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The uptake of amino acids by lipids of *pseudomonas aeruginosa*[*†]

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SUMMARY

The lipids of intact *Pseudomonas aeruginosa* resting cells were extracted and the weights of the lipid fractions determined. The phospholipids were found to comprise a major portion (80%) of the ether-soluble lipids. Chromatograms of the phospholipid complex on silicic acid-impregnated paper revealed at least six fractions. Two-dimensional chromatograms of the aqueous acid hydrolyzate of the phospholipids indicated the presence of at least 13 ninhydrin positive compounds. There was a correlation between the uptake of DL-alanine-1-C^{14}, DL-leucine-1-C^{14}, and DL-phenylalanine-3-C^{14} by phospholipids of the resting cells and utilization of these amino acids by the cell as indicated by manometric studies.

Many investigators have described the unique catabolic pathway of carbohydrate metabolism of various species of the genus *Pseudomonas* (1 to 4). It is also well known that the production of observable amounts of acid in a glucose medium varies and is dependent, to a large extent, on the nitrogenous composition of the medium (5 to 8). An investigation was instigated to study the utilization of protein hydrolyzates and amino acids by *P. aeruginosa* in an attempt to develop better procedures for the identification of this gram-negative bacillus in the diagnostic laboratory. During the course of the investigation it was noted that there was excellent correlation between the utilization of amino acids by *P. aeruginosa* and the uptake of the amino acids by the lipids, and in particular the phospholipids of the cell. The results of a study of the lipids of these cells are reported.

METHODS AND MATERIALS

The strain of *P. aeruginosa* used throughout the study was nonpigmented, cytochrome oxidase-positive (9), and produced observable amounts of acid in a glucose broth medium containing 1% peptone. Resting cells were obtained by growing the culture in a nutrient broth medium containing yeast extract (2 g/150 ml broth) at 37° for 18 hours on a rotary shaking machine which described a circle of one inch diameter at 250 rpm. The cells were harvested by centrifugation and washed several times with cold 0.1 M pH 7.0 phosphate buffer. The cell suspensions were standardized by optical density, viable cells counts, as well as wet and dry weight determinations. Oxygen consumption of the resting cells was measured by conventional Warburg techniques described by Umbreit *et al.* (10).

Uptake of C^{14}-labeled Amino Acids. Resting cells were suspended in pH 7.0 phosphate buffer (0.1 g wet weight/ml) containing 0.5 mg/ml of the amino acid to be studied. The cells were incubated for varying periods of time at 37° on the rotary shaker. Following incubation, the cell suspension was placed in an ice bath and centrifuged at 4°. The cells were washed three or four times with large volumes of cold buffer until the radioactivity of the wash buffer was negligible.

Lipid Extraction. The cells were resuspended in an Erlenmeyer flask containing 200 ml of a chloroform-methanol 2/1 (v/v) solvent mixture and shaken at room temperature for 18 hours. This technique, as originally described by Folch *et al.* (11), was intended for use with macerated or homogenized cells. Following extraction, the cellular residue was separated from the solvent mixture by filtration and the chloroform-methanol extract evaporated to dryness *in vacuo*. The lipids were taken up in petroleum ether and washed with distilled water. The phospholipids were precipitated by the addition of acetone. The acetone-in-

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soluble fraction was reprecipitated several times from petroleum ether and washed thoroughly with distilled water or saline, or both.

The phospholipids were hydrolyzed in aqueous 6 N HCl for 24 hours at 100°. The aqueous hydrolyzate, following repeated evaporation, was chromatographed on Whatman #1 filter paper for identification of the nitrogenous compounds. Duplicate chromatograms were run on all samples. One chromatogram was used for the detection of compounds while corresponding areas were cut from the duplicate but untreated chromatogram for the detection of radioactivity. The radioactivity, determined with the use of a windowless flow counter, was corrected for self-absorption of the paper.

