distilled water, then suspended in triethylamine–succinate–tartrate buffer, pH 5.0, containing 0·01 M KCl,[14, 15] 0·2 M glucose or other substrates, and various concentrations of arsenate, phosphate, or both, labeled with ^{74}As or ^{32}P. The temperature was 25°. With low concentrations of arsenate, an appreciable fraction of that added is taken up by the cells. Consequently, the uptake per cell and the resulting inhibitions depend on the relative volumes of cells and extracellular fluid. For example, with 3×10^{-5} M arsenate, the maximal inhibition of fermentation was 60% with a cell concentration of 2 mg cells/ml suspension, but was only 40% and 15% with 10 and 20 mg/ml respectively. The complex relationships between cell concentrations and arsenate effects were avoided by making all measurements at a yeast concentration of 2 mg/ml. Estimations of cell concentrations were made by centrifugation in calibrated tubes. It was assumed that the packed cells included 25% of trapped water[16] and that the density of the yeast was 1·0.

For measurements of arsenate or phosphate uptake, 10 ml of the cell suspension was taken at various time intervals, placed on a filter disk (H.A. 0·45μ, Millipore Filter Corp.), and the medium was pulled through by suction. The cells on the filter pad were washed with buffer solution to remove intercellular isotope. The cells on the filter were counted with a thin-window GM tube (Nuclear Chicago, model 181A). In a number of experiments the arsenate or phosphate uptake was also measured by disappearance from the medium. The results were not significantly different from those estimated by counting the cells. Thus the washing procedure does not remove a measurable amount of the cellular arsenate or phosphate. The counting errors were less than 5% in all cases.

In order to measure the efflux of arsenate or phosphate, the cells were first "loaded" with isotope by exposure to "hot" phosphate or arsenate plus glucose. After the fixed time interval, the cells were washed free of external isotope and resuspended in "cold" solutions. The loss of isotope from the cells was measured at various times by counting the cells as described above.

The rate of metabolism of the cells was measured in terms of CO_2 output or of O_2 consumption by means of the Warburg technique. In some experiments, the endogeneous metabolism was elevated by a pre-exposure for 60 min to 0·2 M glucose, followed by washing and resuspension without substrate in appropriate arsenate-containing media.

The intracellular distribution of arsenate was determined by a fractionation based on the procedure of Schneider[17] for phosphate. Four fractions were estimated. (1) A cold trichloracetic acid extraction (cold TCA) which contains inorganic arsenate, and presumably simple esters and polyarsenate. For the estimation of inorganic arsenate the ion was precipitated with magnesia mixture by the method of Sacks[18] as used for inorganic phosphate. (2) A hot TCA fraction which contains nucleic acids, inorganic phosphate polymers, and presumably arsenate or arsenate-phosphate polymers. A further separation of nucleic acids was made by absorption-elution on a Norit A column, according to the procedure of Baker and Schmidt.[19] (3) An alcohol-ester extract which contains phospholipids and presumably arsenolipids. (4) An extract of the residue after boiling with 2% NaOH which contains phosphoprotein phosphate and presumably arsenate from arsenoproteins. All fractions were estimated by ^{74}As activities directly or after precipitation with molybdic acid reagent.[18]

RESULTS

A comparison of different metabolites indicates that the uptake of arsenate is dependent primarily on the fermentative pathway, either exogenous or endogeous (Table 1). For example, in starved cells in which endogenous fermentation is exceedingly low,[20, 21] no appreciable uptake occurred. The addition of lactate, ethyl

TABLE 1. THE DEPENDENCE OF ARSENATE UPTAKE ON SPECIFIC SUBSTRATES

Substrate	Condition	Rate of CO_2 output (ml/kg/hr)		Initial rate of arsenate influx (mmoles/kg/hr)
		Control	Arsenate	
None	Aerobic	trace	0	0
	Anaerobic	0	0	0
Endogenous*	Anaerobic	trace	5·1	13·2
Glucose	Aerobic	51·6	20·3	15·9
	Anaerobic	72·0	28·6	16·7
	Aerobic + KCN (2 mmoles/liter)	32·2	4·0	9·4
Fructose	Anaerobic	40·2	15·8	12·7
Mannose	Anaerobic	34·1	13·8	8·9
Sorbose	Anaerobic	0	0	0
Lactate	Aerobic	37·3	36·5	0·3
Ethanol	Aerobic	58·5	38·4	1·6
Acetate	Aerobic	38·2	38·2	0

Substrate concentrations were 0·2 mole/liter in all cases. The rate of arsenate influx was measured with 2×10^{-3} mole/liter of arsenate in the medium.

* Prior to the measurement, the yeast was incubated with 0·2 mole/liter of glucose for 60 min, then washed free of extraneous glucose.

alcohol, or acetate resulted in a minimal uptake, whereas addition of fermentable sugars, glucose, mannose, or fructose resulted in an appreciable uptake, even though the metabolic rates for all the substrates are comparable. Furthermore, the uptake with glucose was as rapid in the absence of 0_2 as in its presence, and cyanide in sufficient concentrations to inhibit the respiration of glucose did not prevent the uptake. With the nonfermentable sugar, sorbose, no uptake was found. In starved cells, endogenous

fermentation is virtually absent, whereas endogenous respiration is low but measurable,[20] yet no arsenate uptake occurs. On the other hand, if the endogenous fermentation is increased to appreciable levels by a prior exposure to glucose[21] followed by washing and exposure to arsenate in the absence of substrate, then the uptake of arsenate is also appreciable. It can therefore be concluded that the uptake of arsenate is specifically associated with the glycolytic pathway of metabolism, either exogenous or endogenous.

In starved cells given arsenate and glucose, the cellular content reached a maximal level after 40 to 150 min, depending on the concentration of arsenate added (Fig. 1).

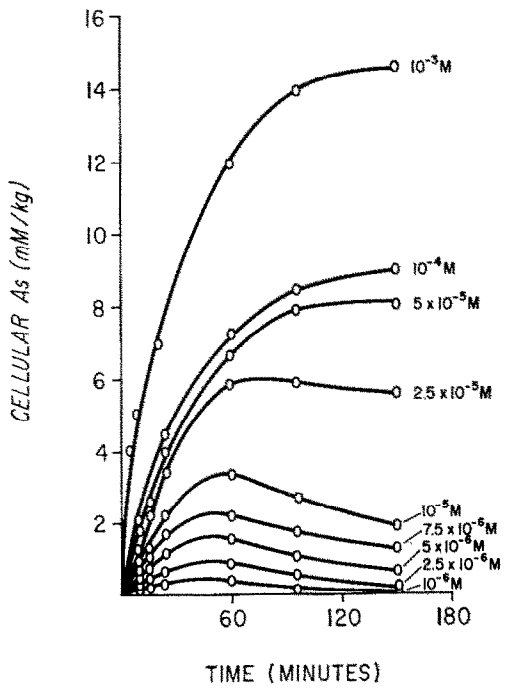

FIG. 1. Time course of net accumulation of arsenate with various arsenate concentrations.

