NITROGENOUS SUBSTRATES OF ENDOGENOUS RESPIRATION IN *PSEUDOMONAS AERUGINOSA*

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ABSTRACT

GRONLUND, AUDREY F. (University of British Columbia, Vancouver, Canada) AND J. J. R. CAMPBELL. Nitrogenous substrates of endogenous respiration in *Pseudomonas aeruginosa*. J. Bacteriol. **86**:58-66. 1963.—The nature of the nitrogenous reserves of *Pseudomonas aeruginosa* that are oxidized during endogenous respiration was studied by following the changes in the chemical constituents and in the distribution of radioactivity of starving cells that had been grown on C^{14}-labeled substrates. The total protein and nucleic acid of Warburg vessel contents decreased during starvation. Deoxyribonucleic acid increased slightly, whereas ribonucleic acid (RNA) decreased. $C^{14}O_2$ was evolved from endogenously respiring cells specifically labeled in the nucleic acid fraction and from cells specifically labeled in the protein fraction. Chemical fractionation of C^{14}-labeled cells showed a decrease in hot trichloroacetic acid-soluble and -insoluble compounds, indicating that the $C^{14}O_2$ arose from the degradation of RNA and protein and not free pool compounds. A decrease in ribosomal RNA and protein was evident from physical fractionations of starved, labeled cells. An enzyme responsible for the initiation of ribosomal degradation was found to be associated with the ribosome fraction. It was concluded that oxidation of the ribonucleoprotein during endogenous respiration may be a general phenomenon in microorganisms.

Reports in the literature suggest that microorganisms contain a non-nitrogenous carbonaceous reserve material which may be utilized during the course of endogenous respiration. Strange, Dark, and Ness (1961) suggested that glycogen served such a role for *Aerobacter aerogenes*, and Doudoroff and Stanier (1959) reported that poly-β-hydroxybutyrate acted in this capacity for *Pseudomonas saccharophila*. Thus, many microorganisms would appear to be similar to higher forms with regard to the storage of specific carbonaceous energy reserves. However, when *P. aeruginosa* was grown in a glucose-ammonium salts medium, no accumulation of poly-β-hydroxybutyrate could be detected (Hogg, *unpublished data*) and no depletion in total carbohydrate was observed after a 2-hr period of starvation (Warren, Ells, and Campbell, 1960).

Ammonia was found to be an end product of the endogenous respiration of *P. aeruginosa* (Warren et al., 1960) and of other microorganisms (Dawes and Holms, 1958; Gronlund and Campbell, 1961); amino acids were implicated as the source of this ammonia. It has been the intent of this work to elucidate the nature of the nitrogenous compound or compounds oxidized during starvation, by following the changes in distribution of the radioactivity in cellular constituents through use of *P. aeruginosa* grown in the presence of C^{14}-labeled substrates.

MATERIALS AND METHODS

Resting-cell suspensions. P. aeruginosa (ATCC 9027) was grown in a glucose-ammonium phosphate-salts medium (Warren et al., 1960) and harvested after 20 hr of incubation at 30 C. The cells were washed twice with 0.85% NaCl (pH 7.4) and resuspended at 10 or 15 times the growth concentration in 0.05 M tris(hydroxymethyl)-aminomethane (tris) buffer (pH 7.4).

Cells were labeled with C^{14} by adding either glucose-U-C^{14} (90 μc/100 ml), uracil-2-C^{14} (71 μc/100 ml, 4.86 or 9.1 mg/100 ml), or proline-U-C^{14} (71 μc/100 ml, with carrier L-proline to give a concentration of 5 mg/100 ml) to growing cultures at 12 hr and continuing incubation until 20 hr.

Glucose-U-C^{14} was purchased from Merck & Co., Ltd., uracil-2-C^{14} from Schwarz BioResearch Inc., and proline-U-C^{14} from both companies.

Cell viability. Samples of endogenously respiring cell suspensions were removed from a large Warburg vessel at specified time intervals

and appropriately diluted with either 0.033 M phosphate buffer (pH 7.4) or 0.033 M phosphate buffer (pH 7.4) plus 0.8% NaCl and 0.1% gelatin. The number of viable cells per ml was determined by plate counts.

Analytical methods. Ammonia was determined by the Conway (1950) microdiffusion technique, protein by the method of Lowry et al. (1951), and ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) by a modification of the techniques of Schneider (1957). RNA assays were corrected for interference by DNA. Oxygen consumption was followed manometrically at 30 C with a conventional Warburg respirometer. Ultraviolet (UV) spectra were determined with a Beckman model DU spectrophotometer. Electrophoresis was carried out using Whatman no. 1 paper, formic-acetic buffer (pH 2; Smith, 1960), and a flat plate water-cooled electrophoresis apparatus with an RSCo model 1911 power supply.