All chromatograms were developed by an ascending technique (12). The solvent systems used to develop the chromatograms were: *n*-butanol-acetic acid-water (13), phenol-water (14), methanol-benzene-*n*-butanol-water (15), and chloroform-methanol plus 2% water or diisobutyl ketone-acetic acid-water (16) or both for silicic acid-impregnated paper.

RESULTS

The results listed in Table 1 show the average weights of the lipid fractions obtained from *P. aeruginosa* cells. It is of interest to note that the phospholipids comprise approximately 80% of the ether-soluble lipids. The lipid content of these cells could be increased by growing the culture in a nutrient medium containing specific phospholipids (phospholipids extracted from *P. aeruginosa* cells). It is obvious from the results shown in Table 2 that while the amounts of the lipid fractions could be increased, the per cent composition of the fractions remained constant.

TABLE 1. RESULTS OF WEIGHT STUDIES OF *P. Aeruginosa*

Fraction	Weights
Wet cells	5.55 g (4 to 7 × 10^{11} viable cells/gm)
Dry cells	1.0079 g (18% ± wet weight)
Ether-soluble lipids	0.0945 g (1.7% ± 0.1% wet weight)
Phospholipids (acetone-insoluble)	0.0707 g (80% ± 5% ether-soluble lipids)
Acetone-soluble lipids	0.0238 g (20% ± 5% ether-soluble lipids)

TABLE 2. COMPARATIVE WEIGHTS OF LIPID FRACTIONS EXTRACTED FROM *P. Aeruginosa* CELLS GROWN IN CONTROL MEDIUM AND PHOSPHOLIPID-ENRICHED MEDIUM

Lipid Fraction	*P. Aeruginosa* Cells Grown in Control Medium*		*P. Aeruginosa* Cells Grown in Phospholipid-Enriched Medium†	
	Weight	Ether-Soluble Fraction	Weight	Ether-Soluble Fraction
	mg	*per cent*	*mg*	*per cent*
Ether-soluble	66.7	100	102.1	100
Acetone-insoluble phospholipid	56.4	84.6	83.1	81.4
Acetone-soluble	8.0	12	9.5	3.9

* 4.19 g wet weight cells from 450 ml broth.
† 5.47 g wet weight cells from 600 ml broth containing 0.215 mg of phospholipid/ml.

It has previously been found in this laboratory (17) and elsewhere (18) that there is a variation in the utilization of different amino acids by resting cells of *P. aeruginosa*. The ability of 18 different amino acids studied to stimulate O_2 consumption of these cells ranged from 18.5 μl O_2 uptake/90 min/mg dry weight for DL-methionine to 74.1 μl for DL-serine. The results listed in Table 3 compare the stimulation of O_2 consumption by *P. aeruginosa* of three representative amino acids. To compare the uptake of these amino acids by the lipids of *P. aeruginosa* cells, 0.5 mg/ml of DL-leucine-1-C^{14}, DL-phenylalanine-3-C^{14}, and DL-alanine-1-C^{14} were added to pH 7.0 phosphate buffer containing a suspension of 0.1 g wet weight resting cells per ml. The cells were incubated on the rotary shaker at 37° for 90 minutes. Following incubation, the cells were washed thoroughly with cold buffer and the lipids

TABLE 3. EFFECT OF THREE REPRESENTATIVE AMINO ACIDS ON THE O_2 UPTAKE OF *P. Aeruginosa*

Amino Acid*	O_2 Uptake†	
	Endogenous	Plus Substrate
DL-Leucine	21.8	32.5
DL-Phenylalanine	20.0	40.7
DL-Alanine	21.2	70.3

* Amino acid concentration: 0.5 mg/ml incubation medium, incubated in Warburg at 37° in phosphate buffer pH 7.0.
† μl/10 minutes/mg dry weight.

extracted as described. The uptake of the C^{14}-labeled compounds by the lipid fractions is shown in Table 4. The figures shown represent μg of amino acids taken up based on their specific radioactivity. These results point out the correlation between the utilization of amino acids by *P. aeruginosa* and the uptake of amino acids by the phospholipid complex of the cell. The phospholipids of heat-killed cells did not take up radioactivity, nor did the phospholipids extracted from *P. aeruginosa* and shaken *in vitro* with C^{14}-labeled amino acids take up radioactivity.

