ARGININE METABOLISM IN PLEUROPNEUMONIA-LIKE ORGANISMS ISOLATED FROM MAMMALIAN CELL CULTURE

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ABSTRACT

Schimke, Robert T. (National Institutes of Health, Bethesda, Md.) and Michael F. Barile. Arginine metabolism in pleuropneumonia-like organisms isolated from mammalian cell culture. J. Bacteriol. 86:195–206. 1963.—Arginine degradation is a significant metabolic process for pleuropneumonia-like organisms (PPLO; Mycoplasma) isolated from cell culture. The conversion of arginine to ornithine in PPLO-contaminated cell culture was rapid, and occurred by the arginine dihydrolase pathway involving arginine deiminase, ornithine transcarbamylase, and carbamyl phosphokinase. In the absence of PPLO contamination, arginine conversion to ornithine was minimal and took place by an arginase activity present in the cell culture, but not in the PPLO. All five PPLO strains isolated from cell culture accomplished the conversion of arginine to ornithine, and contained the requisite enzyme of the arginine dihydrolase system, whereas PPLO-free cell cultures did not. Supplementation of PPLO culture broth with arginine increased the extent of PPLO growth. When the arginine content of the culture limited growth, arginine was completely converted to ornithine. When growth was limited in the presence of excess arginine, citrulline was the major breakdown product. It is suggested that the conversion of arginine to ornithine constitutes a significant, and possibly major, source of adenosine triphosphate for this class of organisms.

During the course of studies of enzymes of arginine metabolism in cell culture (Schimke, 1962a), we were unable to confirm the rapid conversion of arginine to ornithine by cell culture as found by Piez and Eagle (1958) and Manson and Thomas (1960). An explanation for this difference has been suggested by the studies of Rouse and Bonfas (1962) and Kenny and Pollock (1962), which indicate that cell cultures contaminated with pleuropneumonia-like organisms (PPLO; Mycoplasma) rapidly deplete the medium of arginine. Since it has been established that continuous cell cultures are frequently contaminated with PPLO or L forms of bacteria or both (Robinson, Wichelhausen, and Roizman, 1956; Collier, 1957; Pollock, Kenny, and Syvertton, 1960; Barile, Malizia, and Riggs, 1962), the need to re-examine arginine degradation in cell cultures has become evident. This report deals with arginine degradation in a serially propagated cell culture (HeLa-S3), with and without PPLO contamination, and some of the properties of arginine degradation in broth cultures of PPLO isolated from contaminated cell culture.

Two known pathways for arginine degradation resulting in ornithine as a product are:

\[
\text{arginine } \xrightarrow{\text{arginase}} \text{ ornithine } + \text{ urea} \quad (1)
\]

(a.) \[
\text{arginine } \xrightarrow{\text{arginine deiminase}} \text{ citrulline } + \text{ NH}_3 \quad (2)
\]

(b.) \[
\text{citrulline } + \text{ P}_i \xrightarrow{\text{ornithine transcarbamylase}} \text{ ornithine } + \text{ carbamyl phosphate} \]

(c.) \[
\text{carbamyl phosphate } + \text{ ADP } \xrightarrow{\text{carbamyl phosphokinase}} \text{ ATP } + \text{ NH}_3 + \text{ CO}_2
\]

Reaction 1 is the familiar pathway in ureotelic animals (Cohen and Brown, 1960) of arginine degradation mediated by the enzyme arginase. Reaction 2, which constitutes the arginine dihydrolase system first described by Hills (1940), has been described in a number of microorganisms, including Streptococcus (Hills, 1940), Lactobacillus (Walker, 1953), Clostridium (Schmidt,
Logan, and Tytell, 1952), Pseudomonas (Horn, 1933), and yeast (Roche and Laccombe, 1952). It has also been found to occur in two human strains of PPLO (Smith, 1955, 1957).

Evidence will be presented to indicate that arginine breakdown to ornithine in noncontaminated HeLa-S3 is minimal and proceeds by reaction 1. In PPLO-infected HeLa-S3, arginine degradation is extensive and proceeds by reaction 2. All five PPLO isolates obtained from contaminated tissue culture were capable of degrading arginine to ornithine, and contained arginine deiminase, ornithine transcarbamylase, and carbamyl phosphokinase activities. Noncontaminated tissue culture extracts, on the other hand, contained arginase activity, but none of the enzyme activities of reaction 2.