The maximal level did not increase in proportion to the arsenate concentration, but in every case the concentration attained within the cell was very much higher than that in the medium, in an inverse relationship to the arsenate concentration (from 15 to over 1,000 times as high). After the maximal level was attained, losses of arsenate were evident, especially with low initial concentrations in the medium. Losses were also observed at the higher concentrations when experiments were extended for a number of hours.

It is clear that several factors determine the arsenate content of the cells. At the lowest concentration, 1×10^{-6} M, virtually all the arsenate was taken up from the medium, so the limiting factor was the supply of arsenate. With all other concentrations, the depletion of the medium was only partial, ranging from 70 per cent at 5×10^{-6} M to only 4 per cent at 1×10^{-3} M, and the predominant factors that

determined the uptake were the kinetics of inward transport, the development of inhibition of the transport system itself, and the leakage of arsenate from the cells. Each of these factors will be considered separately.

Phosphate uptake by yeast follows typical saturation kinetics that can be described by the Michaelis–Menten equation.[14] The kinetics of arsenate uptake are more difficult to determine because the rate of uptake decreases continuously with time. It has been demonstrated, however, that phosphate and arsenate compete for the same transport system, and that the affinities of the ions, expressed as the Michaelis constants (K_m) are about equal, 4×10^{-4} M.[13] In the present studies it was found that the uptake of arsenate and the effects of arsenate on metabolism were much greater at very low arsenate concentrations (below 1×10^{-5} M) than would be predicted from a transport system with a Michaelis constant of 4×10^{-4} M. The uptake of arsenate was therefore re-evaluated at an extended range of concentrations (from 1×10^{-6} M to 2×10^{-2} M). To avoid the complications of the self-inhibition that continuously develops, the rates of uptake were measured during the first 5 to 7 min, a period during which the uptake was virtually linear with time. The results are expressed in Fig. 2 as reciprocals of

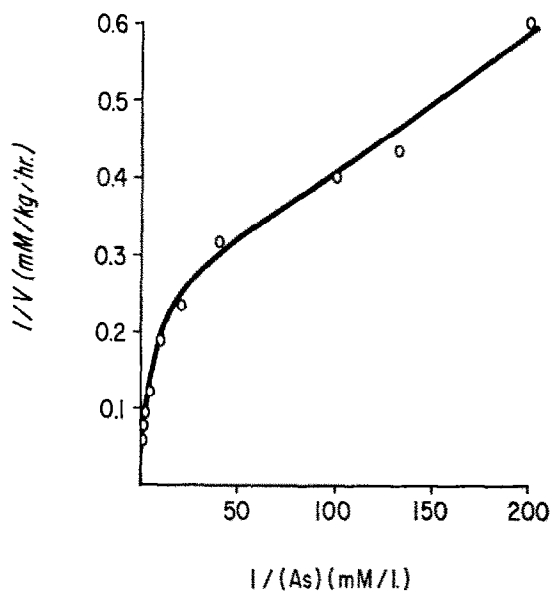

FIG. 2. Relationship between initial rate of arsenate influx and the arsenate concentrations; plot of $1/v$ against 1/(As).

rates and arsenate concentrations according to the Lineweaver–Burk modification of the Michaelis–Menten equation[22] for enzyme kinetics:

$$\frac{1}{v} = \frac{K_m}{V_m} \frac{1}{(\mathrm{As})} + \frac{1}{V_m} \tag{1}$$

The data can be approximately fitted by two lines that merge with each other. The steeper line corresponds approximately to the kinetics reported previously;[13] the intercept indicates a V_m of about 17 mmoles/kg/hr and the slope, a K_m of about 4×10^{-4} M. At lower concentrations of substrate [higher values of 1/(As)], however,

the slope of $1/v$ becomes considerably lower, indicating that the rate of uptake is considerably higher than would be predicted for a transport system with a K_m of 4×10^{-4} M. It was therefore assumed that arsenate transport involves two sites, one with a low and one with a high affinity for arsenate, each site behaving independently according to the Michaelis–Menten equation. On this basis the rate of transport at any particular arsenate concentration is the sum of two Michaelis–Menten terms, one for each site:

$$v = \frac{V_{m_1}(\mathrm{As})}{K_{m_1} + (\mathrm{As})} + \frac{V_{m_2}(\mathrm{As})}{K_{m_2} + (\mathrm{As})} \tag{2}$$

where V_{m_1} and K_{m_1} represent the maximal rates and Michaelis constants for the site of higher affinity (site 1) and V_{m_2} and K_{m_2} are the constants for the site of lower affinity (site 2).

At high concentrations of As, site 1 is virtually saturated, and the condition holds that $(\mathrm{As}) \gg K_{m_1}$. Equation (2) then can be reduced to:

$$v = V_{m_1} + \frac{V_{m_2}(\mathrm{As})}{K_{m_2} + (\mathrm{As})} \tag{3}$$

which can be recast in the form

$$\frac{1}{V_m - v} = \frac{(\mathrm{As})}{V_{m_2} K_{m_2}} + \frac{1}{V_{m_2}} \tag{4}$$

where V_m is the maximal rate with both sites saturated ($V_{m_1} + V_{m_2}$). The value of V_m of 17·2 mmole/kg/hr can be taken from the intercept on the $1/v$ axis of Fig. 2. In Fig. 3, $1/(V_m - v)$ is plotted against (As) for the higher concentrations of arsenate.

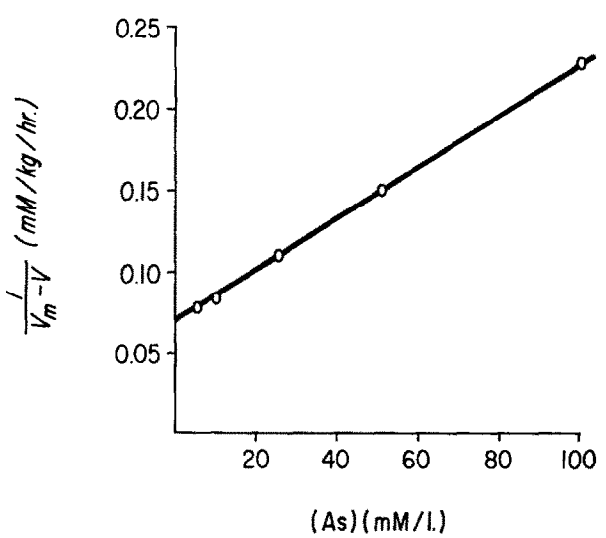

FIG. 3. Analysis of relationship between the initial rate of arsenate influx and the arsenate concentration; a plot of $1/(V_m - v)$ against (As).