Phospholipids extracted from resting cells which had been incubated with DL-alanine-1-C^{14} and DL-leucine-1-C^{14} were chromatographed by the technique described by Westley *et al.* (15). Figure 1 shows the results of chromatograms of the phospholipids extracted from *P. aeruginosa* cells following incubation with DL-leucine-1-C^{14}. Similar results were obtained with phospholipids from DL-alanine-1-C^{14} incubated resting cells. In every instance the radioactivity traveled with the phospholipids (rhodamine G positive). C^{14}-labeled amino acids added to the extracted phospholipids separated on chromatography. The aqueous acid hydrolyzates of the phospholipids chromatographed by this technique or with *n*-butanol-acetic acid-water indicated that the radioactivity was concentrated in the area corresponding to the R_f value of the respective amino acid with which the cells had been incubated. Figure 2 shows the results of radioactive phospholipids developed on silicic acid-impregnated paper (16). The chromatograms were run in triplicate to determine the R_f values of the ninhydrin, choline, and rhodamine G-positive compounds as well as the radioactive areas. It should be noted that the R_f values shown will vary slightly with different lots of silicic acid-impregnated paper. It was of interest to find that with either solvent system used (chloroform-methanol plus 2% water or diisobutyl ketone-acetic acid-water) all of the radioactivity remained with the rhodamine G-ninhydrin-positive spots on or near the origin (R_f <0.05).

Two-dimensional chromatography of the water-soluble acid hydrolyzate of phospholipids extracted from control resting *P. aeruginosa* cells revealed (Fig. 3) that there were at least 13 ninhydrin-positive compounds present. Most of these ninhydrin-positive spots have been identified by the use of known compounds.

TABLE 4. UPTAKE OF C^{14}-LABELED AMINO ACIDS BY THE PHOSPHOLIPIDS OF *P. Aeruginosa*

Fraction	DL-Amino Acid	Fraction Weight	Amino Acid Taken Up*	Amino Acid in 100 mg of Fraction
		mg	*μg*	*μg*
Petroleum ether-soluble lipids	Leucine-1-C^{14}	47.5	17.8	37.5
	Phenylalanine-3-C^{14}	47.9	23.4	48.9
	Alanine-1-C^{14}	72.0	32.1	44.6
Acetone insoluble lipids†	Leucine-1-C^{14}	35.2	5.32	15.1
	Phenylalanine-3-C^{14}	34.1	7.00	20.0
	Alanine-1-C^{14}	58.6	21.3	36.4

Cells incubated 90 minutes at 37° on shaker in phosphate buffer pH 7.0; amino acid concentration: 0.5 mg/ml; cell concentration: 0.1 g wet weight/ml; incubation medium: total volume 30.0 ml.

* μg amino acid taken up based on total amount of radioactivity found in the fraction: 1 μg DL-alanine-1-C^{14} = 5940 cpm; 1 μg DL-leucine-1-C^{14} = 1070 cpm; 1 μg DL-phenylalanine-3-C^{14} = 966.5 cpm.

† Phospholipids.