**Materials and Methods**

**Cell culture.** HeLa-S3 in suspension culture was kindly supplied by L. Levintow, National Institute of Allergy and Infectious Diseases. The cells were maintained at concentrations of 2 to 5 \( \times 10^6 \) cells/ml in Eagle’s (1950) spinner medium supplemented with 5% dialyzed horse serum. The cells were transferred to monolayer by inoculation of 2 to 4 \( \times 10^6 \) cells into Eagle’s (1959) basal medium supplemented with 5% dialyzed horse serum. This cell line in suspension or monolayer was consistently found by cultural methods (Barile, Yaguchi, and Eveland, 1958) to be free from PPLO.

**PPLO.** The strains used were isolated from continuous cell cultures (Barile et al., 1962). They were tissue culture PPLO strain HEP-2 (human epidermoid carcinoma of skin—Fjelde), S1 (human carcinoma of cervix—Eagle), MS (monkey kidney stable—Tytell), CL (human liver—Chang), ERKs (embryonic rabbit kidney stable—Westwood). PPLO were maintained routinely by infecting PPLO-free primary rabbit- kidney cells (Youngner, 1954) grown at 37°C in medium No. 199 (Morgan, Morton, and Parker, 1950) containing 5% rabbit serum. The kidney cells were fed every 3 to 4 days. The supernatant fluids containing from 10^4 to 10^5 PPLO per ml were used as the working PPLO suspension. PPLO were grown in a medium of Brain Heart Infusion (Difco) broth with 15% horse serum and 1% yeast extract added (Barile et al., 1958). The broth cultures were incubated aerobically at 36°C for 1 to 4 days. A dilution-plate count method was used for the estimation of PPLO population. The PPLO suspension was diluted in 0.07 M sodium phosphate (pH 7.4) containing 0.85% NaCl and 0.2% gelatin; 0.1-ml samples were placed on duplicate human blood-yeast agar plates (Barile et al., 1958). The plates were incubated aerobically at 36°C for 7 days. Agar media were examined for PPLO growth with a stereoscopic dissecting microscope. The PPLO colonies were counted, and the population was determined.

**Enzyme assay.** The PPLO of broth cultures were sedimented by centrifugation at 15,000 \( \times g \) for 15 min. The sedimented cells were washed with 0.85% NaCl, resuspended in 1 to 4 ml of 0.1 M potassium phosphate (pH 6.5), and exposed to ultrasonic radiation for 15 min in a Raytheon 10-kc sonic oscillator. The cellular debris was removed by centrifugation, and the resulting extract was dialyzed 3 to 4 hr against 0.1 M potassium phosphate (pH 6.5) or, when phosphate was to be excluded, against 0.1 M imidazole Cl (pH 6.5). All enzyme assays were performed in duplicate. Cell-culture cells (HeLa-S3) were scraped free from the glass with a Teflon policeman, centrifuged for 10 min at 500 \( \times g \), washed with serum-free Eagle’s medium, and disrupted as described for the PPLO suspensions. The resulting extract was used for enzyme assays.

Arginase was assayed as described previously (Schimke, 1962a). Urease was assayed in a system containing (in 1.0 ml): 10 \( \mu \)moles of C^14 urea (New England Nuclear Corp., Boston, Mass.), specific activity 0.02 mc/mmole; 50 \( \mu \)moles of potassium phosphate (pH 6.5); and 1 to 2 mg of extract protein. The incubations were performed for 60 min at 37°C. The C^14O_2 liberated was collected and measured as described previously (Schimke, 1962a). Blanks with boiled extract were also incubated for 60 min.

Arginine deiminase was assayed by measuring the rate of citrulline formation. The assay medium contained (in 0.5 ml): 50 \( \mu \)moles of potassium phosphate (pH 6.5); 50 \( \mu \)moles of L-arginine; and 0.5 to 1.0 mg of extract protein. Samples were withdrawn at 5- to 10-min intervals and assayed for citrulline by the method of Archibald (1944), as modified by Ratner (1955). Controls consisting of extract without L-arginine and L-arginine without extract were included with each assay.