From the intercept, the value for V_{m_2} can be estimated as 14·4 mmoles/kg/hr and from the slope, the value of K_{m_2} is $4{\cdot}4 \times 10^{-4}$ M (in agreement with the value of 4×10^{-4} M reported previously.[13]) By subtraction, $(V_m - V_{m_2})$, the value for V_{m_1} can be calculated as 2·8 mmoles/kg/hr.

At very low concentrations of arsenate, site 2 will be relatively unsaturated and it can be assumed that (As) $\ll K_{m_2}$. Equation (2) can be simplified to

$$v = \frac{V_{m_1}(\mathrm{As})}{K_{m_1} + (\mathrm{As})} + \frac{V_{m_2}(\mathrm{As})}{K_{m_2}} \tag{5}$$

after rearrangement of the terms,

$$1/\left[v - \frac{V_{m_2}(\mathrm{As})}{K_{m_2}}\right] = \frac{K_{m_1}}{V_{m_1}(\mathrm{As})} + \frac{1}{V_{m_1}} \tag{6}$$

This equation is the reciprocal form of the Michaelis–Menten equation for site 1, except that the term $V_{m_2}(\mathrm{As})/K_{m_2}$, representing the rate of transport via site 2, is subtracted from the measured rate; V_{m_2} and K_{m_2} have already been determined from Fig. 3, and (As) is experimentally known. The calculated value of $v - V_{m_2}(\mathrm{As})/K_{m_2}$ is plotted against 1/(As) in Fig. 4. From the intercept and slope the values of V_{m_1} and K_{m_1} are calculated as 2·8 mmoles/kg/hr and 4×10^{-6} M respectively.

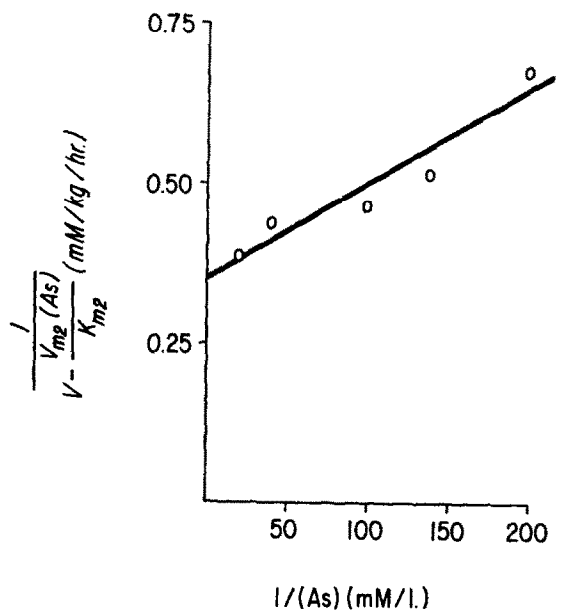

FIG. 4. Analysis of relationship between the initial rate of arsenate and the arsenate concentration; a plot of $1/\left[v - \frac{V_{m_2}(\mathrm{As})}{K_{m_2}}\right]$ against 1/(As).

The kinetic data indicate two transport sites for arsenate with affinities (K_m's) differing by a factor of 100 (4×10^{-6} M compared to 4×10^{-4} M), but the site with the higher affinity has only a small transport capacity (15% of the total) compared to the other.

The second factor that determines the arsenate content of the cells is the efflux of previously accumulated anion. It has previously been reported that absorbed phosphate is not released at a significant rate.[14] This observation was confirmed in the present investigation, both in the absence and in the presence of added arsenate. Nevertheless, an appreciable net loss of previously absorbed arsenate does occur, especially in cells exposed to low concentrations of arsenate (Fig. 1). Such measurements of the net movements of arsenate and phosphate, however, give no indication of the separate contributions of the influx and the efflux. To assess the latter parameters, the following experiments were performed. Pairs of tubes of yeast suspension were prepared which were identical except that one of each pair contained labeled arsenate. At specified times the cells in a pair of tubes were spun down by centrifugation and interchanged so that cells previously exposed to "hot" arsenate were now suspended in "cold" arsenate and vice versa. By measuring the cellular radioarsenate concentrations at different times, the net uptake could be measured and also the rates of efflux and influx (Fig. 5). From such experiments it was found that at all arsenate

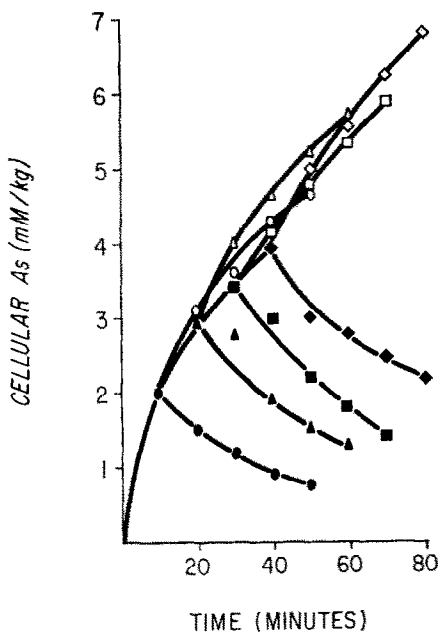

FIG. 5. Influx and efflux of arsenate at different times. Open symbols, influx. Closed symbols, efflux. Arsenate, 4×10^{-5} M.

concentrations tested, a significant efflux was always measurable, even during the early parts of the experiment when the rate of net uptake was greatest. The same kind of experimental procedure carried out with labeled phosphate in the presence of equimolar arsenate (Fig. 6) indicated no measurable efflux of phosphate. It is apparent, therefore, that the efflux of arsenate is a highly specific phenomenon, and that it cannot be attributed to some general damage to the membrane or to a nonspecific increase in permeability.