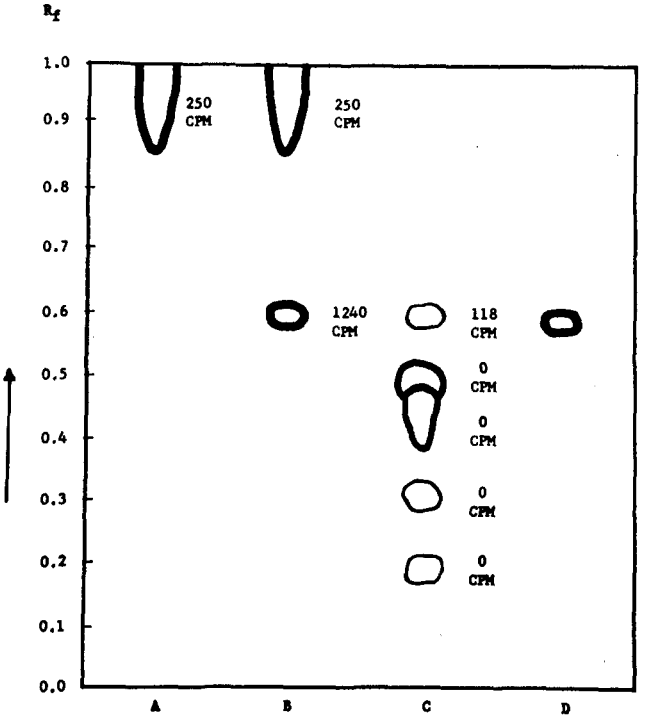

FIG. 1. Chromatographic demonstration of the binding of C^{14}-labeled amino acid to phospholipids extracted from resting cells of *P. aeruginosa*. Solvent system: Methanol-benzene-*n*-butanol-H_2O (2/1/1/1, v/v). (A) Phospholipids extracted from cells incubated with DL-leucine-1-C^{14}. (B) Phospholipids extracted from cells incubated with DL-leucine-1-C^{14} plus DL-leucine-1-C^{14} added to the partially purified phospholipids. (C) Aqueous acid hydrolyzate of phospholipids extracted from cells incubated with DL-leucine-1-C^{14}. (D) DL-leucine control. Phospholipid spots above R_f 0.8 both ninhydrin- and rhodamine G-positive, spots below R_f 0.8 ninhydrin-positive. CPM = counts per minute.

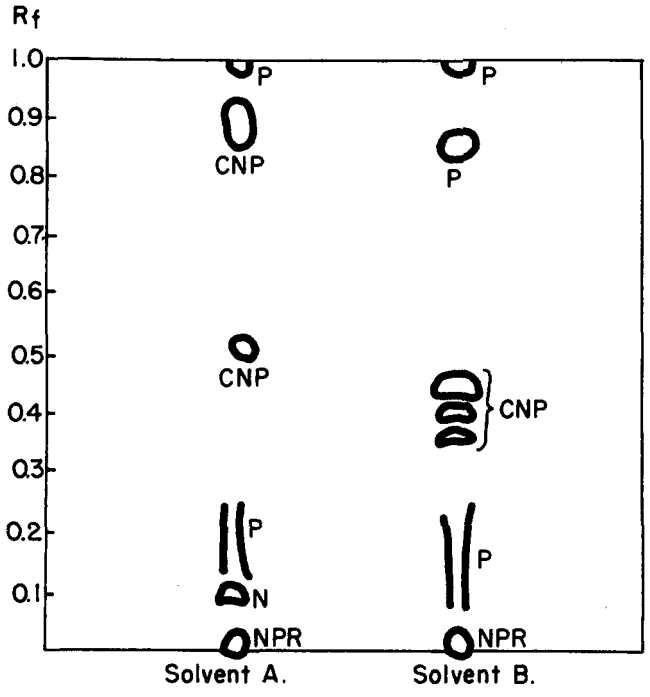

FIG. 2. Chromatographic separation on silicic acid-impregnated paper of phospholipids extracted from *P. aeruginosa* cells incubated with DL-leucine-1-C^{14}. Solvent A: Chloroform-methanol (3/1, v/v) + 2% H_2O. Solvent B: Diisobutyl ketone-acetic acid-H_2O (40/30/7, v/v). Identification of spots. P = rhodamine G-positive; N = ninhydrin-positive; C = choline-positive; R = radioactive area.

