ENDOGENOUS RESPIRATION OF *PSEUDOMONAS AERUGINOSA*[1]

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Classification of bacteria on the basis of their mechanism of degradation of hexoses is currently used for the separation of a number of families into genera and, at least in the case of the genera *Streptococcus* and *Leuconostoc*, the separation correlates with other stable characteristics and appears to be based on an important genetic difference. These bacteria require a carbohydrate for growth and, therefore, their hexose metabolism is of great importance and perhaps of evolutionary significance. However, many bacteria, particularly soil and water forms, do not rely on carbohydrates as a source of energy and so their pathway of hexose metabolism may have had no determining influence on their long term development. For such organisms the pathway by which they degrade their reserve storage products may be an important stable difference. In spite of the importance of knowing the identity of the reserve materials in bacteria and the pathways used in their degradation, little effort has been expended towards obtaining this information. Recently, progress toward this end has been made by Dawes and Holmes (1958) in their studies on *Sarcina lutea*. Most work on this problem has been concentrated on bacterial glycogen and useful information on the factors affecting the accumulation and utilization of this storage product has been reported (Palmstierna, 1956; Dagley and Johnson, 1953).

The nature of the reserve material has practical importance from another point of view; that is, in assessing the quantitative aspects of exogenous substrate degradation. Compounds, which are intermediates in the degradation of the reserve material or are readily converted to such intermediates, may interfere with the utilization of the endogenous reserves. Others may stimulate endogenous respiration, perhaps by supplying a rate limiting reactant. Still others, as appeared to be the case with glucose oxidation by *Pseudomonas aeruginosa*, are not on the pathway of endogenous respiration and the addition of exogenous glucose does not interfere with the rate of endogenous respiration (Norris *et al.*, 1949). It follows, therefore, that the status of the endogenous respiration may differ with each exogenous substrate and so no one calculation can be used for correcting the values obtained when resting cells act on a variety of substrates in an individual Warburg run. This problem was recognized by Blumenthal *et al.* (1957) and discussed by them at some length. The present work was undertaken in an effort to determine the cellular constituents which serve as reserve materials and which are utilized when resting cells of *P. aeruginosa* respire endogenously. Further, it was planned to establish the influence of an oxidizable substrate on these changes.

METHODS

P. aeruginosa (ATCC 9027) was used throughout this work. It was grown in a medium composed of $NH_4H_2PO_4$, 0.3 per cent; K_2HPO_4, 0.2 per cent; glucose, 0.2 per cent; iron, 0.5 ppm. $MgSO_4 \cdot 7H_2O$ was added after sterilization to a concentration of 0.1 per cent. Cells were cultured in 100-ml quantities in Roux flasks.

The following methods were used for the determinations indicated; total carbohydrate, Trevelyan and Harrison (1952); uronic acids, Dische (1950), and by the *p*-anisidine method as described by Block *et al.* (1958); amino acids, Yemm and Cocking (1955); ammonia, by the Conway microdiffusion technique (Conway, 1950); keto acids, Friedemann (1957); proteins, Lowry *et al.* (1951); total nitrogen, titrimetrically after micro-Kjeldahl digestion; ribonucleic acid (RNA), Stuy (1958); deoxyribonucleic acid (DNA), Burton (1956), using the extraction procedure of Stuy; and fat by the method of Salton (1953).

Resting cell suspensions were prepared, from cultures which had been incubated at 30 C for 20 hr, by centrifuging at 900 × G for 10 min, washing twice, and resuspending in 0.033 M phosphate buffer, pH 7.0.

Changes in the chemical composition of cells

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were determined by allowing a 3.7 times growth concentration suspension of washed cells to respire endogenously during a 2-hr period of shaking on a Warburg respirometer at 30 C. Twenty-four ml of the cell suspension were placed in each of two Warburg vessels and after being shaken for 1 hr, a 12-ml sample was removed from each flask and the two samples combined. The procedure was repeated after 2 hr shaking. An additional 24-ml sample was retained for zero time determinations.

Samples (zero time and 1 hr) were stored in ice until the completion of the 2-hr respiration period so that all determinations on the three samples could be performed at the same time. It was assumed (and partially confirmed by ammonia determinations) that storage of the suspensions in ice for this short period of time did not allow any significant changes to occur within the cells.

Samples of suspensions at zero time, 1, and 2 hr, were treated in the following manner before quantitative determinations were performed. All centrifugation procedures were carried out at 900 × G for 10 min. When required, protein was precipitated by the addition of 10 per cent trichloroacetic acid, incubation for 30 min at 30 C, and centrifugation.

Total carbohydrate estimations were made on cell suspensions and extracellular carbohydrate estimations on untreated supernatants of centrifuged suspensions.

