Arse nic-Lipid Complex Formation During the Active
Transport of Arsenate in Yeast

JORGE CERBÓN
Departamento de Bioquímica, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, México 14, D.F. México

Received for publication 16 September 1968

In studying formation of an arsenic-lipid complex during the active transport of 74As-arsenate in yeast, it was found that adaptation of yeast to arsenate resulted in cell populations which showed a deficient inflow of arsenate as compared to the nonadapted yeast. Experiments with both types of cells showed a direct correlation between the arsenate taken up and the amount of As-lipid complex formed. 74As-arsenate was bound exclusively to the phosphoinositide fraction of the cellular lipids. When arsenate transport was inhibited by dinitrophenol and sodium azide, the formation of the As-lipid complex was also inhibited. Phosphate did not interfere with the arsenate transport at a non-inhibitory concentration of external arsenate (10^-9 M). The As-adapted cells but not the unadapted cells were able to take up phosphate when growing in the presence of 10^-6 M arsenate.

It has been well established that arsenate enters the yeast cell via a constitutive transport system which is normally responsible for phosphate uptake; but, in contrast to phosphate, arsenate continuously inactivates the transport system in an irreversible manner, so that the uptake eventually ceases (7). This evidence, together with the possibility of changing the cells from having a highly efficient arsenate transport system [arsenate-sensitive (AsS) cells] to deficient ones [arsenate-resistant (AsR) cells] (1), would allow an objective analysis of the permeation process and perhaps the identification of permeation components by correlating compositional with permeability changes.

There is evidence for mutual cross-resistance between arsenite and arsenate in Pseudomonas pseudomallei (1). Neither proteins, ribonucleic acid, nor deoxyribonucleic acid could be shown to be the receptors for arsenite in P. pseudomallei (1), and arsenite can replace phosphate in the monosaccharide transport system of Nocardia which is completely dependent on this anion (4). It is also known that the lipid extract of this microorganism contains most of the bound arsenic (5). The present study was undertaken to determine whether the same type of As-lipid complex is formed in yeasts during the arsenate transport and, if so, whether its formation is altered in the same way as the arsenate system, by development of arsenate resistance and by the effect of metabolic inhibitors.

MATERIALS AND METHODS

Microorganisms and conditions of culture. AsS cells of Saccharomyces carlsbergensis ATCC 9080 and their corresponding AsR yeast cultures were utilized. The sensitive cells were grown on nutrient broth containing 1% yeast extract plus 1% maltose at 28 C. The AsR cells were obtained by growing the sensitive cells in the same medium to which 10^-4 M arsenate was added. Cultivation was at 28 C under standing conditions. When marked proliferation of microorganisms was observed after 6 days, the cells were separated by centrifugation and inoculated into fresh medium containing the same concentration of arsenate. After three to four passages, the prolonged lag of adapting cultures was reduced, but the rate of growth of the adapted cells did not reach that of the normal ones.

Arsenate resistance was confirmed by growth and glucose uptake in the presence of 10^-4 M arsenate. In the adapted cells, the glucose uptake is not affected by the presence of arsenate, but in the sensitive cells there is a 60% inhibition.

It was also observed that cells adapted to grow on arsenate are not AsR mutants, but phenotypically resistant cells. When a colony of the AsR cells growing in the presence of 10^-4 M arsenate was suspended in fresh medium without arsenate, the resistant character was maintained during 8 hr (approximately 4 generations) and then gradually disappeared. It was not possible to isolate AsR colonies after approximately 12 generations had elapsed.

