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Nature of Endogenous Reserve Material in a Strain of *Pseudomonas fluorescens-putida* Intermediate

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The nature of endogenous reserve materials in various pseudomonads is still incompletely known. Initially Norris, Campbell & Ney (1949) showed that the endogenous respiration of *Pseudomonas aeruginosa* is unaffected by aeration or by the addition of common uncouplers like dinitrophenol or azide, and that it proceeds undiminished in the presence of an exogenous substrate. In subsequent experiments, Warren, Ells & Campbell (1960) showed that the endogenous metabolism of both *P. aeruginosa* and *Pseudomonas fluorescens* involved liberation of ammonia, thus suggesting that the endogenous reserve material is nitrogenous in nature, possibly the cellular protein or the acids of the free amino acid pool. This ammonia could be re-incorporated rapidly into the cell as soon as any oxidizable substrate like glucose was added, suggesting the synthesis of an oxidatively assimilable material (Gronlund & Campbell, 1961). But whether these oxidatively assimilable materials are valid reserves for endogenous respiration is not

clear (Gibbs & Wood, 1952), and as such there is no clear-cut demonstration of any cellular nitrogenous constituent that may be considered to act as an endogenous reserve material in the pseudomonads. The present study was undertaken to investigate the nature of endogenous respiration in an intermediate strain of the *Pseudomonas fluorescens* group and to find out whether any particular cellular component is likely to be the substrate for endogenous respiration in that organism.

EXPERIMENTAL

Materials. Uniformly labelled L-[^{14}C]phenylalanine was purchased from The Radiochemical Centre, Amersham, Bucks. Azaserine was kindly given by Dr John R. Dice of Parke, Davis and Co., Mich., U.S.A., and actinomycin by Dr Junichi Kawamata of Osaka University, Japan.

Growth conditions. Cells of the *Pseudomonas fluorescens-putida* intermediate strain (Ganguly, 1955) were grown on a rotary shaker at room temperature (approx. 30°) in a synthetic medium which contained (g./l.): $(\text{NH}_4)_2\text{HPO}_4$,

2.0; KH_2PO_4 , 1.0; NaCl , 1.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; L-asparagine, 5.0; the pH was adjusted to 6.8–7.0. Sterile glucose (final concn. in the medium, 0.5%) was added separately after autoclaving. These cells, unless otherwise stated, were harvested in the mid-exponential phase after 20 hr. by centrifugation at 0° , washed twice with water and finally suspended in 0.1 M- KH_2PO_4 -NaOH buffer, pH 7.2. Oxygen consumption with such cells was measured by the usual manometric technique (Umbreit, Burris & Stauffer, 1957), and all Q_{O_2} values ($\mu\text{l. of O}_2/\text{hr./mg. dry wt. of bacterial cells}$) have been calculated on the initial rates of uptake. Respiratory quotients (R.Q.) have been determined by the method of Stickland (1956), with 10 mg. dry wt. of cells/flask. Viable counts in different batches of suspensions were noted by diluting a definite portion of the suspension to a fixed volume, plating a particular portion of them on nutrient agar plates and counting the number of colonies after incubation for 24 hr. at 37° .

Analytical estimations of different metabolites. About 10 ml. of cell suspension, containing 10 mg. dry wt. of cells/ml., was shaken in a Warburg respirometer at 37° for different periods (usually 1–4 hr.). A zero-time cell suspension was prepared by removing freshly harvested cold cell suspension immediately into ice at 0° . Since the organism is not very sensitive to cold shock, no change of viability was observed with such suspensions. Samples from different hourly intervals were also stored in ice until the final suspension was ready for simultaneous determination of various metabolites.

Carbohydrate was determined by the anthrone method (Chung & Nickerson, 1954). Total carbohydrate was estimated on cell suspensions, and extracellular carbohydrate was determined on a portion of the supernatant after centrifugation (at 1500g for 15 min.) of the cell suspension. Total keto acids were measured by deproteinizing the suspension with trichloroacetic acid (5%, w/v), centrifuging to remove the precipitated protein and treating a portion of the supernatant with 2,4-dinitrophenylhydrazine as outlined by Friedemann (1957). Extracellular ammonia was determined by distillation into H_2SO_4 of the supernatant of the centrifuged suspension in the presence of alkali in a Markham still, and finally nesslerizing the distillate. Total protein was determined by heating a portion of the suspension at 100° for 10 min. and then precipitating the protein with 10% (w/v) trichloroacetic acid. The precipitate was centrifuged, the pellet dissolved in 0.1 N-NaOH and protein was measured in a portion by the method of Lowry, Rosebrough, Farr & Randall (1951).