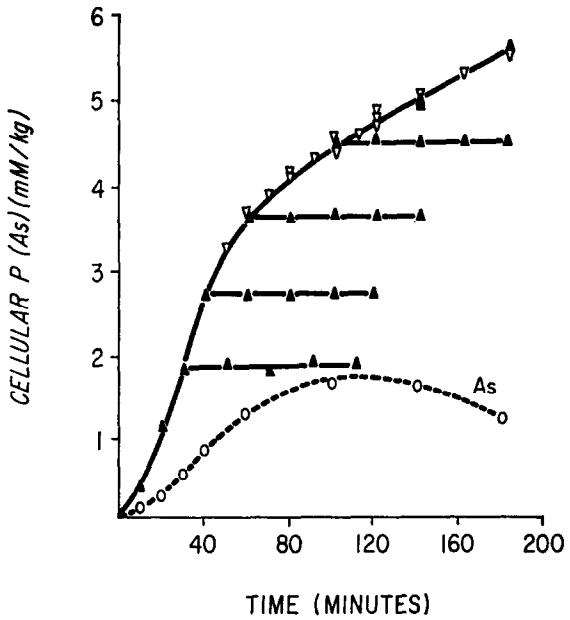

FIG. 6. Influx and efflux of phosphate at different times in the presence of arsenate in equimolar concentration: 2×10^{-5} M. Open symbols, influx. Closed symbols, efflux. The arsenate curve represents the net uptake.

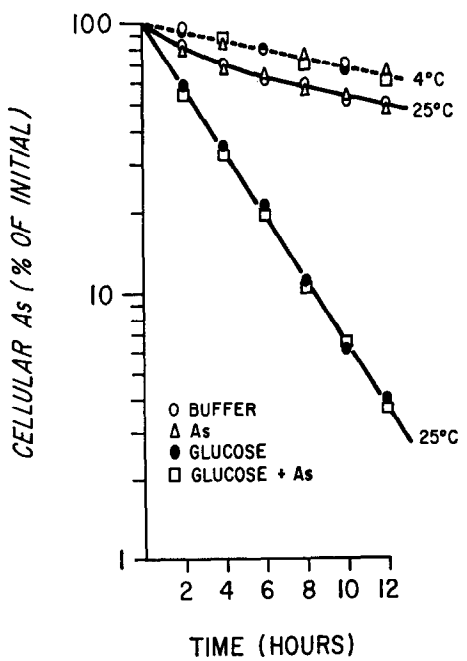

FIG. 7. Effects of glucose, arsenate, and temperature on the efflux of arsenate. Arsenate concentration, 3×10^{-5} M.

In order to determine the kinetics of arsenate efflux in a more exact manner, cells were allowed to take up labeled arsenate from solutions containing glucose plus various concentrations of arsenate. After the uptake was maximal, the cells were quickly washed and placed in experimental solutions containing no ^{74}As, so that the loss of ^{74}As could be measured at various times. The data are plotted in a semi-logarithmic fashion in Fig. 7. At 4° the rate of efflux was the same in the presence or absence of either glucose or arsenate (at the same concentration used in the previous loading period). The data could be fitted by a straight line on the semi-logarithmic plot, indicating that the rate of efflux followed a first-order function. At 25°, the rate of efflux was remarkably increased in the presence of glucose, but external arsenate

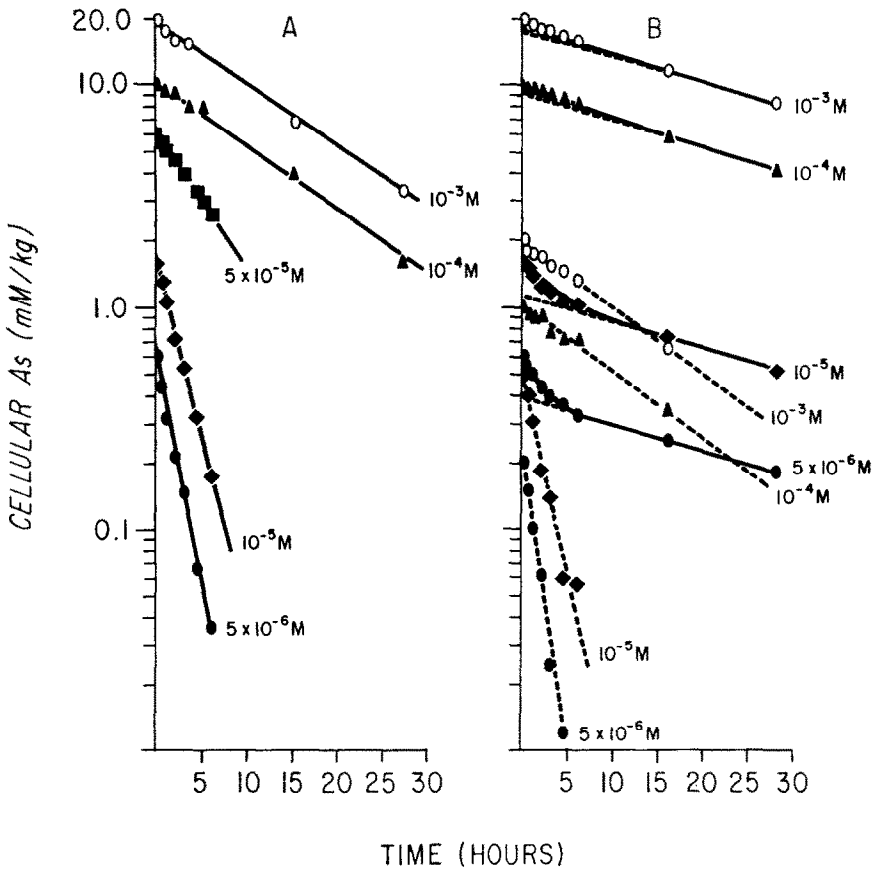

FIG. 8. Efflux of arsenate in the presence of glucose (A) and in the absence of glucose (B) after the loading with different concentrations of arsenate.

was without effect. The data could be fitted by a single rate constant involving at least 96% of the cellular arsenate. In the absence of glucose the rate of efflux was higher initially but after 4 hr achieved a rate constant about the same as that at 4°.

Similar experiments with or without glucose were carried out after exposure to an array of arsenate concentrations during the loading period (Fig. 8). In the presence of

glucose, the data can be fitted by a series of straight lines on a semilogarithmic plot, with the slope inversely related to the arsenate concentration used in the loading period. In the absence of glucose the rate constants for efflux were independent of the arsenate concentrations except for an initial period. If the slow component is subtracted, the resulting data, representing the initial rapid components, are found to fit single logarithmic functions, with rate constants that increase inversely with the arsenate concentration. In fact, the slopes are very close to those for equivalent concentrations in the presence of glucose. Arsenate efflux seems to be divisible into two components: one that is relatively slow, and independent of temperature and of metabolism, with first-order rate constants that are independent of the concentration of arsenate used during the loading period; and another that is relatively rapid, dependent on temperature and on metabolism, with first-order rate constants in inverse relationship to the concentration of arsenate used during the loading period. The small initial concentration-dependent component in the absence of glucose in Fig. 7 and 8 probably results from a residual high rate of endogenous fermentation related to the previous exposure to glucose in the loading period.