Arsenate transport in AsS and AsR yeast. In order to avoid the complex relationship between cell concentrations and arsenate effects, all the experiments were made with cell concentrations ranging from 4 to 5 mg (dry weight) of cells per ml of suspension and,
usually 10^{-4} M arsenate. The cells were grown during 48 to 72 hr on the above-mentioned medium with or without arsenate. They were washed with ice-cold water, then centrifuged, and resuspended to about 4 to 5 mg (dry weight) of cells per ml. Samples (1 ml) of each suspension were utilized for dry weight determination. Five-milliliter samples of the suspensions were sedimented by centrifugation, suspended in 4.0 ml of bidistilled water, and shaken at 28 C in a water bath for 5 to 10 min; then 1.0 ml of a solution containing radioactive ^{14}As-arsenate of known specific activity and 1% glucose as energy source were added. If glucose is omitted from the incubation medium, arsenate uptake is decreased by at least 50%. The cells were incubated for various periods of time (5, 10, and 30 min) at 28 C under standing conditions. The limit exposure time of 30 min was chosen to avoid extensive solubilization of cellular lipid material, the maximum of uptake as well as the maximum blocking of arsenate transport in yeast occurs between the first 20 to 30 min (7). After incubation, the cell suspensions were placed in an ice bath and centrifuged at 6 C; the cells were washed three times with large volumes (90 to 100 times the cellular volume) of cold water or saline solution until the radioactivity of the last washing liquid was negligible. The cells were suspended in 10 ml of Bray's solution (3), and the radioactivity was estimated in a liquid scintillation counter (Nuclear Chicago 720 series). Uptake is expressed as micromoles of inorganic arsenate per gram (dry weight) of cells, calculated from the known specific activity of the extracellular anion.

Measurement of Incorporation of ^{14}As into polar lipids. After incubation of the cells as described above, the washed yeast pellets were extracted by a procedure closely similar to that described by Nikaido (12). After incubation, ice cold trichloroacetic acid (final concentration 5%, w/v) was added to the pellet and the suspension was kept in ice for 20 min. The precipitated material was collected by centrifugation and washed five more times with 5% cold trichloroacetic acid. The precipitate was then extracted with 3.0 ml of methanol at 55 C for 15 min, cooled, extracted with 6 ml of chloroform, vigorously stirred, and filtered through a plug of glass wool into a 12-ml vial. A sample of each extract was taken for determination of radioactivity and the remainder was dried under nitrogen and redissolved in 200 μl of a chloroform-methanol mixture in preparations for thin-layer chromatography.

Thin-layer chromatography of the polar lipids. Thin-layer chromatography of the polar lipids was carried out with the use of Silica Gel G (Brinkman) as the adsorbent on a chloroform-methanol-acetic acid-water (170:40:16:8, v/v) solvent system in a lined jar. The components were first revealed by placing the developed chromatogram in a jar saturated with iodine vapors; the iodine-positive spots were marked and the yellow color was eliminated with hot air. The presence of " ^{14}As was detected by removing the marked zones from the plates with a spatula and suspending the material in Bray's solution. The radioactivity was determined in a liquid scintillation counter. The presence of amino groups was detected with ninhydrin, and the choline-containing lipids were detected by the Dragendorff's reagent.

To confirm the identity of the observed phospholipids, paper chromatography was also performed on silica acid-impregnated paper according to the method of Marinetti (11). Chromatograms were developed with disobutyl ketone-acetic acid-water (40:20:3, v/v) and revealed with Rhodamine 6G.

In both systems, the following commercial standards were utilized as reference: L-a-lecithin, L-a-acephalin, phosphatidyl-L-serine, and phosphatidylinositol. The last two were utilized after purification by thin-layer chromatography.

Inositol assay. The microbiological method as described by McKibbin (10) was utilized for inositol assay.

Materials. ^{14}As (0.64 μmole/mc) and ^{32}P (7.0 μmole/mc) were purchased from the Radiochemical Centre, England, as sodium arsenate and sodium orthophosphate solutions. The standard lipids were purchased from the Sigma Chemical Co., St. Louis, Mo.