Extracellular amino acids were determined in portions of supernatant from the centrifuged suspension by the method of Yemm & Cocking (1955), with glycine as standard. The free amino acid content of bacteria was determined by heating the cells, obtained after centrifuging the original cell suspension, and finally resuspending the cells in distilled water, in a boiling-water bath at 100° for 15 min., centrifuging and then treating the supernatant with the ninhydrin reagent of Yemm & Cocking (1955). However, for determinations of both intracellular and extracellular amino acids, NH_3 was previously liberated by heating the sample with few drops of N-KOH (pH to 9.0) for 20 min. at 100° , cooling and finally adjusting the pH to 7.0.

Total lipids were measured by hydrolysing the centrifuged cells with 6 N-HCl and finally extracting with ether as described by Salton (1953).

For the determination of RNA and DNA, 5 ml. of cell suspension was centrifuged at 0° , the cells were washed first with 10% (w/v) trichloroacetic acid at 0° and then extracted with 5% (w/v) trichloroacetic acid at 90° for 20 min., as recommended by Stuy (1958), and finally centrifuged. Portions of the supernatant were used for determination of RNA by the orcinol reaction as outlined by Stuy (1958), and for the determination of DNA by the diphenylamine reaction of Burton (1956).

Growth of cells with labelled proteins. To avoid ambiguity in the results relating to 'labile storage material' or 'stable reserve material' (Danforth & Wilson, 1961), 100 μmoles of uniformly labelled L- ^{14}C phenylalanine (10 μC) were added during inoculation to 200 ml. of a 1% (w/v) casein hydrolysate medium. After about 18 hr. such cells were harvested by centrifugation at 0° , suspended in 0.1 M- KH_2PO_4 -NaOH buffer, pH 7.2, and divided into two different fractions. One fraction was held at 0° , while the other was incubated at 37° for 4 hr. in a Warburg respirometer to deplete its endogenous reserve material. These cells were then centrifuged at 0° , and fractionation of cell materials from both the batches was carried out according to the method of Park & Hancock (1960) with the omission of a few steps as directed by Halvorson (1958). Essentially, the method consisted of the following steps:

(a) Cold-trichloroacetic acid-soluble fraction. Cell suspensions (0.5 ml., containing approx. 5 mg. dry wt. of cells) from both batches (control undepleted and endogenous-depleted, both taken from cells grown with L- ^{14}C phenylalanine) were centrifuged, and the cells were washed twice with cold water and finally extracted for 10 min. at 0° with 3 ml. of 5% (w/v) trichloroacetic acid. Cells were then centrifuged at 2000g for 15 min. and the supernatant was assayed for radioactivity.

(b) Ethanol-soluble fraction. Centrifuged cells from both the batches were extracted with 3 ml. of 75% (v/v) ethanol for 10 min. at room temperature (approx. 30°) and again centrifuged. Supernatants from both the batches were assayed for radioactivity.

(c) Hot-trichloroacetic acid-soluble fraction. The residues were again suspended in 3 ml. of 5% (w/v) trichloroacetic acid and heated at 90° for 6 min. and cooled. After centrifugation at 2000g for 15 min. the supernatants were decanted and assayed for radioactivity.

(d) Trichloroacetic acid-precipitable fraction. The residues from each batch were suspended in 1 ml. of water; to each of them 0.5 ml. of 30% (w/v) trichloroacetic acid was added, and the mixtures were kept for 1 hr. at 37° . They were then centrifuged and the supernatants were discarded. The residues in both cases were dissolved in 0.1 N-NaOH and finally assayed for radioactivity.

Radioactivity was determined after oxidation with persulphate as described by Chen & Lauer (1957) in portions from the above samples made up to a fixed volume. After the oxidation with persulphate, the liberated $^{14}\text{CO}_2$ was trapped in KOH and precipitated as $\text{Ba}^{14}\text{CO}_3$ in the presence of carrier carbonate. These were then thinly plated on stainless-steel planchets as described by Sakami (1956). Radioactivity on these planchets was determined with a windowless gas-flow type of Geiger-Müller counter with an M-5 semiautomatic sample changer (model 182 x scaler; Nuclear-Chicago); corrections for self-absorption, dead-time and background counts were applied.