Chemical fractionations of whole cells. The fractionation procedure described by Duncan and Campbell (1962) was followed, with the exception that the hot trichloroacetic acid extraction was carried out at 90 C for 9 min. When required, RNA and DNA were separated by a modification of the Schmidt-Thannhauser technique (Hutchison, Downie, and Munro, 1962).

Preparation and physical fractionation of cell-free extracts. At zero time and after a 3-hr starvation period on the Warburg respirometer, the supernatant fluid was separated from the cells by centrifugation and the cell pellet resuspended in a minimal volume of distilled water. The cells were lysed in tris buffer (pH 8.0) by a lysozyme-ethylenediaminetetraacetic acid (EDTA) treatment, and the viscosity of the resulting lysate was decreased by the addition of deoxyribonuclease and magnesium ions. Whole cells that survived this treatment were removed by centrifugation at 900 × *g* for 10 min. The resultant cell-free extract was centrifuged for 30 min at 25,000 × *g* in a refrigerated centrifuge. The pellet, consisting of "membranes" (Campbell, Hogg and Strasdine, 1962), was resuspended in distilled water. The supernatant fluid (25,000 × *g*) was centrifuged at 110,000 × *g* for 2 hr in a Spinco model L preparative ultracentrifuge. The resulting pellet ("ribosomes") was suspended in distilled water by homogenization with a Teflon Potter homogenizer or dissolved in 0.5 N NaOH.

Radioactivity measurements. Duplicate samples of whole cells and the chemical and physical fractions were plated at infinite thinness on stainless steel planchets and dried with a heat lamp. Radioactive carbon dioxide was released from the reaction mixture in a Warburg vessel by the addition of HCl and was collected in the center well, which contained 20% KOH and a folded strip of filter paper. The KOH and filter papers were removed, the center wells washed, and the radioactive samples diluted to an appropriate volume with distilled water and plated directly. Radioactivity was measured with a model 181 Nuclear-Chicago scaler with an automatic gas-flow counter having a thin end-window Geiger tube. Corrections were made for background and for coincidence as required.

Radioactive areas on electrophoretograms were located by passing strips through a Nuclear-Chicago model C 100 B Actigraph II with a gas-flow counter, a model 1620 B Analytical Count Ratemeter, and Chart Recorder.

Measurement of ribosome degradation. Cell-free extracts of 20-hr cells were prepared by means of a Hughes press (Hughes, 1951), and the viscosity of the cell-free extract was decreased by a 60-sec period of treatment in a 10-kc Raytheon sonic oscillator. The "ribosome" fraction was obtained by the previously described differential centrifugation procedure and was suspended in 0.05 M tris buffer (pH 7.4) by means of a Potter homogenizer. Assays were carried out in 1-ml volumes, by use of 0.05 M tris buffer (pH 7.4) and ribosomes. Reactions were stopped at zero time and after a 60-min incubation at 30 C by the addition of 1 ml of 1.4 N perchloric acid (PCA). After centrifugation, the increase in ultraviolet-absorbing material in the supernatant fluid was measured with a Beckman model DU spectrophotometer.

Results and Discussion

Cell viability during starvation. The total cell count of a dense population of endogenously respiring *P. aeruginosa* was followed for 3 hr to determine whether a significant amount of cell lysis occurred during this period. Cell lysis would introduce the possibility that the survival of remaining cells was due to their utilization of material released during lysis. In an effort to determine the diluent which exhibited the least deleterious effects on the starved cells during plating procedures, samples of a cell suspension

[approximately 5 mg (dry weight)/ml], at ten times the growth concentration, were removed at hourly intervals from a Warburg vessel and suspended in different buffers. Cells suspended in gelatin-NaCl-phosphate buffer exhibited an overall decrease in viability of 36% in 3 hr, whereas a portion of the same starved-cell suspension diluted with 0.033 M phosphate buffer showed no decrease in viability (Table 1). It was concluded, therefore, that no significant number of cells were lysed in the Warburg vessels during the 3-hr test period used in this and subsequent experiments. This is consistent with the results of Warren et al. (1960), who found no decrease in viable cells of less dense populations of *P. aeruginosa* after a 2-hr interval of starvation. The effects of various physical and chemical conditions on the survival of starved bacteria have been amply stressed by Postgate and Hunter (1962).

Ultraviolet-absorbing material in Warburg supernatant fluids. The appearance of increasing amounts of UV-absorbing material in the suspending fluid of endogenously respiring cells was noted (Fig. 1). During the first hour of starvation, the UV-absorbing material increased approximately 265% over the zero-time value, an additional 56% during the second hour, and 40% during the third hour. This material showed maximal absorption at 260 mμ, minimal absorption at 240 mμ, reacted positively with the orcinol reagent and negatively with diphenylamine, and, therefore, was concluded to consist primarily of RNA or RNA degradation products, or both. After electrophoresis, actigraphs of Warburg supernatant fluids from glucose-U-C^{14}-labeled cells, followed by elution of radioactive areas and UV spectroscopy, confirmed the presence of high molecular weight 260-mμ absorbing material that did not migrate in an electric field. In addition, nucleotides, nucleosides, and possibly free bases were detected. In a like manner, the presence of apparently lesser quantities of "protein" and free amino acids was established.