Ornithine transcarbamylase and carbamyl phosphokinase were assayed by the methods of
Jones (1962) for measuring citrulline formation. Carbamyl phosphokinase was assayed by coupling with ornithine transcarbamylase isolated from rat liver (Burnett and Cohen, 1957). Carbamyl phosphate, lithium salt, was obtained from Calbiochem.

**Arginine, citrulline, ornithine, and urea.** PPLO cultures were prepared for estimation of arginine, ornithine, and urea as follows. PPLO were removed by centrifugation at 15,000 × g for 10 min. Protein in the supernatant fluid was precipitated with trichloroacetic acid at a final concentration of 10%. The trichloroacetic acid was removed by extraction with ether. Arginine was determined by the method of Sakaguchi (1925) as modified by Van Pilsum et al. (1956). Citrulline was determined by the method of Archibald (1944) as modified by Ratner (1955). Ornithine was measured by enzymatic conversion to citrulline with ornithine transcarbamylase and added carbamyl phosphate, as outlined by Burnett and Cohen (1957). In some experiments, it was desirable to separate ornithine from citrulline for accurate quantitative estimates. This was accomplished by passing the solution through a column (0.3 x 1.0 cm) of Amberlite CG-50 (NH4+ form) which retains only ornithine. The ornithine could then be readily eluted with 2 M NH4OH. The ammonia was removed by vacuum distillation. Urea, as derived from C14-arginine, was measured as described in the urease assay.

**Radiochemicals.** Guanido-C14-L-arginine was obtained from the ChemTrac Corp., Cambridge, Mass.; uniformly labeled C14-L-arginine from Nuclear Chicago Corp., Chicago, Ill.; and ureido-C14-L-citrulline from New England Nuclear Corp., Boston, Mass. All radiochemicals were subjected to column chromatography before use to insure absolute purity. Arginine was chromatographed on an Amberlite CG-50 (NH4+ form) column (1 x 15 cm) with a linear 0 to 2.4 N NH4OH gradient (see Fig. 1 and 2). Citrulline was chromatographed on a Dowex-50 x 2 (H+ form) column (0.5 x 10 cm) with a linear 0 to 3 N HCl gradient (see Fig. 3). Samples were counted in a Nuclear Chicago gas-flow counter. Similar techniques were used for determinations of radioactive products of arginine breakdown in cell culture (Fig. 1, 2, and 3). The measurement of C14O2 derived from ureido-C14-citrulline (see reaction 2 involving ornithine transcarbamylase and carbamyl phosphokinase) was accomplished in a manner similar to that used for the urease assay.

Protein was measured by the method of Lowry et al. (1951), using crystalline bovine albumin as standard.

**Results**

**C14-L-arginine breakdown in HeLa-S3 tissue culture with and without PPLO contamination.** Figures 1 and 2 show the breakdown products of uniformly labeled C14-L-arginine in the medium of noninfected HeLa-S3 and HeLa-S3 infected with a mixture of five cell-culture PPLO strains. Three peaks of radioactivity were found on chromatography of samples of medium on Amberlite CG-50 columns. The first peak represents the radioactive products of arginine breakdown not adsorbed by the resin. The second and third peaks are ornithine and arginine, respectively, and are eluted with the linear 0 to 2.4 N NH4OH gradient. The pattern of arginine products is similar in the PPLO-free HeLa-S3 and in medium without any cells (Fig. 1). In contrast, the PPLO-contaminated cells (Fig. 2) show extensive arginine breakdown. The decrease in arginine was not accompanied by an equivalent increase in ornithine, particularly in the flask incubated for 12 hr (Fig. 2). In this case, a substantial amount of radioactivity was not retained on the column.