RESULTS

Effect of growth periods, glucose concentration and period of starvation on the rate of endogenous respiration. Endogenous respiration in the organism was first ascertained by harvesting cells after different growth periods and noting the Q_{O_2} values of the cells. Since during the growth cycle of the organism the exponential phase started from 10 hr. after inoculation and continued up to about 50 hr., cells of 24 hr. and 48 hr. growth periods represent those of mid-exponential phase and late exponential phase respectively. The stationary phase continues up to about 80 hr., after which a steady decrease in the viability of the cells is noted. The rate of oxygen uptake with glucose as the substrate was also noted with each batch of cells of different growth periods. The results are given in Table 1. The maximum rate of endogenous respiration is shown by cells harvested after a 24 hr. growth period, i.e. by the cells harvested from the mid-exponential phase. Cells grown for shorter periods were, however, not tested for their content of endogenous reserve material. With aging this capacity changes, and cells harvested after 120 hr. are practically devoid of endogenous respiration.

A R.Q. of 0.54 ± 0.05 is found throughout. Such low R.Q. values indicate that no single substance, namely carbohydrate, lipid or protein, serves as the sole endogenous reserve, but a number of complex products may be the oxidizable material, whose oxidation furnishes energy to the organism with a R.Q. of 0.55.

Table 1 shows that, although the R.Q. of glucose oxidation is different from that of the endogenous respiration, both of them remained fairly constant throughout the entire period of aeration (up to 2 hr.). Also, batches of cells from different growth periods seemed to possess the same R.Q., and were thus possibly utilizing the same reserve material.

Table 2 presents the results from experiments where the glucose concentration was varied and the resultant oxygen uptake for oxidizing these different concentrations was noted. The oxygen uptake expressed as a percentage of the theoretical uptake varies from 79 to 125 if endogenous uptake is supposed to be completely suppressed during glucose catabolism. If endogenous respiration is taken as operative to the same degree during the oxidation of different amounts of glucose, a more or less constant value (54–58 % of the theoretical value) is obtained for glucose oxidation. Since the

Table 1. *Effect of different growth periods on the rate of endogenous respiration by resting cells of Pseudomonas fluorescens-putida*

Oxygen uptake was measured under identical conditions after harvesting cells, grown for different periods, on the same day. Uptake was noted for the first 2 hr. at 37°. For the oxidation of glucose, 20 μ moles of glucose were added to each flask, and the oxygen uptake was noted for the first 2 hr. These uptake values have not been corrected for endogenous respiration.

Growth period (hr.)	Phase of growth	Endogenous respiration value		Respiration value with glucose oxidation	
		(Q_{O_2})	(R.Q.)	(Q_{O_2})	(R.Q.)
24	Mid-exponential	20.3	0.52	86.5	0.65
48	Late exponential	17.8	0.49	63.8	0.69
72	Stationary	11.2	0.55	51.0	0.64
96	Negative exponential	5.1	0.57	32.3	0.68

Table 2. *Effect of different amounts of glucose on the rate of endogenous respiration by cells of Pseudomonas fluorescens-putida*

Cells were harvested after growth for 20 hr. The complete system contained, in a total volume of 3 ml., the following: cells, equivalent to 3.3 mg. dry wt., suspended in 100 μ moles of KH_2PO_4 -NaOH buffer, pH 7.2, and different concentrations of glucose (when added). The centre well contained 0.2 ml. of 20% (w/v) KOH on filter paper. The temperature was 37°, the gas phase air and the shaker speed 120 strokes/min. Preincubation for 10 min. was allowed before the contents of the side arm were tipped in. Oxygen uptake was noted for 220 min.

Substrate added	Amount of glucose added (μ moles)	Total O_2 uptake (μ l.)	Theoretical O_2 uptake (μ l.)	O_2 uptake (% of theoretical)	O_2 uptake after subtraction of endogenous value (μ l.)	O_2 uptake (% of theoretical after subtracting endogenous value)
None	—	192	—	—	—	—
Glucose	2	338	269	125	146	54
	4	505	538	93	313	58
	6	641	807	79	449	55

time allowed for incubation was adequate (nearly 4 hr.) for the degradation of almost all the added glucose it is apparent that a major portion of the glucose (42–46 %) was being oxidatively assimilated during the incubation period for synthesis of cellular constituents.