TABLE 1. *Effect of endogenous respiration on cell viability*

Time of starvation	Diluent*	
	Gelatin-NaCl-phosphate buffer	Phosphate buffer
hr		
0	28.3	27.5
1	21.7	25.8
2	20.5	30.7
3	18.2	28.5

* Results expressed as viable cells/ml × 10^{-9}.

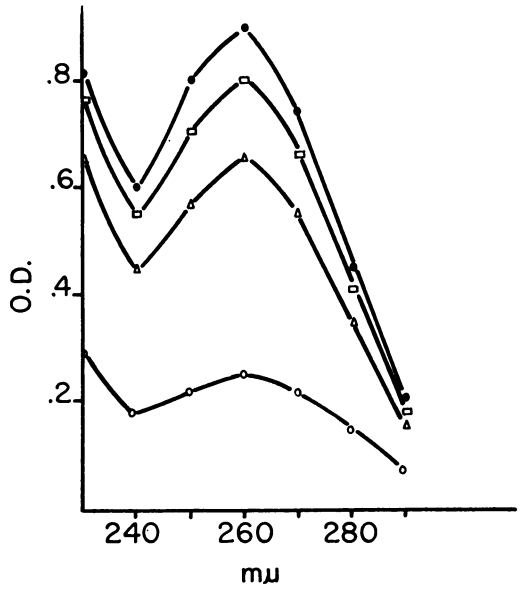

FIG. 1. *Increase in ultraviolet-absorbing material in the supernatant fluids of Warburg reaction mixtures of endogenously respiring cells. Samples were diluted 3:7 with distilled water before measurement. Zero time control, ○; starvation time: 1 hr, △; 2 hr, □; 3 hr, ●.*

The release of both nucleic acids and amino acids by a variety of microorganisms respiring in the absence of an exogenous substrate has been reported by many workers (Strange et al., 1961; Borek, Ryan, and Rockenbach, 1955). It is thought, however, that the release of these compounds from at least *Bacillus cereus* (Urba, 1959), *Escherichia coli* (Horiuchi, 1959), and *Saccharomyces cerevisiae* (Higuchi and Uemura, 1959) is not the result of cell lysis. Although amino acids were released by nongrowing cells of *B. cereus* throughout a 2-hr period, Urba (1959) found less than 1% of the total catalase activity present in the suspending medium. This supported the contention that protein breakdown was not dependent on cell lysis but was largely intracellular. In view of the viable cell counts, it must be concluded that this is also the case with *P. aeruginosa*, and the release of UV-absorbing material, then, is related to endogenous respiration.

Change in protein and nucleic acid content. The determination of protein and nucleic acids on Warburg cup contents after a 3-hr period of starvation showed a decrease in total protein and RNA, a slight increase in DNA, but a net decrease in total nucleic acids (Table 2). An increase in DNA at the apparent expense of RNA has been reported in phosphate-deficient *E. coli* (Horiuchi, 1959).

These results suggested that protein and RNA may serve as endogenous substrates. A decrease in both of these constituents during starvation has been observed with *A. aerogenes* (Strange, 1961), a decrease in amino acids and protein with *Sarcina lutea* (Dawes and Holms, 1958) and a slime mold (Wright and Anderson, 1960), and a decrease in RNA with *E. coli* (Dawes and Ribbons, 1962).

Effect of magnesium and phosphate ions on endogenous respiration. Bowen, Dagley, and Sykes (1959) reported that a ribonucleoprotein component of *E. coli* was influenced considerably by environmental conditions. When cells were incubated in phosphate buffer, the ribonucleoprotein component was depleted but was reformed on the addition of magnesium ions. The addition of either 3.33×10^{-2} M phosphate or magnesium to endogenously respiring *P. aeruginosa* caused a decrease in the total amount of UV-absorbing material appearing in the supernatant fluids. In both instances there was a continual increase in this material with time, the greatest increase occurring during the first hour of incubation.

The influence of these ions on oxygen consumption and on ammonia production was followed at hourly intervals (Table 3). The presence of inorganic phosphate stimulated oxygen uptake but slightly depressed ammonia production. Primarily, this was probably due to a slight shift in the ratio of oxidation of RNA to oxidation of protein. The depression of endogenous activity in the presence of magnesium ions is in accord with ribonucleoprotein serving as an endogenous substrate, as magnesium has a major stabilizing influence on ribonucleoprotein. Wade (1961) demonstrated the inhibitory effect of magnesium ions on the endogenous degradation of the RNA of *E. coli*.