To characterize further the more acidic arginine breakdown product(s) not retained on the Amberlite CG-50 column, the nonabsorbed material from the 12-hr sample was chromatographed on Dowex-50 × 2 (H+ form) with a linear 0 to 3 N HCl gradient (total volume 400 ml). The elution pattern shown in Fig. 3 shows a number of discrete peaks, with the majority of radioactivity presented in a single peak. The fractions from this peak were pooled, concentrated, and further chromatographed on paper in four solvent systems (I. Smith, 1960): phenol–ethanol–water–ammonia (75:20:9:1); phenol–water (80:20); methanol–pyridine–water (20:5:1); and butanol–water–pyridine (1:1:1). In each case, a single ninhydrin spot was present which also contained the radioactivity, and which co-chromatographed with authentic citrulline. In addition, the test material produced a chromogen identical to that obtained with citrulline when tested by the colorimetric method of Archibald (1944). When tested by the arsenolysis reaction with purified ornithine transcarbamylase from
liver (conditions similar to those in Table 4), the test material was converted to a compound that co-chromatographed with ornithine, and which was retained on an Amberlite CG-50 column.
Thus, it is shown that the major product of arginine breakdown not retained on the Amberlite CG-50 column of Fig. 1 and 2 is citrulline.

The presence of urea in fractions 12 through 15 from the Dowex-50 \( \times 2 \) column (Fig. 3) was located by incubating samples of each fraction with urease and measuring \( \mathrm{C}^4 \mathrm{O}_2 \) formation. It is evident that urea is not a major product of arginine breakdown in PPLO-infected tissue culture. The other radioactive peaks have not been further identified, and probably result from assimilation of \( \mathrm{C}^4 \mathrm{O}_2 \) liberated during citrulline breakdown (reaction 2).

The calculated stoichiometry of arginine breakdown in these experiments is shown in Table 1. In the absence of any cells, arginine depletion occurred to an extent of 1.9% of the original arginine present. This was accompanied by the appearance of urea and ornithine. The formation of ornithine and urea was not observed in Eagle’s medium devoid of serum. This conversion in the absence of any cells is tentatively attributed to arginase activity present in the added serum. The addition of PPLO-free cells increased the formation of urea and ornithine only slightly. The depletion of arginine in the noninfected cells was largely accounted for by

\[
\begin{array}{c|c|c|c|c|c|c}
\text{Condition} & \text{Time} & \text{Arginine depleted} & \text{Urea found} & \text{Ornithine found} & \text{Citrulline found} & \text{Arginine present in a cell protein} \\
\hline
\text{No cells} & 24 \text{ hr} & 1.9 \% & 1.5 \% & 1.6 \% & 0 \% & 8.5 \% \\
\text{HeLa-S3} & 24 \text{ hr} & 11.1 \% & 2.6 \% & 2.9 \% & 0 \% & 8.5 \% \\
\text{HeLa-S3 + PPLO} & 12 \text{ hr} & 77.9 \% & 42.1 \% & 21.6 \% & 8.5 \% & \\
\text{HeLa-S3 + PPLO} & 24 \text{ hr} & 98.3 \% & 83.1 \% & 13 \% & 5.8 \% & \\
\end{array}
\]

* Cell protein was prepared for counting by the method of Siekevitz (1951), but \( \mathrm{C}^4 \) arginine was used in the trichloroacetic acid washes.
† Not determined.

<table>
<thead>
<tr>
<th>Culture</th>
<th>Arginase activity</th>
<th>Urease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected HeLa-S3</td>
<td>3.2</td>
<td>0</td>
</tr>
<tr>
<td>PPLO-HeLa-S3</td>
<td>1.1</td>
<td>0</td>
</tr>
</tbody>
</table>

* Enzyme assays were performed in cells grown in parallel with those used for the experiments indicated in Fig. 1. Specific activity expressed as \( \mu \)moles of product per mg of protein per hr.

The extensive breakdown of arginine to ornithine by PPLO-infected tissue culture is not the result of an arginase activity. For the data of Table 1 to be the result of arginase, an active urease would be required to degrade urea to ammonia and carbon dioxide, thereby accounting for the lack of equivalence of urea and ornithine formed. Table 2 indicates that urease was not present in extracts of either PPLO-free or PPLO-contaminated HeLa-S3. It was also found that the uninfected cell culture contained more ar-
TABLE 3. Stoichiometry of arginine deiminase of PPLO extracts

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Arginine* (umoles/ml)</th>
<th>Citrulline (umoles/ml)</th>
<th>NH₃† (umoles/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>-18.2</td>
<td>+17.6</td>
<td>+16.8</td>
</tr>
<tr>
<td>30</td>
<td>-35.7</td>
<td>+34.4</td>
<td>+33.9</td>
</tr>
<tr>
<td>60</td>
<td>-47.2</td>
<td>+46.2</td>
<td>+50.2</td>
</tr>
</tbody>
</table>

* Initial arginine concentration was 50 μmoles/ml.
† Ammonia was determined by the method of Bessman and Bessman (1955).