Table 3 records the increased rate of oxidation of glucose with diminished endogenous respiration. Oxidation of glucose is nearly completely suppressed with cells having a high rate of endogenous respiration, but it increases considerably with a decreased rate of endogenous respiration. The viable count shows that there is no net gain or loss of viability during the period of shaking. The increased rate of substrate oxidation is thus not due to an increase in the total cell population. It seems that the undepleted cells utilize the endogenous reserve materials as the primary substrates, and that only when these reserve materials are exhausted to a marked extent do the cells utilize exogenously added substrates. However, since the total oxygen uptake in the presence of glucose increases considerably during endogenous metabolism, it is clear that a mere competition for the available enzymes for glucose catabolism cannot explain the observed findings of suppression of glucose oxidation by undepleted cells. Mandelstam (1958) has shown that, in *Escherichia coli*, protein-degrading enzymes become much more active in non-growing cells than when these cells were growing exponentially. A similar situation may also exist in the present organism where, owing to change in environmental conditions, protein-degrading enzymes may become more active, whereas the carbohydrate-degrading enzymes may perhaps become essentially non-functional, and only after sufficient proteins have been degraded do the non-functional enzymes become active again. The increase in the functional activity of the glucose-catabolizing enzymes, as reflected in the increased total oxygen uptake, may also be due to

an increased synthesis of these enzymes from the degradation products of cellular proteins. Such increased synthesis of an inducible enzyme in *E. coli* has been shown by Mandelstam (1958), and it may be that, in the absence of any such inducer, an increase in the net synthesis of constitutive glucose-catabolizing enzymes takes place in the present pseudomonad.

Effect of different inhibitors on endogenous respiration. To investigate the nature of the endogenous reserve, the effect of different inhibitors on endogenous respiration was tested. A rather wide range of concentrations of the inhibitors was employed. At low concentrations (0.1 mM) dinitrophenol, iodoacetate, fluoroacetate or arsenite had practically no effect on the rate of endogenous respiration, although all of them, especially iodoacetate and dinitrophenol, at relatively high concentrations caused a marked inhibition of respiration (Table 4). Relatively high concentrations (10 mM) of azide did not inhibit, however. Since endogenous respiration is believed to furnish energy for the maintenance of the cells' integrity under starvation conditions, the inhibitory effect of high concentrations of these compounds is likely to be a reflexion of an inhibition of the production of suitable substrates for endogenous respiration. That this energy may be derived from the breakdown of carbohydrates through the tricarboxylic acid cycle, at least partially, is seen from the observed inhibitory effect of iodoacetate, arsenite and fluoroacetate. Actinomycin and azaserine, which are known to interfere with the synthesis of proteins or ribonucleic acids (Kawamata & Imanishi, 1961; Lavenberg, Melnick & Buchanan, 1957), are also slightly inhibitory. Of the azapurines tested, azaxanthine, azaguanine, azauracil and 6-mercaptopurine were without effect, whereas azaserine caused some inhibition (10–15 %) in the initial stages (up to 1 hr.), after which the inhibitory effect declined and rapid respiration ensued. It

Table 3. *Rate of glucose oxidation by cells of Pseudomonas fluorescens-putida with depleted endogenous reserves*

Cells were harvested at the end of growth for 20 hr., and different batches were aerated for 0, 2, 3 and 4 hr. The complete system contained, in a total volume of 3 ml.: cells of different batches equivalent to 2.3 mg. dry wt. suspended in 100 μ moles of potassium phosphate buffer, pH 7.2, and 10 μ moles of glucose (when added). The centre well contained 0.2 ml. of 20 % (w/v) KOH on filter paper. The temperature was 37°, the gas phase air and the shaker speed 120 strokes/min. Preincubation for 10 min. was allowed before the contents of the side arm were tipped in. The uptake was noted for 1 hr.

Period of starvation (hr.)	O ₂ uptake			Viable count (cells/ml.)
	No addition (μ l.)	Glucose added (μ l.)	Net increase (μ l.)	
0	61	78	17	0.87×10^{11}
2	47	138	91	0.82×10^{11}
3	38	207	169	—
4	36	222	186	0.84×10^{11}

may be that turnover of RNA is necessary initially to facilitate the breakdown of products of endogenous metabolism for energy-generating purposes.