TABLE 2. *Change in total protein and nucleic acids of Warburg cup contents during 3 hr of endogenous respiration*

Time	Protein	RNA	DNA
	μg/ml	*μg/ml*	*μg/ml*
Zero time.....	3,126	1,112	384
Endogenous....	2,882	935	417
Net change.....	−244	−177	+33

TABLE 3. *Effect of magnesium and phosphate ions on endogenous respiration*

Respiration	Time	Control	Plus Mg^{++}	Plus PO_4^{-}
	hr			
Oxygen uptake	0–1	4.75	4.15	5.17
(μmoles/ml)	1–2	4.0	3.72	3.86
	2–3	3.19	3.23	3.40
Total in 3 hr		11.94	11.10	12.43
Ammonia pro-	0–1	0.93	1.03	0.98
duction (μM/	1–2	0.80	0.82	0.66
ml)	2–3	1.19	0.71	0.93
Total in 3 hr		2.92	2.55	2.57
O_2/NH_3	At 3 hr	4.10	4.35	4.84

Specificity of incorporation of radioactive proline and uracil. To confirm the oxidation of cellular protein or RNA, cells were labeled with uracil-2-C^{14}, proline-U-C^{14}, or glucose-U-C^{14} with the intent of measuring radioactivity in the carbon dioxide evolved during endogenous respiration. In view of the ability of *P. aeruginosa* to utilize a large number of individual nitrogenous compounds as the sole source of carbon and nitrogen, the specificity of the labeling of nucleic acid with uracil-2-C^{14} was determined by a modification of the Schmidt-Thannhauser technique, and the specificity of the labeling of protein when proline-U-C^{14} was substrate was determined by the procedure of Duncan and Campbell (1962).

Cells labeled with uracil-2-C^{14} retained only 2.7% of the radioactivity in the "protein" residue (Table 4). This is well within the experimental limitations of the fractionation procedure, as has been demonstrated by Hutchison et al. (1962); in the interpretation of subsequent results of fractionation procedures, radioactive uracil has been considered to be confined solely to the nucleic acid fraction. The appearance of approximately 30% of the total radioactivity in the DNA fraction illustrates the probable ability of this organism to interconvert pyrimidine nucleotides (Potter, 1960).

Proline-U-C^{14}-labeled cells retained only 3.4%

TABLE 4. *Distribution of radioactivity in cells grown on uracil-2-C^{14}*

Fraction	Counts per min per ml $\times 10^{-3}$	Per cent of total C^{14}
Cold trichloroacetic acid-soluble extract of NaOH hydrolysate (RNA)	130.0	69.7
Hot trichloroacetic acid-soluble extract (DNA)	51.75	27.7
Hot trichloroacetic acid residue (protein)	5.11	2.7

TABLE 5. *Distribution of radioactivity in cells grown on proline-U-C^{14}*

Fraction	Counts per min per ml $\times 10^{-3}$	Per cent of total C^{14}
Cold trichloroacetic acid-soluble	24.7	2.5
Acid-ethanol-soluble	104.0	10.5
Hot trichloroacetic acid-soluble	33.5	3.4
Hot trichloroacetic acid residue	825.4	83.5

of the total radioactivity in the nucleic acid fraction, and again this has been considered as contaminating radioactivity (Table 5). It would appear, therefore, that the proline-U-C^{14} was incorporated exclusively into protein.

Evolution of radioactive carbon dioxide. The relative amount of radioactivity evolved as $C^{14}O_2$ by endogenously respiring cells labeled with glucose-U-C^{14}, uracil-2-C^{14}, or proline-U-C^{14} was determined and expressed as per cent of total radioactivity in the Warburg flask (Table 6). After zero-time values were subtracted from the results obtained with glucose-U-C^{14}-labeled cells, 3.31% of the total radioactive carbon was recovered as $C^{14}O_2$. An even greater percentage of radioactive CO_2 was evolved from cells labeled with C^{14}-proline or -uracil. Because of the specificity of labeling with these two compounds, this clearly illustrates that both nucleic acid and protein were oxidized and, therefore, must serve as endogenous substrates in *P. aeruginosa*.

From Table 2 it can be seen that RNA decreased during endogenous respiration whereas DNA increased; further, the UV-absorbing material in Warburg supernatant fluids was identified as RNA. Therefore, it may be concluded that the radioactivity in $C^{14}O_2$ from uracil-2-C^{14}-labeled cells arose solely from RNA. As 70% of the total label appeared in the RNA fraction (Table 4), the apparent amount of RNA oxidized would be 8.05% rather than 5.64%. This value may be lower than the actual percentage of RNA oxidized, for the rate of oxidation of pyrimidines is somewhat slower than that of purine and purine degradation products (Fig. 2). An analogous situation may exist with proline-U-C^{14}-labeled cells.