Studies of arginine breakdown to ornithine by other cell-culture lines, including KB (obtained as a spinner culture from Microbiological Associates, Bethesda, Md.), and a strain of L cells supplied by R. A. Fleishman, National Institute of Allergy and Infectious Diseases, have indicated that the formation of ornithine by these cell lines occurs to an extent of not more than 2% of the arginine present in the medium (0.6 mM) as determined by colorimetric estimates.

Studies with isolated PPLO. The following studies were undertaken to demonstrate that arginine is actively degraded by way of reaction 2 in extracts of PPLO, i.e., by the combined action of arginine deiminase, ornithine transcarbamylase, and carbamyl phosphokinase.

Arginine conversion to citrulline (arginine deiminase). Dialyzed extracts of a mixed culture of the five strains grown in broth were capable of degrading arginine to citrulline and ammonia in stoichiometric amounts (Table 3) in a system consisting of 0.05 M potassium phosphate (pH 6.5), 0.05 M L-arginine, and 0.7 mg of protein, in a volume of 1 ml. With the same dialyzed PPLO extracts in a system consisting of 50 μmoles of potassium phosphate (pH 6.5) and 25 μmoles of C¹⁴-argininosuccinic acid (Schimke, 1962a), citrulline was not detected by color reaction or paper chromatography. Thus, it is shown that the conversion of arginine to citrulline was not due to a reversal of the bacterial and animal biosynthetic pathway (see review of Cohen and Brown, 1960) involving argininosuccinate synthetase and argininosuccinase.

Conversion of citrulline to ornithine (ornithine transcarbamylase and carbamyl phosphokinase).

Table 4 indicates that, in extracts of PPLO dialyzed against 0.05 M imidazole Cl (pH 6.5), adenosine diphosphate (ADP), inorganic phosphate, and Mg²⁺ were required for the conversion of citrulline to ornithine as determined by C¹⁴O₂ formation, and that arsenate could substitute for these requirements. Table 5 indicates that, in a system similar to that used in Table 4, for every mole of citrulline converted to ornithine, 1 mole of carbon dioxide and 1 mole of ammonia were formed.

Since the previous studies were performed with mixed cultures of all five PPLO strains, studies of enzymes of arginine degradation were undertaken for each strain. Table 6 indicates that extracts of each PPLO isolate contain the necessary enzymes.

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount (μmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrulline*</td>
<td>-8.6</td>
</tr>
<tr>
<td>Ornithine†</td>
<td>+8.6</td>
</tr>
<tr>
<td>NH₃‡</td>
<td>+7.9</td>
</tr>
<tr>
<td>CO₂‡</td>
<td>+8.8</td>
</tr>
</tbody>
</table>

* Reaction medium initially contained 25 μmoles of ureido-C¹⁴-l-citrulline.
† Ammonia estimated by the method of Bessman and Bessman (1955).
‡ Estimated as C¹⁴O₂.
for conversion of arginine to ornithine via citruline, but essentially no arginase activity. No attempt was made to quantitate the specific activities with the strains CL, MK, and S3 because of the small amount of material available, leading to quantitatively inaccurate values. However, the specific enzyme activities were of the same order of magnitude as those found for ERKS and HEp-2.

Characteristics of arginine breakdown by intact PPLO in isolated culture. Table 7 indicates that the breakdown of arginine by PPLO does not require the presence of tissue culture cells, but occurs readily when the organisms are grown in PPLO broth medium. The five PPLO strains were grown for 4 days, at the end of which time samples of the culture were used for estimates of PPLO population and arginine, citrulline, and ornithine deter-

**TABLE 6. Activities of arginase, arginase deiminase, ornithine transcarbamylase, and carbamyl phosphokinase in extracts of PPLO originally isolated from contaminated cell cultures**

<table>
<thead>
<tr>
<th>PPLO strains</th>
<th>Arginase</th>
<th>Arginase deiminase</th>
<th>Ornithine transcarbamylase</th>
<th>Carbamyl phosphokinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERKS</td>
<td>0.005</td>
<td>3.0</td>
<td>2.1</td>
<td>0.50</td>
</tr>
<tr>
<td>HEp-2</td>
<td>0.005</td>
<td>2.6</td>
<td>3.1</td>
<td>0.27</td>
</tr>
<tr>
<td>CL</td>
<td>0.005</td>
<td>+†</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MS</td>
<td>0.005</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S3</td>
<td>0.005</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* Enzyme activities are expressed as µmoles of product formed per mg per min.
† Enzyme activities are only noted as being present. The small quantity of each sample made accurate quantitative estimates of special activity impossible.