Metabolic activity might influence arsenate efflux either by altering its transfer through the membrane or by altering its distribution and localization within the cell. It has already been pointed out that the efflux is specific for arsenate as compared to phosphate and that it is independent of the transport system directed into the cell. It seems unlikely that the cell would possess an additional arsenate-specific, outwardly directed active transport system. Furthermore, first-order kinetics of Figs. 7 and 8 suggest a diffusion-limited process. It seems more likely that the cell membrane has a finite permeability to arsenate and that the rate of efflux is determined by the concentration of free arsenate in the cell in contact with the membrane. The effect of metabolism on efflux in relation to the arsenate load (Fig. 8) would then result from an altered rate of metabolic turnover of arsenate, and consequently an altered concentration adjacent to the membrane.

Presumably arsenate competes with phosphate for esterification reactions within the cell, but unfortunately no detailed studies have been carried out concerning its distribution, nor have fractionation procedures been developed. In order to gain at least some primitive knowledge concerning the changes in arsenate distribution as a function of arsenate concentration, a simple fractionation procedure was adopted, based on methods for phosphate (see Methods). The amount of labeled arsenate was estimated in each of four fractions at different times, for yeast loaded at four concentrations of arsenate corresponding to those used in the experiment of Fig. 8. Arsenate was found in all fractions, but the predominant one was the cold TCA extract (Fig. 9). At the time when the arsenate content of the cells was maximal, this fraction contained 80% to 90% of the arsenate. Between 10% and 20% of this fraction could be precipitated with magnesia mixture which is specific for inorganic phosphate. This 10 to 20% may represent the inorganic arsenate content of the cell, and the rest of the cold TCA fraction probably represents soluble esters and polyarsenate. Most of the remainder of the arsenate was in the hot TCA fraction, with small but definite amounts in the lipid fractions and only traces in the proteins. The relative sizes of the latter fractions were much higher when the cells were loaded at low concentrations of arsenate. The hot TCA extract was treated with Norit A which specifically absorbs nucleotides, but only at high arsenate concentrations was any significant amount of arsenate (about

3% of the total cellular arsenate) adsorbed on Norit A. No further attempts at fractionation or identification were made.

The third factor that determines the cellular content of arsenate is the continuous reduction in transport capacity. It was previously reported that at concentrations of arsenate of 1×10^{-4} M and higher, the inhibitory effect reached virtually 100% in

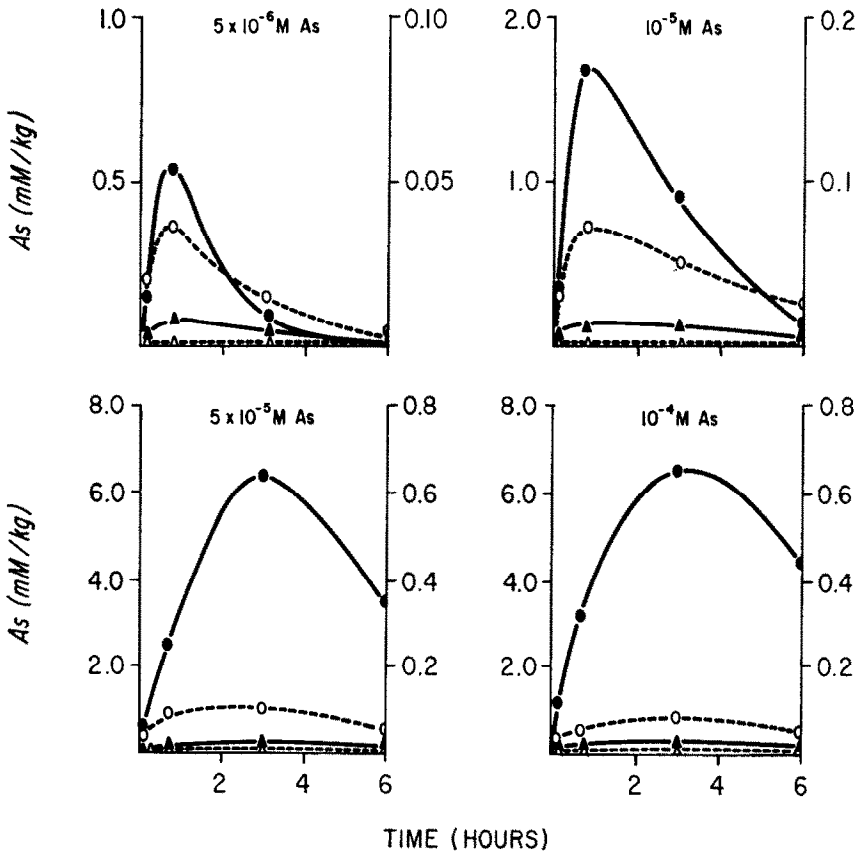

FIG. 9. Time course of arsenate distribution in four fractions with the exposure to four different concentrations of arsenate. The abscissa on the left of the graph represents the values for the cold TCA-soluble (●) and hot TCA-soluble (▲) fractions, and that on the right the lipid (○) and protein (△) fractions.

each case, but that the inhibition developed more rapidly with higher concentrations, following first-order kinetics in each case.[13] The present experiments were extended to considerably lower concentrations of arsenate. To avoid the complications of the continuously decreasing rate of transport that occurs in the presence of arsenate, cells were exposed to a particular concentration of arsenate and at time intervals samples of the suspension were washed free of arsenate and given a test concentration of ^{32}P-labeled phosphate to measure the transport capacity. (The reduction in the transport capacity for phosphate is identical with the reduction in the transport capacity for arsenate.) The washing produced no reversal of the inhibition, and the degree of inhibition was stable for at least several hours. By using a series of phosphate concentrations in the test period, a kinetic analysis of the inhibitory effect

indicated that the affinity constant (K_m) for phosphate was not altered, but that the maximal rate (V_m) was reduced—behavior typical of either a noncompetitive or of an irreversible inhibition. In order to evaluate the inhibitory effect in greater detail, a single test concentration of phosphate was chosen, at 1×10^{-3} M, sufficiently high to give approximately the maximal rate of transport. The inhibition of phosphate transport, with any given concentration of arsenate, develops over a period of about an hour to a final stable value (Fig. 10). The time course is approximately first order,