The complete oxidation of all the ribosomal RNA of uniformly labeled cells would result in the evolution of 13.5% of their radioactivity. Therefore, under the experimental conditions where

TABLE 6. *Radioactivity evolved as $C^{14}O_2$*

Cells labeled with	Fraction	Zero-time control*	Endogenous after 3 hr of starvation*	Increase in $C^{14}O_2$ in 3 hr
				%†
Glucose-U-C^{14}	CO_2	23.72	115.3	3.31
	Cells‡	2,878.0	2,625.0	
Proline-U-C^{14}	CO_2	19.45	88.7	4.05
	Cells	1,529.0	1,586.0	
Uracil-2-C^{14}	CO_2	3.85	15.24	5.64
	Cells	204.0	188.5	

* Expressed as counts per min per ml $\times 10^{-3}$.
† Of total C^{14}.
‡ Cells plus supernatant fluid.

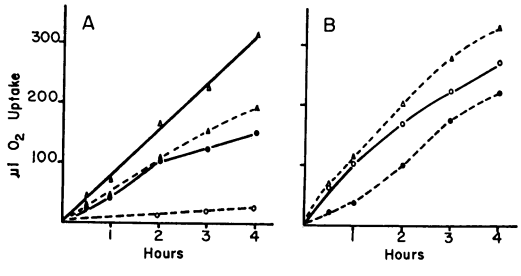

FIG. 2. *Oxidation of purines, pyrimidines, and purine degradation products by washed-cell suspensions of Pseudomonas aeruginosa. Each Warburg cup contained 5 μmoles of substrate and 2 ml of cells at ten times the growth concentration in 0.05 M tris buffer (pH 7.4). Endogenous oxygen uptake values have been subtracted. A, purines and pyrimidines: △, adenine; ▲, guanine; ●, uracil; ○, cytosine. B, purine degradation products: △, allantoin; ○, uric acid; ●, xanthine.*

8.05% of the RNA was oxidized, one may suggest that the oxidation of this amount of ribosomal RNA would yield 1.08% of the total radioactivity of uniformly labeled cells as $C^{14}O_2$. In the same way, the $C^{14}O_2$ from the proline-U-C^{14}-labeled cells constituted 4.05% of the total cellular protein which, if the cells had been uniformly labeled, would have contributed 2.32% of the cellular $C^{14}O_2$ released. The cells grown with glucose-U-C^{14} may be considered to be uniformly labeled, and the $C^{14}O_2$ released from these cells as a result of the oxidation of cellular protein and ribosomal RNA would, according to the above reasoning, amount to 3.40%. The experimental value obtained was 3.31%, thus confirming the absence of carbohydrate endogenous reserves.

Distribution of radioactivity in cell fractions. To ensure that the radioactivity in $C^{14}O_2$ from proline-U-C^{14}- and uracil-2-C^{14}-labeled cells did not arise merely from free intracellular "pools" of proline or uracil, whole cells were fractionated according to the modified procedure of Duncan and Campbell (1962) at zero time and after 3 hr of starvation. The change in distribution of radioactivity in the various fractions, as well as the influence of 3.33×10^{-2} M magnesium on this change, was determined (Table 7). As the chemical fractionation could not be carried out on cells used for $C^{14}O_2$ determinations, Table 7 is a composite of two separate experiments.

TABLE 7. *Distribution of C^{14} in chemical fractions of whole cells after 3 hr of starvation**

Fraction	Cells labeled with radioactive	Zero time	Endogenous	Plus Mg^{++}
		%	%	%
CO_2	Glucose	—	3.32	3.29
	Uracil	—	5.64	4.97
	Proline	—	4.05	4.67
Warburg supernatant fluid	Glucose	10.80	14.70	7.34
	Uracil	3.29	10.65	4.22
	Proline	4.89	7.94	4.50
Cold trichloroacetic acid	Glucose	3.34	2.58	3.36
	Uracil	7.20	8.90	11.05
	Proline	3.35	2.70	3.24
Acid-ethanol	Glucose	7.73	8.39	6.70
	Proline	9.45	10.25	9.15
Hot trichloroacetic acid	Glucose	14.10	11.70	13.42
	Uracil	90.40	74.20	79.70
Hot trichloroacetic acid residue	Glucose	66.90	59.90	65.90
	Proline	82.60	74.40	78.80

* Results expressed as per cent of total radioactivity.