**TABLE 7. Arginine breakdown by PPLO in broth cultures**

<table>
<thead>
<tr>
<th>Strain</th>
<th>PPLO/ml at time of harvest</th>
<th>Amount (µmoles/ml)</th>
<th>Arginase</th>
<th>Citrulline</th>
<th>Ornithine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broth only.</td>
<td></td>
<td>12.4*</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>ERKS</td>
<td>5.6 X 10⁶</td>
<td>-12.21</td>
<td>+0.12</td>
<td>+11.80</td>
<td></td>
</tr>
<tr>
<td>HEp-2</td>
<td>6.7 X 10⁷</td>
<td>-9.87</td>
<td>+0.37</td>
<td>+9.00</td>
<td></td>
</tr>
<tr>
<td>MK</td>
<td>10⁸</td>
<td>-2.79</td>
<td>+0.02</td>
<td>+2.54</td>
<td></td>
</tr>
<tr>
<td>CL</td>
<td>10⁴, &lt;10⁶</td>
<td>-1.64</td>
<td>+0.01</td>
<td>+1.46</td>
<td></td>
</tr>
<tr>
<td>S3</td>
<td>10⁴, &lt;10⁴</td>
<td>-0.02</td>
<td>+0.01</td>
<td>+0.41</td>
<td></td>
</tr>
</tbody>
</table>

* Original arginine content of broth medium.

minations. In both cultures of growing PPLO strains isolated from cell culture, arginine was converted essentially completely to ornithine.

In contrast to the findings with PPLO growing in broth, Smith (1955) reported that resting, human PPLO strain O7 degraded arginine to citrulline, but not ornithine, although extracts of these cells were capable of converting citrulline to ornithine (Smith, 1957). Experiments of a type similar to Smith’s (1955), using washed PPLO resuspended in phosphate buffer containing 0.05 M L-arginine, are shown in Fig. 4 for strains ERKS and HEp-2. It is evident that in both strains there is a rapid degradation of arginine, and that both ornithine and citrulline constitute significant degradation products. In strain ERKS, ornithine ac-
Table 8. Viable population count of ERKS and HEP-2 maintained at 37°C in phosphate buffer after centrifugation and washing

<table>
<thead>
<tr>
<th>Strain</th>
<th>PPLO/ml (plate count) at</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 hr</td>
</tr>
<tr>
<td>ERKS</td>
<td>2.2 × 10^8</td>
</tr>
<tr>
<td>HEP-2</td>
<td>2.4 × 10^8</td>
</tr>
</tbody>
</table>

Counts for 75% of the consumed arginine, whereas in HEP-2, ornithine accounts for 40% of the degraded arginine. Citrulline accounts for the remaining arginine breakdown.

The degradation of C14-citrulline added to either ERKS or HEP-2 under the conditions of Fig. 4 is negligible. This finding is in confirmation of the finding of Smith (1955) that citrulline does not enter these cells. It indicates that the breakdown of citrulline to ornithine is not the result of lysis of PPLO cells during preparation, releasing into the suspending medium active extracts capable of degrading citrulline, but rather that the conversion has occurred within the cells.

The results (Fig. 4) indicating significant citrulline formation in PPLO agree with Smith’s (1955), and are in contrast to the findings in broth cultures (Table 7). However, experiments with centrifuged PPLO are not comparable with those in which PPLO growth occurs. As shown in Table 8, the centrifugation and washing of PPLO markedly reduces viability during the subsequent 3-hr incubation period in phosphate buffer. In other studies, PPLO maintained in the phosphate buffer without centrifugation and washing did not show any decrease in population over a 24-hr period.