Total quantitative determinations of uronic acids were carried out with cell suspensions. For qualitative paper chromatography, washed cells were suspended in distilled water to give a 12 × growth concentration and treated in a 10-kc Raytheon sonic oscillator for 12 min. A sample of the extract was hydrolyzed with 2.5 N H_2SO_4 in a sealed ampule for 17 hr at 80 C. Chromatograms were run on hydrolyzed and nonhydrolyzed samples. For keto acid determinations, samples of the suspension were treated with an equal volume of trichloroacetic acid, centrifuged, and the supernatants examined. For protein determinations, 1 ml of each suspension was boiled for 20 min to release intracellular amino acids. After cooling, the samples were treated with 0.5 ml trichloroacetic acid. The precipitates obtained after centrifugation were dissolved in 1 ml of 0.1 N NaOH by heating and a 1:10 dilution was made with distilled water. To determine amino acids quantitatively, 3 ml of each suspension were centrifuged and 1 ml of trichloroacetic acid was added to 2.5 ml of supernatant. After further centrifugation, the supernatants were used for extracellular amino acid determinations. Three ml of distilled water were added to each of the precipitates; the resulting suspensions were boiled for 20 min, treated with 1.5 ml of trichloroacetic acid, centrifuged, and the supernatants used for the determination of intracellular amino acids. Corrections were made for the presence of intra- and extracellular ammonia. For the determination of ammonia, 1.5-ml samples of the suspensions were centrifuged and the supernatants used for extracellular ammonia estimations. The precipitates were suspended in 1.5 ml of distilled water and the resulting suspensions were boiled for 20 min and centrifuged. The supernatant solutions were used for intracellular ammonia determinations. Dry weight determinations were carried out in duplicate on 5-ml amounts of each suspension by drying to constant weight at 100 C. Corrections were made for the presence of phosphate buffer.

For the determination of RNA and DNA, a 4-ml sample of each suspension was centrifuged and the pellet gently suspended in 2 ml cold 8 per cent trichloroacetic acid. After 30 min the sediment was centrifuged and the supernatant removed. Each pellet was resuspended in 1.5 ml cold 8 per cent trichloroacetic acid. The tubes were placed in a water bath at 90 C for 20 min, cooled, and centrifuged. The supernatant was removed, diluted to 4 ml, and analyzed for RNA and DNA.

The chromatography procedures employed for amino acids were those of Mandelstam (1958). Solutions of intracellular and extracellular amino acids were obtained by the method already described but with the trichloroacetic acid treatment omitted. The zero time sample was 30 ml of a 5 times growth concentration cell suspension and the second sample was obtained by shaking a 30-ml sample of the suspension on the respirometer for 2 hr. The solutions obtained were placed on columns of Dowex 50 H^+ and washed with 50 ml of distilled water. The amino acids were then eluted with 1.5 N NH_4OH, and the eluates concentrated to 1 to 2 ml in a flash evaporator. The concentrated solutions were evaporated to dryness *in vacuo* over concentrated H_2SO_4. The residues were taken up in

TABLE 1

Viable count of Warburg vessel contents when respiring endogenously and when respiring in the presence of various substrates

Time	Endogenous	Glucose	Succinate	Fructose	Malate
hr					
0	17.3*	16.9	18.7	16.1	17.3
1	16.8	15.0	15.2	14.7	14.3
2	17.3	16.1	17.6	15.3	19.4

* Viable count per ml ($\times 10^9$).

0.3-ml quantities of distilled water, and 50-μl amounts spotted on Whatman no. 3 filter paper. The chromatograms were run for 22 hr with the solvent system: butanol-acetic acid-water (63:10:27). The sheets were then dried, sprayed with 0.5 per cent ninhydrin in acetone-acetic acid (90:10), allowed to dry at room temperature, and developed at 65 C for 10 min.

RESULTS

In an effort to ensure that neither growth nor cell autolysis had occurred to any appreciable extent during the course of the experiments, viable cell counts were determined over a 2-hr period during which 1-ml quantities of a 10 times growth concentration cell suspension were shaken in Warburg flasks at 30 C in an atmosphere of air. Parallel experiments were carried out with various substrates added to the vessels.

No significant changes occurred in viable cell count during the 2-hr period, with or without exogenous substrate (table 1). It is assumed, therefore, that the changes in the chemical composition of the cells and suspending fluid which were found to occur during respiration, were not attributable to death and, hence, changed permeability properties of the cells, or to lysis of the cells.

When chemical analyses were carried out on endogenously respiring resting cell suspensions and on the suspending fluids, a number of changes were apparent. The pronounced release of ammonia indicated that a substrate of endogenous respiration was nitrogenous. The source of the ammonia is not certain but it is reasonable to suggest that it could be amino acids. Analysis of the suspensions for keto acids did not show the production of detectable amounts during the 2-hr period of respiration. This would indicate that keto acids resulting from the deamination of any amino acids were oxidized to completion.

The changes in RNA and DNA were within the range of experimental error and it would appear that there were no significant changes in these constituents. The uronic acid content as determined by the carbazole test of Dische (1950) decreased. However, no free or combined uronic acid could be detected by the *p*-anisidine test and it is assumed that the carbazole test is not sufficiently specific to give useful information on the chemical composition of the organism. There was no detectable change in the fat content or total protein during the 2-hr period (table 2).