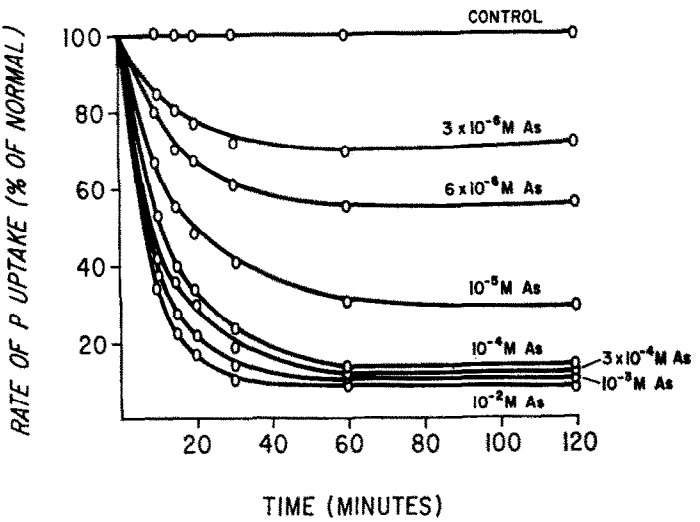

FIG. 10. Time courses of the blocking of transport by arsenate in various concentrations.

with half-times about the same at concentrations below 1×10^{-4} M, and with decreasing values at concentrations above 1×10^{-4} M. The final inhibition increases with arsenate concentration to a maximum of over 90% at 1×10^{-4} M. Thus the inhibitions at low concentrations of arsenate are characterized by a constant half-time of development and by an increasing maximal effect, whereas those at high concentrations are characterized by a constant maximal effect (90% inhibition) and a decreasing half-time.

As arsenate uptake proceeds, an inhibition of metabolism also develops, reaching a maximum in 20 to 40 min. The maxima are plotted against the arsenate concentration in Fig. 11. The extent of inhibition was identical under aerobic or anaerobic conditions, so the blocking effect is presumably in a common pathway, probably the glycolysis chain. The curve is unusual inasmuch as most of the effect was obtained with concentrations of arsenate of 2×10^{-5} M or less. Yet concentrations of arsenate as high as 1×10^{-2} M could inhibit only to the extent of 60%. The limitation on the extent of inhibition must be related to the organization of the intact cell, because in broken cells prepared with a French press, arsenate inhibition of metabolism approached 100%.

Although large amounts of arsenate can be taken up during endogenous fermentation of cells pretreated with glucose (Table 1), such arsenate-loaded cells after they are washed free of arsenate have a normal rate of fermentation for at least 2 hr when

tested against glucose. The same amount of arsenate taken up during exogenous metabolism would result in an inhibition of 60%.

Another, perhaps related, feature of the inhibition of metabolism by arsenate was found in experiments designed to test its reversibility. After various times of exposure to arsenate, half of each suspension was washed three times in buffer solution, a

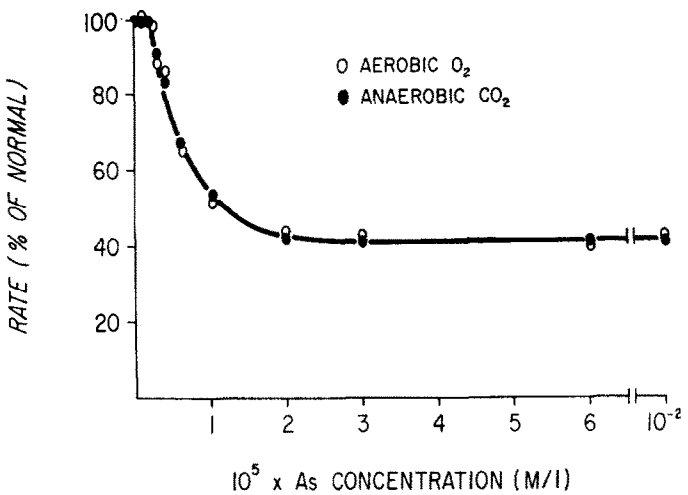

FIG. 11. Final extent of inhibition of metabolism as a function of the concentration of arsenate.

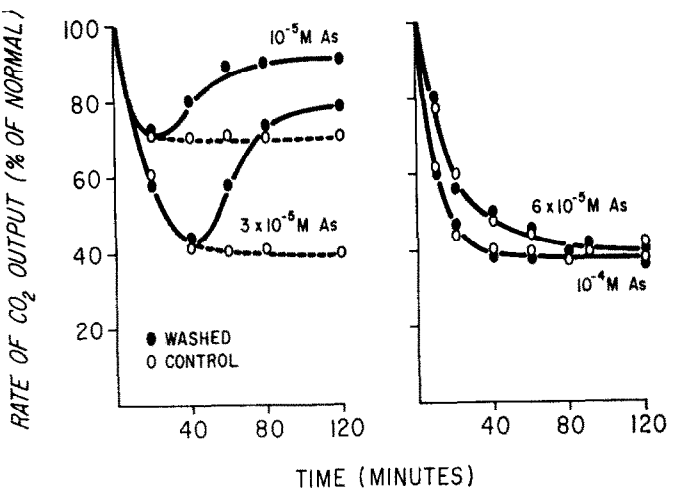

FIG. 12. Time course of inhibition of fermentation by arsenate and its reversal by washing and resuspension in arsenate-free solutions.

procedure requiring about 20 min. These cells were then suspended in glucose buffer, and their metabolic rate was compared with the yeast cells that remained in the original arsenate medium. The results are shown in Fig. 12. In the yeast continuously exposed to arsenate, the rate of metabolism decreased over a period of time to a new level. In the cells that were washed, a reversal of inhibition was observed at the lower

concentrations of arsenate (1×10^{-5} and 3×10^{-5} M), but not at the higher concentrations (6×10^{-5} and 10×10^{-5} M) even though the maximal inhibition was the same (60%) for the 3, 6, and 10×10^{-5} M. The reversibility after washing followed a distinct time pattern. For example, in the experiment with 3×10^{-5} M arsenate, no reversal was observed after 20 or 40 min of exposure to arsenate, although inhibition was maximal at the latter time. After 60 min, however, washing resulted in a partial reversal, from 60 to 40% inhibition. After 80 min the reversal was considerably greater, from 60% to 25%. In other experiments, the cells were washed with buffer containing arsenate alone or glucose alone. In either case, the reversal was the same as washing with buffer. If, after washing, the cells were suspended in glucose plus arsenate, the reversal was transient. If the cells were washed with arsenate plus glucose and resuspended in the same medium, no reversal occurred. The full inhibition is maintained only if both glucose and arsenate are present. Thus some changes in the state of the cell occur, with respect to arsenate inhibition, after 40-min exposure. This change in state is unmasked provided that, during the washing procedure, glucose and arsenate are not present together. On the other hand, the washing is ineffective if the concentration of arsenate is excessively high (6×10^{-5} M or higher).