Effects of arginine on growth of PPLO strain ERKS in broth. It has been found that supplementation of the infusion broth with 0.05 M additional arginine increases the yield of PPLO, whether measured by turbidimetric techniques, population titer, or protein content of the sedimented PPLO. Figure 5 shows a growth curve of ERKS (chosen for its rapid growth rate) obtained by standard turbidimetric technique (Klett-Summerson photoelectric colorimeter, 540-nm wavelength). In the unsupplemented broth, which contained 12 to 13 mM of arginine, growth ceased abruptly at a time when the arginine of the medium was totally depleted (see Fig. 6). If arginine was added after the cessation of growth, growth again commenced, and continued at a rate and to an extent similar to that occurring when arginine was added at the onset of the experiment. Arginine supplementation does not increase the rate of growth, but merely the extent. The addition of other potential energy sources, glucose and glutamine (by reversal of the glutamine synthetase reaction) at 0.05 M concentrations, did not result in a more rapid rate or greater extent of growth than that which occurred without supplementation. In other experiments, the addition of arginine peptides to the broth in the form of a partial hydrolysate of salmon (Gale, 1945) did not stimulate growth to any greater extent than the limit of the added arginine, in either the presence or absence of added glucose. This finding is in contrast to the results of Gale (1945), who found that arginine peptides were far superior to arginine in stimulating Streptococcus growth.

Table 9 shows the relationship among Klett optical density readings, population titer, and protein content of cells, and indicates that the use of turbidimetric analysis of ERKS population is valid.

Figure 6 indicates the products of arginine breakdown by ERKS growing in broth under conditions where: (i) arginine limits growth at two concentration levels, and (ii) growth ceases in the presence of excess arginine. Samples of broth were removed and analyzed for arginine, ornithine, and citrulline, by both colorimetric and radioactive techniques similar to those used for Fig. 4. It is evident that during all phases of active growth,
or when growth is limited by arginine, ornithine is the sole product of arginine breakdown. However, when the PPLO cells are no longer growing, but when excess arginine remains, arginine breakdown continues, now being accompanied by the formation of citrulline rather than ornithine. Thus, the arginine breakdown product obtained will depend on the growth phase of the PPLO culture and the presence of excess arginine in the medium. In other experiments where glucose or glutamine were present in the medium without arginine supplementation (Fig. 5), arginine was also converted entirely to ornithine.

**DISCUSSION**

The absence of arginine degradation to ornithine in HeLa, KB, and L cells indicated in these studies is in contrast to the reports of Piez and Eagle (1958), who used a HeLa strain, and Manson and Thomas (1960), who used L cells. The arginase content of the cell line would appear to have little influence on conversion of arginine to ornithine in the culture medium, since Sanford et al. (1961) failed to find urea formation in a cell line with far higher arginase levels than the cell lines used in this study. These findings of little arginine breakdown by arginase of cell cultures can be accounted for largely by the high concentrations of arginine required for significant activity of the enzyme ($K_m$ of arginase for arginine = 1 to 2 x 10^{-4} M L-arginine), concentrations never approached with the arginase concentrations employed in standard culture media. We therefore conclude that extensive degradation of arginine to ornithine is not an inherent property of tissue culture, but rather is an artifact due to PPLO contamination. It is of note that Manson and Thomas (1960) found that arginine breakdown by L cells was accompanied by the formation of 1 mole of ornithine and 2 moles of ammonia, a stoichiometry consistent with the pathway of arginine breakdown by PPLO.

The source of the extensive contamination of cell cultures with PPLO is unknown, although the suggestion of Rothblat and Morton (1959) that PPLO contamination results from transformation of bacterial contaminants to stable L forms seems likely (Barrie et al., 1962). Our studies would indicate that streptococci, or bacterial species capable of arginine degradation, are potential sources of such PPLO contamination. Neimark and Pickett (1960) previously remarked on the similarities of carbohydrate metabolism in PPLO and streptococci. This similarity is further demonstrated by the studies with arginine breakdown presented in this paper.

In addition to the artifact of arginine degradation by PPLO, the additional artifact of arginine breakdown by the added horse serum has been en
accumulates only without the line growth, sluggish man PPLO a 1962). limited would exists Neimark a such (ATP) phosph ate ginine breakdown associated with the formation of adenosine degradation of tyrosine breakdown in PPLO strain 07.