Under all test conditions there was a significant decrease in the radioactivity in the "protein residue" and the hot trichloroacetic acid-soluble fraction. This confirms that the $C^{14}O_2$ from uracil- and proline-labeled cells was not a result of oxidation of "pool" material but was due to the degradation of both protein and RNA. It would appear that protein and RNA are degraded more rapidly than they can be oxidized and, therefore, are excreted into the suspending fluid. In each test sample, the "free amino acid pool" decreased during endogenous respiration, whereas the "nucleic acid precursor pool" increased. This suggests a preference for oxidation of amino acids or a greater ability of the organism to oxidize amino acids. From a comparison of the amount of RNA and protein in the cell and the apparent percentage of these components oxidized as indicated by $C^{14}O_2$ data, approximately twice as much protein as RNA was oxidized. In the presence of magnesium, relatively less RNA was degraded, and the degradation products were retained, to a greater extent, in the cold trichloracetic acid-soluble pool rather than being excreted into the suspending fluid. From the $C^{14}O_2$ values, it can be seen that magnesium ions have a slight inhibitory effect on RNA oxidation and a slight stimulatory effect on protein oxidation. Starvation in the presence of inorganic phosphate gave generally similar results; however, some as yet uninterpreted differences were evident.

Variously labeled cells were disrupted by the lysozyme-EDTA technique at zero time and after respiring endogenously for 3 hr, and were fractionated by differential centrifugation (Table 8). The commercial lysozyme preparation contained ribonuclease as a contaminating enzyme, as was shown by the release of nucleotides from commercial RNA incubated with lysozyme. The ribonuclease accounts, in part, for the abnormally low percentage of radioactivity in the ribosome fraction and the abnormally high percentage of radioactivity in the supernatant fluid (110,000 ×

TABLE 8. *Distribution of C^{14} in physical fractions of cells after 3 hr of starvation**

Fraction	Cells labeled with radioactive	Zero time	Endogenous	Plus Mg^{++}
		%	%	%
CO_2	Glucose	—	3.32	3.29
	Uracil	—	5.64	4.97
	Proline	—	4.05	4.65
Warburg supernatant fluid	Glucose	10.80	14.77	7.34
	Uracil	3.29	10.65	4.22
	Proline	4.89	7.97	4.50
"Membranes"	Glucose	16.10	10.55	8.79
	Uracil	7.22	6.24	7.10
	Proline	10.91	8.92	8.86
"Ribosomes"	Glucose	2.28	1.18	1.36
	Uracil	1.92	1.11	0.93
	Proline	4.79	3.14	1.97
Supernatant fluid (110,000 × *g*)	Glucose	64.80	66.10	76.30
	Uracil	87.80	75.50	82.50
	Proline	77.20	75.90	81.40

* Results expressed as per cent of total radioactivity.

g). The majority of the radioactivity in the supernatant fluid (110,000 × *g*) of uracil-2-C^{14}-labeled cells was cold trichloroacetic acid-soluble, indicating that it was low molecular-weight material. This is in contrast to the data obtained from the chemical fractionation of whole cells in which the lysozyme treatment was omitted. From Table 7 it can be seen that the majority of the radioactivity from the chemical fractionation procedure was contained in material insoluble in cold trichloroacetic acid.

All test samples showed a significant decrease in the protein and the RNA of the ribosome fraction, demonstrating the occurrence of ribosomal degradation during endogenous respiration. While following the turnover of protein and RNA in *E. coli*, Mandelstam and Halvorson (1960) found that the ribosomes contributed almost half the amino acids and essentially all the ribonucleotides passing through the free pool during starvation. However, no reference was made to the oxidation of these compounds.

The increase in protein in the supernatant fluid (110,000 × *g*) of cells incubated in the presence of magnesium ions and the apparently only slight decrease in RNA may be attributed to the solubilization of particulate material with a lower level of excretion of these materials into the Warburg suspending fluid. The appearance of nucleic acid in the "membrane" fraction can be accounted for by the presence of a small number of "bound" ribosomes as has been discussed previously (Campbell, Gronlund, and Duncan, 1963). The general decrease in the radioactivity of this fraction is again the result of degradation of ribonucleoprotein.

Ribosomal degradation. As ribosomes were shown to be degraded during starvation, the ribosomal fraction was examined for the presence of an enzyme responsible for the initiation of the degradative process. When ribosomes were incubated in tris buffer (pH 7.4), there was an increase in cold PCA-soluble material having a maximal absorption at 260 mμ (Fig. 3). EDTA completely inhibited this increase in UV-absorbing material, which tends to rule out the presence of a ribonuclease and the presence of the "V" route of ribosomal breakdown as described by Wade (1961) in *E. coli*. The addition of inorganic phosphate resulted in a 21-fold increase in activity, strongly implicating poly-