RESULTS

Uptake of radioactive arsenate into AsS and AsR yeast and the arsenic-lipid complex formation. As shown in Fig. 1, the As-lipid complex formed in the AsS cells was approximately twice that in the AsR cells, coinciding with the nearly twofold increment influx of arsenate into the
cells. The increased labeling of lipid could be associated with a rapid influx of $^{74}\text{As}$ inorganic arsenate into the cells. The overall uptake of radioactivity from the incubation media was always higher in the sensitive than in the resistant cells, in agreement with previous results of others using P. pseudomallei (1).

Efflux of $^{74}\text{As}$-arsenate from previously loaded AsS and AsR cells. Since the AsR yeasts were not apparently affected in their glucose metabolism by the presence of $10^{-3}$ M arsenate, it is inferred that these cells could have an adequate energy supply for an active efflux, and the lower intracellular level of arsenate found in them could be due to this fact and not to a decreased permeability. Further studies were made to evaluate the effect of As resistance on the rate of efflux of arsenate from preloaded cells. The cells were first incubated for 30 to 35 min with known concentrations of $^{74}\text{As}$ arsenate plus 1% glucose in order to load them at two different levels ($10^{-3}$ M and $10^{-7}$ M arsenate). The cells were then washed free of external isotope and suspended in non-radioactive solutions of the same composition and concentration used during the loading periods, or in distilled water. The loss of isotope from the cells was determined by measuring the radioactivity of the cells at 10, 20, and 35 min.

It was observed (Fig. 2) that the outflow mechanism is similar in both types of cells and that it is not affected either by the arsenate concentration utilized in the loading period or by the presence of external arsenate during the outflow. The intracellular arsenate concentration in the AsS cells preloaded with $1.1 \times 10^{-7}$ M arsenate was four times higher than that found in the suspending medium; conversely, in the AsR cells it was four times lower than in the suspending medium.

Characteristics of the phospholipids in AsS and AsR yeasts. Table 1 shows the characteristics of the phospholipids extracted with chloroform-methanol (2:1, v/v) from both AsS and AsR yeasts. The same five spots were found in each case. When utilizing $^{74}\text{As}$-arsenate, the maximal radioactivity was present in spot B. Material remaining at the origin after chromatographic development included inorganic arsenate, other non-lipid compounds, and polyphosphoinositides. Adequate washing of the lipid extract before chromatographic spotting completely removed the inorganic arsenate from the origin of the chromatograms but did not eliminate the arsenate bound to the inosside spots A and B.

Spots A and B were identified as phosphoinositides by their chromatographic behavior and by their inositol content as determined microbiologically. Spot C was very weak and coincided with the phosphatidyl-serine standard; spot D was Dragendorff-positive, ninhydrin-negative, and its $R_p$ was identical to the lecithin standard. Spot E was identified as phosphatidyl-ethanolamine.

Spot B from yeast lipids was similar in its chromatographic behavior to the previously described As-lipid complex from Nocardia (5). Inositol was demonstrated also in the As lipid isolated from Nocardia, which was also Dragendorff-negative; these properties confirm their identification as phosphoinositides.

Arsenate-phosphate competition. It has been suggested that the yeast membrane is more permeable to arsenate than to phosphate and that arsenate causes a continuous and irreversible inactivation of the phosphate transport system which cannot be relieved by washing the cells with solutions containing glucose or phosphate, or both (7). Then, as arsenate is transported, there must be a parallel irreversible decrease in the functionality of the carrier. To obtain evidence about these points, experiments were con-
formation. To study the role of active transport in the inhibition of glucose uptake, the uptake of 74As and labeling of the phospholipids was investigated. It was observed (Table 2) that, in the presence of 10^{-2} M arsenate, there was no P uptake nor incorporation of phosphatidylcholine into lipid, in spite of the fact that glucose uptake was inhibited only to an extent of 55 to 60%. Under the reverse conditions (10^{-2} M 74As in the presence of 10^{-2} M phosphate), however, there was 74As uptake and formation of the 74As-lipid complex, indicating that phosphate was unable to compete with arsenate for uptake and specific labeling of the phosphoinositides.