All five of the PPLO strains isolated from contaminated tissue culture degrade arginine to ornithine (Tables 7) and contain the requisite enzymes (Table 6). The widespread occurrence of arginine depletion of culture media in the presence of PPLO is further indicated by the studies of Powelson (1961), which consistently demonstrated arginine depletion with several cell lines by use of PPLO isolated from sheep and avian sources.

The physiological significance of the active degradation of arginine to ornithine by PPLO is unknown. The studies of Bauchop and Elsden (1960) suggest that, in Streptococcus faecalis, arginine breakdown by this same pathway is associated with the formation of adenosine triphosphate (ATP) used in cellular multiplication. That such a function for arginine breakdown in PPLO exists would appear plausible in view of the apparent limited oxidative capacity of PPLO (Lynn, 1960; Neimark and Fickett, 1960; review of Razin, 1962). P. F. Smith (1960) concluded that the possibility of arginine breakdown constituting a significant energy source seemed unlikely in human PPLO strains because of the lack of conversion of arginine to ornithine in resting cells, and the sluggish activity of the conversion of citrulline to ornithine in cell-free extracts. Our studies indicate that arginine is in fact converted to ornithine (Table 7, Fig. 4 and 6). During active PPLO growth, arginine is degraded to ornithine without the accumulation of citrulline. Citrulline accumulates only under resting conditions, i.e., in washed cells in phosphate buffer (Fig. 4) and in broth after growth has ceased under conditions where arginine is not limiting growth (Fig. 6). We propose that such results can be explained on the premise that arginine breakdown does, indeed, represent a source of high-energy phosphate. The continual breakdown of ATP that occurs in biosynthetic processes of growth provides a continual supply of ADP which pulls the reversible carbamyl phosphokinase reaction (Jones and Lipmann, 1960) in the direction of carbamyl phosphate breakdown. The continual removal of carbamyl phosphate in turn pulls the ornithine transcarbamylase reaction in the direction of citrulline breakdown. When growth ceases, and the ATP is no longer utilized, the breakdown of citrulline stops. The breakdown of arginine to citrulline, on the other hand, continues with the resultant formation of citrulline, which can now be demonstrated in the medium. The inability of washed and sedimented cells to complete arginine breakdown to ornithine (Fig. 4; Smith, 1955) may well result from lack of growing conditions or from damage to the organisms as suggested by loss of viability (Table 8) and, hence, decreased or lost ability to utilize ATP.

Although it is suggested that arginine conversion to ornithine constitutes a significant source of high-energy phosphate (ATP) for PPLO, and indeed cannot take place without formation of ATP, the extent to which this conversion constitutes the only or major source of high-energy phosphate is unknown. The continual breakdown of arginine and limitation of growth by this breakdown in PPLO is similar to the degradation of proline by a prolineless mutant of Escherichia coli (Stone and Hoberman, 1953) and of tyrosine by S. faecalis (Kihara, Klatt, and Smell, 1952). In these previous cases, as well as in the breakdown of arginine by Streptococcus when grown in a glucose-containing broth (Gale, 1945), peptides containing the required amino acid were more effective in promoting growth than the free amino acid, a finding ascribed to a protective effect of the peptide on degradation of the amino acid. In PPLO, on the other hand, peptides containing arginine did not result in growth greater than occurs from the added arginine. If arginine did indeed furnish the major source of ATP, one would predict that the addition of peptides containing arginine would not stimulate growth to any greater extent than the free amino acid itself.
That such is the case would suggest that arginine breakdown is a major source of ATP for at least the one Mycoplasma organism studied extensively in this paper.

Irrespective of speculation concerning the significance of arginine breakdown in PPLO, it is clear that the addition of arginine to broth cultures results in a two- to threefold increase in cellular yield of PPLO (Fig. 5, Table 9). Similar increased yields of PPLO in contaminated cell cultures have been found after increasing the arginine content of the medium. This finding indicates the prime importance of arginine metabolism of PPLO, and the desirability of using a PPLO growth medium supplemented with arginine.

We are presently investigating whether the arginine dihydrose pathway exists in all PPLO organisms. To date, we have found complete lysis of PPLO, and the desirability of using a PPLO growth medium supplemented with arginine.

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