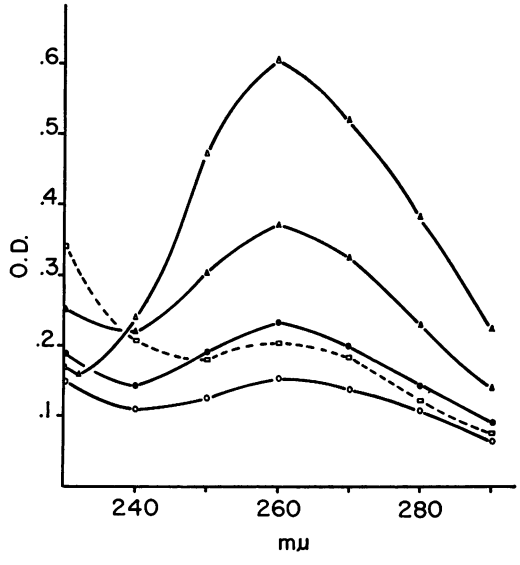

FIG. 3. *Degradation of ribosomes by a ribosomal enzyme as indicated by the increase in PCA-soluble ultraviolet-absorbing material in a reaction mixture containing ribosomes and tris buffer (pH 7.4). Test,* ▲, *control,* ●; *with* 6×10^{-2} *M* P_i (△) × *1/5, control* = ○; *with 1 mg/ml of EDTA, test and control* = □.

nucleotide phosphorylase (Grunberg-Manago and Ochoa, 1955) as the degradative enzyme causing the following reaction:

$$RNA + nP_i \rightarrow nXDP$$

where XDP represents purine or pyrimidine nucleoside diphosphates. Polynucleotide phosphorylase has been previously reported to be associated with the ribosomal fraction of this organism (Strasdine, Hogg, and Campbell, 1962). The breakdown of endogenous RNA by polynucleotide phosphorylase has been shown to occur in other organisms (Wade and Lovett, 1961; Ogata, Imada, and Nakao, 1962), and this, rather than biosynthesis of RNA, may be the true function of the enzyme.

When cells respire endogenously and a net decrease in protein concentration takes place, this must represent a situation of absolute minimal protein synthesis. As the rate of protein synthesis is proportional to the number of ribosomes (Kennell and Magasanik, 1962), under starvation conditions ribonucleoprotein is in excess and presents a logical endogenous substrate capable of supplying the maintenance energy requirements of the cell. The ribosome concentration would appear to be more than adequate to serve this function as well as acting as a source of free amino acids and nucleotides required for the synthesis of new enzymes or a greater quantity of enzymes specifically concerned with endogenous respiration.

Under the specified experimental conditions, *P. aeruginosa* did not store or utilize a carbohydrate reserve material but oxidized RNA and protein during starvation. It is conceivable that under different growth conditions, particularly with limiting nitrogen, the organism may store a carbohydrate reserve which can be drawn upon in the absence of exogenous substrates either to the exclusion of, or in addition to, RNA and protein. However, no evidence for the storage of such materials under conditions of limiting nitrogen has been found in the oxidative assimilation studies of Duncan and Campbell (1962).

The oxidation of ribosomal material by microorganisms during endogenous respiration may be a general phenomenon. As yet this is not supported by adequate data, but reports of the production of ammonia and the degradation of ribosomes by a large variety of microorganisms during endogenous respiration tends to support such a supposition. However, if an organism stores a carbohydrate reserve which is also oxidized during starvation, and the organism does not require organic nitrogen for synthesis, then the evolution of ammonia may not be detected until the carbohydrate reserve has been depleted. Dawes and Ribbons (1962) reported the excretion of ammonia from endogenously respiring *E. coli* only after the glycogen reserve had been utilized. This does not necessarily infer that glycogen is oxidized to the exclusion of nitrogenous compounds, but it may imply that any ammonia produced initially is assimilated during the oxidation of glycogen. The assimilation of endogenously produced ammonia during the oxidation of exogenous glucose has been reported (Gronlund and Campbell, 1961).

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LITERATURE CITED

BOREK, E., A. RYAN, AND J. ROCKENBACH. 1955. Nucleic acid metabolism in relation to the lysogenic phenomenon. J. Bacteriol. **69**:460–467.

BOWEN, T. J., S. DAGLEY, AND J. SYKES. 1959. A ribonucleoprotein component of *Escherichia coli*. Biochem. J. **72**:419–425.

CONWAY, E. J. 1950. Microdiffusion analysis and volumetric error, 3rd ed. Crosby, Lockwood and Sons Ltd., London.

CAMPBELL, J. J. R., A. F. GRONLUND, AND M. G. DUNCAN. 1963. Endogenous metabolism of *Pseudomonas*. Ann. N.Y. Acad. Sci. **102**:669–677.

CAMPBELL, J. J. R., L. A. HOGG, AND G. A. STRASDINE. 1962. Enzyme distribution in *Pseudomonas aeruginosa*. J. Bacteriol. **83**:1155–1160.