DISCUSSION

Current investigations involving lipids extracted from various tissues and microorganisms reveal that there is yet a great deal to be learned about the phospholipids and their role in metabolism. Haining *et al.* (19) working with mitochondria of rat tissues have shown that the degree of amino acid uptake by the lipid fraction of mitochondria varied with the type of compounds studied, but that they were taken up without degradation or alteration. Hendler (20) concluded from his studies with hen oviduct that the lipids appeared to be an intermediate carrier of amino acids entering the cell. He characterized the lipid amino acid "bond" as being highly labile in contrast to the stable bond linking an amino acid component of a nucleic acid fraction. Hokin and Hokin (21) have proposed, as a result of their studies, a mechanism of transport of zymogens across lipid membranes of the cells, with the phospholipids acting as some type of intermediate in this mechanism. Hunter and Goodsall (22) concluded from their studies with *Bacillus megaterium* that phospholipid-amino acid complexes may be involved in the transfer of amino acids from the site of amino acid activation to the site of protein synthesis. They found that following incorporation studies and extraction of phospholipids from cells of *B. megaterium*, the radioactivity incorporated did not separate from the phospholipids when chromatographed on silicic acid-impregnated paper.

The data presented in the present study support the existence of a phospholipid-amino acid complex as a part of the lipid component of *P. aeruginosa*. Repeated experiments confirmed the observations that resting cells of *P. aeruginosa* varied in their ability to utilize different amino acids, and this variation was reflected in the uptake of C^{14}-labeled amino acids by the phospholipid complex of the cell. Furthermore, it would appear that amino acids are taken up only by phospholipid complex of metabolically active cells. The phospholipids of heat-killed cells or partially purified phospholipids extracted from the cells do not take up C^{14}-labeled amino acids.

Two-dimensional chromatograms run on the aqueous acid hydrolyzates of phospholipid complexes extracted from *P. aeruginosa* cells indicated the presence of at least 12 ninhydrin-positive compounds in addition to choline. Chromatograms of aqueous acid hydrolyzates of the phospholipid complexes extracted from resting

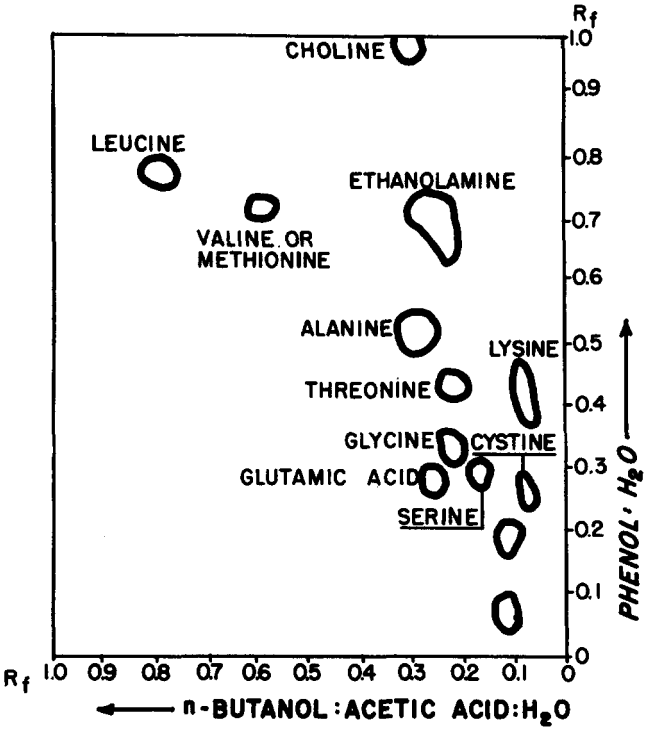

FIG. 3. Two-dimensional chromatogram of the aqueous acid hydrolyzate of phospholipids extracted from normal resting cells of *P. aeruginosa*. All spots with the exception of choline were ninhydrin-positive.

cells previously incubated with an amino acid showed an increase in the intensity of the ninhydrin-positive spot which corresponded to the R_f value of the amino acid with which the cells were incubated.

The identification of the phospholipids in this study was based on their solubility properties in various lipid solvents. The authors are aware of the possibility that these microorganisms might have a large content of novel lipids not related to the phospholipids.

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