Calculations were made, based on the experiment of table 2, to determine whether or not the changes in cellular constituents would account for the observed oxygen utilization. Since there were essentially no changes in the carbohydrate, fat, RNA, or DNA content of the cells, and there was a marked production of ammonia, it was assumed that protein or amino acids were the major fuel of endogenous respiration. Glutamic acid was taken as representative of the amino acids as 1 mole of glutamate requires 4½ moles of oxygen for complete oxidation, a reasonable average for a mixture of amino acids. The data apparently account for all of the oxygen consuming changes which occurred in the cell. The experiments were repeated on six occasions with similar results.

A similar experiment was performed with glucose added to the Warburg vessels at zero time. Since endogenous respiration was the subject of investigation, only changes in the ammonia content of the cells and the suspending fluid were followed (table 3). It would appear that, in the presence of glucose, the ammonia was rapidly incorporated into cellular material. This was confirmed by the second half of the experiment in which the cells were allowed to respire in the absence of added substrate for 1 hr before glucose was added. It can be seen that all of the external ammonia was reincorporated within 1 hr. In the presence of glucose, no increase in intracellular carbohydrate could be detected indicating that the carbohydrate which was assimilated was not incorporated as polysaccharide reserve.

The free amino acids of the internal pool and of the suspending fluid were separated by paper chromatography. The number and nature of the amino acids released to the external medium

TABLE 2

Changes in cell constituents during endogenous respiration

Time	Protein	Carbohydrate		Amino Acids*		NH_3	
		Total	Extracellular	Intracellular	Extracellular	Intracellular	Extracellular
hr	*mg/100 mg dry weight of cells*						
0	56.0	3.59	0.334	0.204	0.040	0.016	0.070
1	56.9	3.59	0.669	0.077	0.214	0.045	0.296
2	56.9	3.59	0.527	0.106	0.211	0.057	0.465

Observed O_2 uptake = 2448 μl; calculated O_2 uptake on basis of NH_3 produced = 2590 μl.

* Expressed as amino nitrogen.

TABLE 3

Influence of glucose on production of ammonia from endogenous reserves

	Time	Ammonia	
		Intracellular	Extracellular
	hr	*mg/100 mg dry weight of cells*	
Resting cells + glucose	0	0	0.013
	1	0	0
	2	0	0
Resting cells with glucose added at 1 hr	0	0.004	0.044
	1	0.022	0.125
	2	0.015	0

corresponded to those present in the internal pool. A total of 9 to 10 distinct compounds were apparent and no particular amino acid appeared to be preferentially utilized during the 2-hr period of the experiments. Some of the amino acids identified were glycine, glutamic acid, alanine, and cysteine. Serine and leucine also appeared to be present. None of the isolated amino compounds either internal or external could be further hydrolyzed with 6 N HCl and so apparently peptides were not present in detectable quantities.

DISCUSSION

The data recorded here indicate that when cells of *P. aeruginosa* were grown on a glucose-ammonium phosphate medium, the major, if not the sole storage product which they accumulated and which was oxidized during endogenous respiration, was nitrogenous and probably protein in nature. No changes in protein content were detected, probably because the percentage of the cellular protein used during 2-hr of respiration was negligible. The data confirm the conclusion of Norris *et al.* (1949) that the pathway of endogenous metabolism does not compete for enzymes with the pathway for glucose metabolism in *P. aeruginosa*. It is apparent, however, that the presence of an oxidizable substrate, such as glucose, changes the general economy of the cell by allowing it to reincorporate an end product of endogenous respiration, namely ammonia. This reincorporation of ammonia is at the expense of oxidizable products of glucose degradation and undoubtedly accounts for much of the substrate which is shunted into oxidative assimilation.

Work on other organisms has also implicated nitrogenous, or at least noncarbohydrate material, as an endogenous substrate. Stickland (1956) found that aeration of baker's yeast reduced the endogenous oxygen uptake but did not reduce the polysaccharide reserves. Dawes and Holmes (1958) observed the production of ammonia during the endogenous respiration of *S. lutea*, and also found that the level of oxygen uptake fell as the free intracellular amino acid pool was depleted.

The release of amino acids to the external medium during endogenous respiration was in agreement with the results of Mandelstam (1958) who found that free amino acids accumulated in the external medium when cells of *Escherichia coli* were shaken in phosphate buffer. Chloramphenicol interfered with the release of these amino acids in Mandelstam's experiments, which would indicate the protein breakdown preceded the excretion of amino acids.

SUMMARY

Resting cells of *Pseudomonas aeruginosa* were allowed to respire endogenously in air for 2 hr, during which chemical changes in the cells and in the suspending fluid were followed. It was found that other than carbon dioxide, ammonia was the only product of endogenous respiration which could be detected. No keto acids accumulated and it was concluded that the ammonia arose as the result of complete oxidation of amino acids. Calculations based on this assumption showed that ammonia production could account for all of the oxygen consumed. In the presence

of glucose, the ammonia was reincorporated into cellular material thus explaining, at least to some degree, the phenomenon of oxidative assimilation in this organism.

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