Phosphate competes with arsenate for transport into the cell and also reduces the rate at which the inhibition of the transport develops.[13] Phosphate also prevents the arsenate inhibition of metabolism in a competitive manner. For example, in Fig. 13

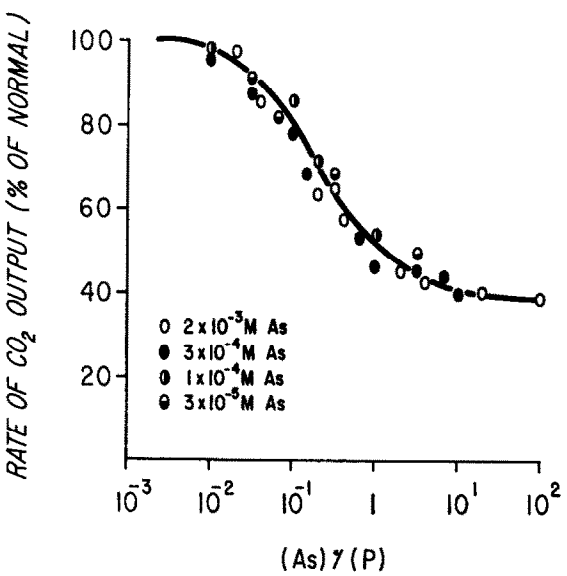

FIG. 13. Relative rates of anaerobic CO_2 output as a function of the ratio of arsenate to phosphate concentrations.

the degree of inhibition in the presence of mixtures of arsenate and phosphate is independent of the absolute concentration of arsenate (over a hundredfold range), provided that the ratio of arsenate to phosphate is constant. The midpoint of the curve occurs at an arsenate-to-phosphate ratio of 1 to 2·5. Similar curves with the same midpoint fit the data on inhibition of metabolism, on reduction in transport

capacity, and for the rate of arsenate transport into the cell (Fig. 14). It is probable, therefore, that the inhibitory effects of arsenate are altered by extracellular phosphate at the level of the competition for transport into the cell, and consequent reduction in the delivery of arsenate to the metabolic and transport centers.

The inhibitory effects of arsenate on metabolism and on transport are also reduced if the glucose concentration is low and, as in the case of addition of phosphate, the

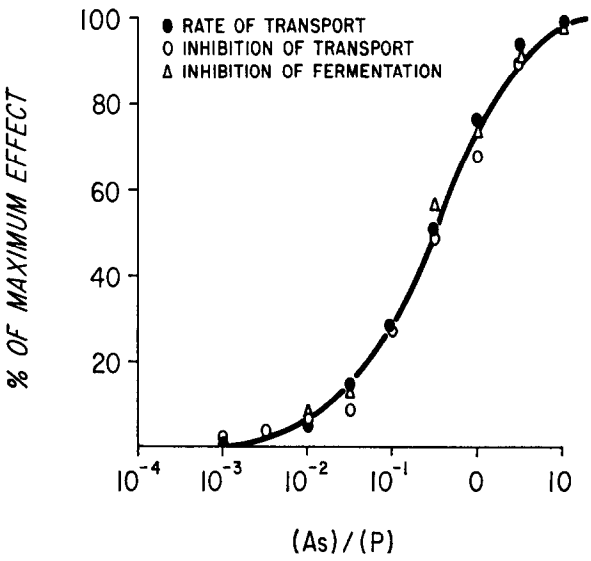

FIG. 14. Comparison of rate of arsenate uptake, blocking of transport, and inhibition of fermentation as a function of the ratio of arsenate to phosphate concentrations; 10^{-4} and 3×10^{-4} M phosphate concentrations are used with varying arsenate concentrations.

curves for the rate of arsenate transport can be superimposed on the inhibition curves. Thus the reduced effect of arsenate at lower concentrations of glucose can be attributed to a reduced rate of delivery of arsenate into the cell. The midpoint of the curves, with an arsenate concentration of 10^{-4} M, occurs at a glucose concentration of 6×10^{-3} M, which is approximately the Michaelis constant for glucose uptake and metabolism. Thus the arsenate uptake and the degree of the subsequent inhibitory effects are directly proportional to the rate of glucose metabolism.

DISCUSSION

In the present paper data are presented concerning the kinetics of arsenate transport, the kinetics of development of inhibition of transport and of metabolism, the final degree of inhibition, the efflux of arsenate, and the cellular content and distribution of arsenate. Despite the obvious complexities of the systems involved, certain relationships can be established or presumed.

An analysis of the kinetics of arsenate influx suggests that two kinds of membrane transport sites are involved, with affinities differing by a factor of about 100 (K_m's of

4×10^{-6} and 4×10^{-4} M). Although the capacity of the sites of high affinity (K_m of 4×10^{-6} M) is only about 15% of the total capacity, they can deliver sufficient arsenate into the cell to achieve the maximal inhibitory effects. Two such effects have been studied, the inhibition of metabolism and the inhibition of phosphate transport. In each case, the inhibition curves (the maximal inhibition for a given concentration of arsenate) correspond approximately to the curve for saturation of the transport site of K_m 4×10^{-6} M (Fig. 15). At arsenate concentrations producing the maximal

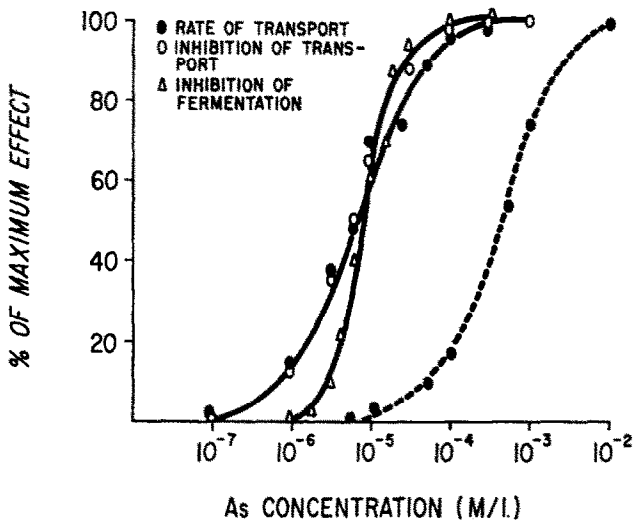

FIG. 15. Comparison of rate of arsenate uptake, blocking of transport, and inhibition of glycolysis as a function of the concentration of arsenate in the exposure system. The rate of arsenate uptake is analyzed in terms of two transport systems with differing affinities for arsenate.

inhibitory effect, over 80% of the arsenate uptake occurs via the sites of low K_m, and with inhibitions of 50% or less, the proportion is over 90%. An increased rate of delivery of arsenate at higher concentrations, via the other transport site (K_m, 4×10^{-4} M) does, however, result in a more rapid development of the inhibition.