Changes of metabolite concentration during endogenous respiration. To discover whether there was a change in the content of carbohydrate, protein, lipid, nucleic acid or amino acids of the free amino acid pool of the cell, an assay of the content of these materials was conducted before and after various periods of endogenous respiration. To cause appreciable depletion, cells endogenously depleted for 2 and 4 hr. were used for such assays. However, an inherent difficulty in such estimations was the wide variation of the results. It was almost impossible to pipette equal amounts of cells from each suspension for a comparative study. Besides, cells of different batches showed variations in their carbohydrate, protein and lipid contents, and so any change due to endogenous respiration was also variable. Nevertheless, by conducting a number of measurements with such accuracy as can be expected under these conditions, it was possible to obtain an average value that represented the change in the content of any particular constituent of the cell during endogenous metabolism of the pseudomonad. The results are shown in Table 5. Since the total amount of lipid (27.5 % on dry wt. basis) was always found to be the same in all the samples, its value has not been included in the Table.

The results in Table 5, when examined critically, show some slight changes in the different cell con-

stituents during endogenous metabolism. Both protein and carbohydrate decreased, but not to any appreciable degree, and were presumably well within experimental error. Apparent inconsistency was only found in the total content of the free amino acid pool. Although this pool decreased in all the experiments with cells incubated for 2 hr., with cells after 4 hr. incubation it was found to decrease in some experiments whereas in others it showed the same value or even sometimes a greater one than cells at zero time. However, when cells after incubation for 4 hr. showed an amino acid pool content similar to or increased above those from the zero-time system, they invariably had a smaller cellular protein content than the 2 hr. or the zero-time cells. It was apparent that, to allow continuation of respiration, cellular protein was partly being degraded to replenish the free amino acid pool, which really acted as the primary substrate of endogenous respiration. However, since only 68 % of the total cell constituents has been accounted for, it may be that the substances comprising the remaining 32 % may contribute partly to the endogenous reserve material of the organism.

To study the ways in which the free amino acid pool is replenished by intracellular nitrogenous constituents, cells grown in media containing L-[¹⁴C]phenylalanine were used. After being harvested by centrifugation and washed thoroughly with water, cells were aerated for 4 hr. in a Warburg respirometer to deplete their endogenous reserves, while a control unaerated suspension was kept

Table 4. *Effect of different inhibitors on the rate of endogenous respiration by cells of Pseudomonas fluorescens-putida*

Cells were harvested at the end of growth for 20 hr. and suspended in potassium phosphate buffer, pH 7.2. Oxygen consumption was measured as described in the text. Neutralized inhibitors, when added, were pre-incubated with the cells for 15 min., after which oxygen consumption was noted.

Inhibitor added	Concn. of inhibitor (mM)	Q_{O_2}	Inhibition (%)	Stimulation (%)
None	0.0	16.0	—	—
Dinitrophenol	0.1	22.3	—	39
	10	2.9	82	—
Iodoacetate	0.1	17.2	—	7.5
	1.0	12.5	22	—
	10	2.5	84	—
Azide	1.0	17.8	—	11
p-Chloromercuribenzoate	0.01	17.8	—	11
None	0.0	21.3	—	—
Arsenite	1.0	20.8	2	—
	10	13.9	35	—
Azide	10	22.6	—	6
Fluoroacetate	0.1	21.5	—	0
	10	17.3	18	—
Actinomycin	50 µg./ml.	20.7	3	—
Azaserine	50 µg./ml.	19.2	10	—

Table 5. *Changes in different cellular constituents during endogenous respiration by Pseudomonas fluorescens-putida*

Cells, both depleted of endogenous reserve materials as well as undepleted, were analysed for the content of total protein, total and extracellular carbohydrates, amino acids, nucleic acids and total keto acids as described in the Experimental section. The $O_2:NH_3$ ratio was determined after noting total oxygen uptake and the total quantity of NH_3 produced by these cells. Values for total protein, carbohydrate, amino acids, nucleic acids and NH_3 are expressed in mg./100 mg. dry wt. of cells, and are averages of eight determinations with different batches of cells. Protein was measured with crystalline albumin as standard, carbohydrates with glucose as standard, amino acids with glycine as standard, and RNA and DNA with yeast RNA and DNA respectively as standards. Total keto acids are expressed as μ moles of pyruvate/100 mg. dry wt. of cells. The values given in parentheses represent the range of values for each set of eight determinations.