Effect of metabolic inhibitors on the arsenate active transport system and the As-lipid complex formation. To determine whether cellular metabolic energy is important in the active transport of arsenate and the 74As labeling of the lipids, metabolic inhibitors were added to the incubation mixtures in the presence of noninhibitory amounts of 74As (10^{-2} M). Table 3 shows that both 10^{-2} M dinitrophenol (DNP) and 10^{-2} M sodium azide (NaN_{3}), but not 10^{-3} M potassium cyanide (KCN), were effective inhibitors of both the arsenate uptake and the As-lipid complex formation. KCN inhibited the glucose respiration in yeasts (7) but did not prevent the active transport nor the formation of the arsenic-lipid complex. Kaback and Stadtman have found that DNP and azide, but not cyanide, produced significant inhibition of both concentative proline uptake (8) and the labeling of phospholipids during glycine uptake in Escherichia coli (9). Working with P. pseudomallei, Beppu and Arima (1) found that there was no inhibition of the arsenate transport

---

**Table 1. Characterization of phospholipids in AsS and AsR yeasts**

<table>
<thead>
<tr>
<th>Spot</th>
<th>R_{p}</th>
<th>R_{b}</th>
<th>Rhodamine 6G</th>
<th>Ninhydrin</th>
<th>Dragen-dorr</th>
<th>Inositol</th>
<th>Identity of components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Origin</td>
<td>0.0</td>
<td>0.0</td>
<td>Blue ±</td>
<td>-</td>
<td>+</td>
<td>Polyporphoinositides</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>0.25</td>
<td>0.10</td>
<td>Blue ±</td>
<td>-</td>
<td>+</td>
<td>Phosphoinositide</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>0.35</td>
<td>0.17</td>
<td>Blue ±</td>
<td>-</td>
<td>-</td>
<td>Phosphatidylserine</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.40</td>
<td>0.20</td>
<td>Blue ±</td>
<td>-</td>
<td>-</td>
<td>Phosphatidylcholine</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>0.48</td>
<td>0.38</td>
<td>Yellow -</td>
<td>-</td>
<td>-</td>
<td>Phosphatidylethanolamine</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>0.75</td>
<td>0.50</td>
<td>Yellow +</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Thin-layer chromatography. R_{p} values were measured on Silica Gel G plates, developed with chloroform-methanol-acetic acid-water (170:40:16:8, v/v).

* Paper chromatography. R_{b} values were measured on silicic acid-impregnated paper developed with diisobutyl ketone-acetic acid-water (40:20:3, v/v); the spots were observed under ultraviolet light after Rhodamine 6G treatment.

Inositol content was determined by microbiological assay on individually extracted spots of several thin-layer plates.

---

**Table 2. Competition between arsenate and phosphate on the lipid labeling and the arsenate uptake in AsS yeasts**

<table>
<thead>
<tr>
<th>Strain and experimental conditions</th>
<th>Cellular uptake</th>
<th>Label in total lipids</th>
<th>Label in spot B</th>
</tr>
</thead>
<tbody>
<tr>
<td>AsS yeasts + 32P without carrier</td>
<td>139.1</td>
<td>3.6</td>
<td>0.48</td>
</tr>
<tr>
<td>AsS yeasts + 32P + 10^{-2} M arsenate</td>
<td>16.0</td>
<td>0.1</td>
<td>0.03</td>
</tr>
<tr>
<td>AsS yeasts + 74As without carrier</td>
<td>106.2</td>
<td>0.7</td>
<td>0.50</td>
</tr>
<tr>
<td>AsS yeasts + 74As + 10^{-2} M phosphate</td>
<td>114.2</td>
<td>0.6</td>
<td>0.50</td>
</tr>
</tbody>
</table>

* The cells were exposed for 30 min at 23 C in 1% glucose containing 74As-arsenate (2 X 10^{-4} M) or 32P-orthophosphate (5.2 X 10^{-4} M), with or without 10^{-2} M competing anion. The results are expressed as picomoles per gram (dry weight) of yeasts.