The sequence of events after exposure to arsenate is best illustrated by examining the consequences of addition of a single concentration of arsenate, say 4×10^{-6} M. The first transport site (K_m 4×10^{-6} M) will be about half saturated, and the second will be virtually free of arsenate. The initial rate of uptake will be about 1·5 mmoles/kg/hr. The rate of uptake will fall continuously with time, for two reasons. First, the arsenate of the medium will be partially depleted, with the concentration falling to approximately 1×10^{-6} M in an hour. Second, the transport capacity will be gradually reduced by a noncompetitive type of inhibition. By the end of an hour, the transport capacity will be reduced to a new stable value of about 50% of the initial capacity. Because of these two factors, the actual rate of arsenate influx after 1 hr is reduced to 0·25 mmoles/kg/hr or about one sixth of the initial rate of uptake. The rate of metabolism also decreases with about the same time course, reaching a stable level of 80% of normal after 1 hr. Although arsenate transport continues at a reduced level, the arsenate content of the cell reaches a peak at 1 hr, and thereafter

declines because the rate of efflux has been increasing and after 1 hr exceeds the rate of influx. By the end of 2 hr, the arsenate content is only about half that at 1 hr.

It is evident from the observations cited above that a stable level of inhibition is established after an hour of exposure, despite relatively large changes that continue to occur in the rate of efflux, the arsenate content of the cell, and the arsenate concentration in the medium. It must be concluded, therefore, that the final degree of inhibition is not a direct function of any one of these parameters, but that it is related to the arsenate level in some cellular compartment which contains only a small fraction of the total arsenate, and which retains a constant amount of arsenate despite the large changes that occur in efflux and total arsenate content. Evidence that only a fraction of the arsenate content is involved in the inhibition of transport had also been presented in a previous paper[13] in which it was demonstrated that the same degree of inhibition could be attained even though the total arsenate content varied by a factor of ten. Direct evidence of compartmentalization is given in the present paper by the data on fractionation in Fig. 9. Not only is arsenate found in several chemical fractions, but the relative amount in each fraction varies with time and with the initial arsenate concentration. Undoubtedly, arsenate substitutes for phosphate in many metabolic reactions, in many parts of the cell, but the present state of knowledge concerning phosphate and arsenate pools permits only the most general speculations concerning the particular pools and reactions involved in the inhibitory effects.

The inhibition of transport and of metabolism do not reflect the action of arsenate on the same cellular sites because the inhibition of metabolism can be reversed by washing the cells free of external arsenate, whereas the inhibition of transport cannot. The reversal with washing is not associated with any measurable loss of arsenate beyond the small amount that would leak out in the same period (20 min) even if the cells were not washed. Presumably, the arsenate concentration in contact with the metabolic sites depends on a steady state involving new arsenate transported into the cell from the medium and its continued dispersion and turnover into other parts of the cell. When the inflow is shut off at various times by removing the external arsenate plus glucose, the degree of reversal probably reflects changes in the pool size and the rate of metabolic turnover of arsenate of that compartment. If the cell is exposed to high concentrations of arsenate, the redistributions of cellular arsenate cannot result in a sufficient reduction in the arsenate level in the inhibitory pool, and no reversal can be accomplished by washing.

In considering the relationships between transport and metabolism, certain facts and presumptions can be stated. The metabolism can be inhibited only if arsenate is delivered into the cell by the transport system. As arsenate is taken up, however, the capacity of the transport system is continuously reduced, and the delivery of arsenate is slowed down or may virtually cease. Consequently, as the rate of transport is increased with higher concentrations of arsenate, the rate of inactivation of the transport system is also increased, and the total delivery of arsenate to the cell is to a large degree self-limited. Such a limitation may account for the fact that even with the highest arsenate concentrations, the maximal inhibition is only 60%, whereas in broken cells it is virtually 100%. The reverse dependence, of the transport on metabolism, has also been demonstrated. Arsenate is taken up in appreciable amounts only when the glycolytic–metabolic pathway is activated. However, the inhibition of glycolysis by arsenate is not the cause of the inactivation of transport. First, the

metabolism can be inhibited to the extent of only 60%, and the remaining rate of metabolism is sufficient to maintain an appreciable rate of transport. Second, the metabolic effects can be reversed by washing, with no reversal of the inactivation of the transport system. Even though the inhibitions of transport and of metabolism appear to involve the effects of arsenate on two independent sites, they may be connected. It seems more than a coincidence that each effect is related to a small part of the arsenate pool which does not change in size in parallel with the total content of arsenate, and that the two inhibition curves together with the saturation of one of the transport sites fall into the same range of arsenate concentrations (Fig. 15). Perhaps a fraction of the arsenate entering the cell is involved sequentially in the inhibition of transport and in the inhibition of metabolism.

Although the rate of arsenate efflux may be appreciable, leading to a large net loss of cellular arsenate, such loss does not seem to influence the inhibition of transport or of metabolism. The efflux must therefore derive from a major fraction of the cellular arsenate, but not from the compartments responsible for the inhibitions. The efflux is of interest, however, from another point of view. It is highly specific to arsenate as compared with phosphate. In fact, the efflux of phosphate is not measurable over a period of several hours even in arsenate-poisoned cells. In this respect the efflux pattern is different from the influx pattern in which both arsenate and phosphate are taken up in competition for a common transport system. Not only do the influx and efflux pathways differ in specificity, but a high rate of efflux is found in cells in which the transport system (influx) is almost completely inhibited. Because of the high degree of specificity, the efflux cannot be attributed to some nonspecific increase in permeability. Nor is it an exchange system of some kind such as exchange–diffusion, for its rate is uninfluenced by the presence of arsenate in the external solution. It is suggested that the yeast membrane is more permeable to arsenate than to phosphate, and that metabolic activity increases the rate of efflux appreciably by increasing the pool of diffusible arsenate in contact with the membrane, because of a more rapid turnover and redistribution of arsenate in the cell.

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