Time of respiration (hr.)	Carbohydrate			Amino acids		Nucleic acids		Ammonia		O ₂ :NH ₃ ratio	Total keto acids
	Total protein	Extra- cellular		Extra- cellular	Free amino acid pool	RNA	DNA	Extra- cellular	Intra- cellular		
		Total	cellular								
0	23.8 (±2.4)	5.28 (±0.35)	0.066 (±0.01)	0.13 (±0.05)	0.782 (±0.12)	7.42 (±0.65)	2.50 (±0.08)	0.178 (±0.03)	0.032 (±0.01)	8.25	0.0
2	22.7 (±2.9)	4.87 (±0.28)	0.208 (±0.03)	0.18 (±0.03)	0.590 (±0.08)	7.29 (±0.81)	2.50 (±0.11)	0.371 (±0.09)	0.067 (±0.01)	—	0.08 (±0.02)
4	20.3 (±1.6)	4.72 (±0.52)	0.522 (±0.06)	0.37 (±0.03)	0.804 (±0.21)	7.41 (±1.2)	2.48 (±0.14)	0.395 (±0.07)	0.089 (±0.02)	7.95	0.41 (±0.06)

Table 6. *Fractionation of cells of Pseudomonas fluorescens-putida grown in the presence of L-[^{14}C]-phenylalanine before and after 4 hr. endogenous respiration*

Uniformly labelled L-[^{14}C]phenylalanine-grown cells were divided into two batches: one unaerated control cells, the other aerated for 4 hr. to deplete its endogenous reserve. Fractionation of the cells and radioactivity determinations were done as described in the Experimental section.

Fraction	Distribution of radioactivity (counts/min.)	
	Unaerated control cells	Aerated endogenous depleted cells
Cold-trichloroacetic acid-soluble	410	890
Ethanol-soluble	187	98
Hot-trichloroacetic acid-soluble	827	845
Trichloroacetic acid-precipitable	8600	7492
Suspension medium	129	427
	10153	9752

at 0°. Cells from both the batches were then fractionated (Table 6).

It is clear that when the pseudomonad is grown in the presence of L-[^{14}C]phenylalanine the maximum incorporation occurs in the trichloroacetic acid-precipitable fraction (proteins), although both hot-trichloroacetic acid-soluble (nucleotides) and cold-trichloroacetic acid-soluble (free amino acid pool) fractions also incorporate radioactivity to some extent. After endogenous depletion for 4 hr. the radioactivity in the cold-trichloroacetic acid-soluble fraction increases twofold, whereas radioactivity from the trichloroacetic acid-precipitable fraction diminishes. This suggests that trichloroacetic acid-precipitable materials (proteins) are degraded to form cold-trichloroacetic acid-soluble materials (free amino acid pool), some of which are oxidized leading to an increase in radioactivity of the suspension medium and in respired carbon dioxide. Since this respiratory carbon dioxide has not been measured, the low yield of radioactivity with the depleted cells may be ascribed to this loss in respired carbon dioxide.

DISCUSSION

Attempts have been made to study the nature of the possible storage material which is utilized by *P. fluorescens-putida* during endogenous respiration. The present pseudomonad, like other members of its genus, can respire for a long time with an average Q_{O_2} of 15.0. Its low R.Q. (0.54 ± 0.05), production of ammonia, depletion of free amino acid pool and release of carbohydrates to the

external medium, and above all a high oxygen:ammonia ratio, all suggest that a mixture of carbohydrates, proteins and possibly other materials serves as endogenous substrates. Although Gronlund & Campbell (1961) have shown that both *P. aeruginosa* and *P. fluorescens* utilize nitrogenous compounds as substrates for endogenous respiration, no unequivocal demonstration of the nature of the nitrogenous compound was possible. They showed, however, that the free amino acid pool possibly served as the initial substrate which, on replenishment from nitrogenous constituents of the cell, could help in the continuation of the endogenous respiration. This second phase is absent in *Sarcina lutea*, where it has been shown that, when the free amino acid pool is depleted to nearly one-half, the endogenous metabolism falls to a negligible level (Dawes & Holms, 1958). Evidently a mechanism whereby the free amino acid pool could be enriched by degradation products of cellular proteins is absent in this organism. Various other bacteria also utilize these amino acid or peptide pools, and some have been shown to degrade proteins, carbohydrates, RNA, fat and other characteristic polymers of the cell (Dawes & Ribbons, 1962). In this sense, the present pseudomonad resembles several other organisms in having no specific reserve storage material but rather drawing on several components of the cell.