DAWES, E. A., AND W. H. HOLMS. 1958. Metabolism of *Sarcina lutea*. III. Endogenous metabolism. Biochim. Biophys. Acta **30**:278–293.

DAWES, E. A., AND D. W. RIBBONS. 1962. Effect of environmental conditions on the endogenous metabolism of *Escherichia coli*. Biochem. J. **84**:978.

DOUDOROFF, M., AND R. Y. STANIER. 1959. Role of poly-β-hydroxybutyric acid in the assimilation of organic carbon by bacteria. Nature **183**:1440–1442.

DUNCAN, M. G., AND J. J. R. CAMPBELL. 1962. Oxidative assimilation of glucose by *Pseudomonas aeruginosa*. J. Bacteriol. **84**:784–792.

GRONLUND, A. F., AND J. J. R. CAMPBELL. 1961. Nitrogenous compounds as substrates for endogenous respiration in microorganisms. J. Bacteriol. **81**:721–724.

GRUNBERG-MANAGO, M., AND S. OCHOA. 1955. Enzymatic synthesis and breakdown of polynucleotides; polynucleotide phosphorylase. J. Am. Chem. Soc. **77**:3165–3166.

HIGUCHI, M., AND T. UEMURA. 1959. Release of nucleotides from yeast cells. Nature **184**:1381–1383.

HORIUCHI, T. 1959. RNA degradation and DNA and protein synthesis of *E. coli* in a phosphate deficient medium. J. Biochem. (Tokyo) **46**: 1467–1480.

HUGHES, D. E. 1951. A press for disrupting bacteria and other microorganisms. Brit. J. Exptl. Pathol. **32**:97–109.

HUTCHISON, W. C., E. D. DOWNIE, AND H. N. MUNRO. 1962. Factors affecting the Schneider procedure for estimation of nucleic acids. Biochim. Biophys. Acta **55**:561–570.

KENNELL, D., AND B. MAGASANIK. 1962. The relation of ribosome content to the rate of enzyme synthesis in *Aerobacter aerogenes*. Biochim. Biophys. Acta **55**:139–151.

LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.

MANDELSTAM, J., AND H. HALVORSON. 1960. Turnover of protein and nucleic acid in soluble and ribosome fractions of non-growing *Escherichia coli*. Biochim. Biophys. Acta **40**:43–49.

OGATA, K., A. IMADA, AND Y. NAKAO. 1962. Formation of 5′ nucleotides by degradation of endogenous RNA in a *Bacillus* by its own polynucleotide phosphorylase. Agr. Biol. Chem. (Tokyo) **26**:586.

POSTGATE, J. R., AND J. R. HUNTER. 1962. The survival of starved bacteria. J. Gen. Microbiol. **29**:233–263.

POTTER, V. R. 1960. Nucleic acid outlines. Vol. 1, Structure and metabolism, p. 166. Burgess Publishing Co., Minneapolis.

SCHNEIDER, W. C. 1957. Determination of nucleic acids in tissues by pentose analysis, p. 680. *In* S. P. Colowick and N. O. Kaplan [ed.], Methods in enzymology, vol. 3. Academic Press, Inc., New York.

SMITH, I. [ed.]. 1960. Chromatographic and electrophoretic techniques. Vol. 2, Zone electrophoresis, p. 170. Interscience Publishers, Inc., New York.

STRANGE, R. E. 1961. Induced enzyme synthesis in aqueous suspension of stained stationary phase *Aerobacter aerogenes*. Nature **191**:1272–1273.

STRANGE, R. E., F. A. DARK, AND A. G. NESS. 1961. The survival of stationary phase *Aerobacter aerogenes* stored in aqueous suspension. J. Gen. Microbiol. **25**:61–76.

STRASDINE, G. A., L. A. HOGG, AND J. J. R. CAMPBELL. 1962. A ribosomal polynucleotide phosphorylase in *Pseudomonas aeruginosa*. Biochim. Biophys. Acta **55**:231–232.

URBA, K. C. 1959. Protein breakdown in *Bacillus cereus*. Biochem. J. **71**:513–518.

WADE, H. E. 1961. The autodegradation of ribonucleoprotein in *Escherichia coli*. Biochem. J. **78**:457–472.

WADE, H. E., AND S. LOVETT. 1961. Polynucleotide phosphorylase in ribosomes from *Escherichia coli*. Biochem. J. **81**:319–328.

WARREN, R. A. J., A. F. ELLS, AND J. J. R. CAMPBELL. 1960. Endogenous respiration of *Pseudomonas aeruginosa*. J. Bacteriol. **79**:875–879.

WRIGHT, B. E., AND M. L. ANDERSON. 1960. Protein and amino acid turnover during differentiation in the slime mold. 1. Utilization of endogenous amino acids and proteins. Biochim. Biophys. Acta **43**:62–66.