---

**Table 3. Effect of metabolic inhibitors on the arsenate uptake and the As-lipid complex formation in AsS yeasts**

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>Cellular uptake (picomoles/g, dry wt)</th>
<th>Label in spot B (picomoles/g, dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AsS yeasts + glucose + 74As</td>
<td>102.0</td>
<td>0.61</td>
</tr>
<tr>
<td>AsS yeasts + glucose + 74As + DNP</td>
<td>24.6</td>
<td>0.08</td>
</tr>
<tr>
<td>AsS yeasts + glucose + 74As + NaN_{3}</td>
<td>36.4</td>
<td>0.07</td>
</tr>
<tr>
<td>AsS yeasts + glucose + 74As + KCN</td>
<td>107.0</td>
<td>0.65</td>
</tr>
</tbody>
</table>

* The cells were exposed for 30 min at 23 C in 1% glucose containing 2 X 10^{-4} M 74As-arsenate and, when indicated, 5 X 10^{-4} M DNP, 5 X 10^{-4} M sodium azide or 10^{-3} M potassium cyanide. 
in the presence of DNP or azide; however, these workers used $10^{-4}$ M arsenate, a concentration which is toxic per se and may have masked possible inhibitory effects of DNP or azide.

**DISCUSSION**

In studying the active transport of arsenate in yeast, an apparent involvement of the polar lipid phosphoinositides in arsenate (phosphate) movements through the yeast membrane was found. This finding was substantiated by correlating several different states of arsenate flux with the $^{74}$As labeling of the phosphoinositide fraction. It was found that yeasts adapted to grow in the presence of $10^{-2}$ M arsenate show a deficient arsenate influx and a reduced As-lipid complex formation. The difference in isotope labeling by $^{74}$As could be due, however, to the difference in the intracellular levels attained which were 2 to 18 times higher in the As-sensitive cells than in the As-resistant cells, depending upon the extra-cellular arsenate concentration in the suspending media. In the cases in which the arsenate uptake was inhibited by DNP or NaN, there was also an inhibition in the formation of the As-lipid complex. The fact that, among all the cellular lipids, only the isotope fraction becomes labeled with $^{74}$As suggests a specific interaction.

The yeast membrane is more permeable to arsenate than to phosphate, as evidenced by experiments in which $2 \times 10^{-9}$ M arsenate was transported by AsS yeasts in the presence of $10^{-1}$ M phosphate, whereas the transport of $5 \times 10^{-4}$ M phosphate was completely inhibited by $10^{-1}$ M arsenate. This suggests the existence of high affinity sites for arsenate transport which are modified during arsenate adaptation, thus making phenotypically AsR yeasts with a deficient arsenate inflow system. The presence of those highly specific sites for arsenate transport, on which phosphate does not have any effect, were detected by others (2, 6, 7). With the use of higher arsenate concentrations ($10^{-4}$ M to $10^{-2}$ M), however, phosphate-arsenate competition can be detected, although this is further complicated by interference of arsenate with metabolic pathways. The glucose uptake by the AsR yeasts was apparently normal. Furthermore, AsR yeasts develop, modify, or expose new sites for phosphate uptake which are, in turn, not completely inhibited by arsenate, since there was a slow but significant uptake of $10^{-4}$ M phosphate in a medium containing $10^{-2}$ M arsenate.

These facts alone do not establish that a phospholipid or arsenolipid is part of the transport system for these anions. In order to know whether the As-lipid complex formation maintains any relationship with the efficiency of the arsenate transport system, a detailed study of the characteristics of the AsR cells, including their phospholipid metabolism, is being undertaken and will be reported elsewhere.

Unpublished experiments by this author have shown that, although phosphate uptake is completely inhibited by $10^{-3}$ M arsenate in the AsS cells, there is an increment in the rate of phospho- lipid biosynthesis (utilizing endogenous phosphate) during arsenate transport which is not as evident in the AsR cells.

**LITERATURE CITED**