Although it has been demonstrated that protein breakdown may account, at least partially, for the continuation of endogenous respiration in the pseudomonad, evidence for the demonstration of carbohydrate breakdown is not conclusive enough to make a clear-cut conclusion. However, indirect evidence suggests that carbohydrates may also be simultaneously degraded during endogenous metabolism. A high oxygen:ammonia ratio points to this possibility (Gronlund & Campbell, 1961). A small decrease in the total carbohydrate content of the cell with endogenous respiration, together with the fact that soluble carbohydrates are released from the cells in increasing quantity during endogenous metabolism and that this metabolism is inhibited to a large extent by iodoacetate, fluoroacetate or arsenite (Table 4), all are indicative of a concurrent carbohydrate degradation. The increase in Q_{O_2} value in the presence of low concentrations of dinitrophenol or azide indicates that some resynthesis of the endogenous substrates from an intermediate on the catabolic route goes on side by side with endogenous degradations, and that these inhibitors cause an excess of oxygen uptake by selectively inhibiting this concurrent resynthesis of the endogenous substrates from those intermediates (Midwinter & Batt, 1960). The high rate of inhibition of endogenous respiration by 10 mM-dinitrophenol is possibly due to the inhibition of

protein degradation, as has been found for other organisms (Mandelstam, 1958).

That the liberation of ammonia, amino acids or carbohydrates from the cell, or the resultant decrease in protein or free amino acid pool, is not due to lysis of the organisms has been shown from the viable counts of the cells (Table 3). It must be stressed, however, that such counts are only approximate, and attempts to detect ultraviolet-absorbing materials (Strange, Dark & Ness, 1961) or any stable intracellular enzyme (Urba, 1959) failed because of the lack of suitable methods. Nevertheless, the approximate viable counts indicate that no appreciable lysis occurs during the 4 hr. incubation period of endogenous respiration.

SUMMARY

1. A study has been made with *Pseudomonas fluorescens-putida* to find out the possible nature of endogenous reserve materials, the oxidation of which in the absence of exogenous nutrients furnishes energy for the maintenance of the cellular integrity of the organism.

2. It has been shown that cells after 24 hr. of growth possess the maximum amount of endogenous reserve material, and that oxidation of this reserve material proceeds even in the presence of exogenous substrates.

3. A study of the effect of inhibitors suggests that both carbohydrate- and protein-degradative reactions are of utmost importance in the continuation of the endogenous respiration.

4. Quantitative estimation of cellular constituents before and after endogenous respiration shows that there is a very small decrease in the total content of proteins and carbohydrates, and a comparatively greater decrease in the content of the free amino acid pool, especially during the initial stages of endogenous respiration. An increase in the production of extracellular ammonia, amino acids and carbohydrates also occurs.

5. Cells grown with uniformly labelled L-[^{14}C]-phenylalanine show that radioactivity in the intracellular proteins decreases with increasing respiration, and a replenishment of the pool amino acids occurs from these degradation products.

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The Action of Pepsin on Protein Fractions from Horse Antisera to Diphtheria Toxin

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The work on the action of enzymes on antitoxic horse sera described in the patents of Parfentjev (1936, 1938, 1939) and in the publications of Pope (1938, 1939*a*, *b*) stimulated a considerable amount of work on horse antitoxins and their peptic digestion in the years immediately following. From this it appeared that when horses are immunized with diphtheria toxin the γ -globulin increases slightly in the early stages and the β_2 -globulin (or T-globulin) to a greater extent in the later stages, and that antitoxic activity is associated with both (Kekwick & Record, 1941). The β -antitoxin has a long flocculation time and a low *in vivo/in vitro* ratio, and gives floccules containing only half the antibody nitrogen found in floccules from the γ -globulin antitoxin, which has a short flocculation time and a high *in vivo/in vitro* ratio. On peptic digestion the antitoxin gives a product with the short flocculation time of a γ -globulin, but with the low *in vivo/in vitro* ratio of β -globulin (Kekwick, Knight, Macfarlane & Record, 1941). Sera high in their proportion of γ -globulin give poor yields of antitoxin on digestion, and it was suggested that digested antitoxin comes from the β -globulin. On the other hand, on digestion, the β_2 -globulin peak disappears and is replaced by a γ -globulin peak (van der Scheer & Wyckoff, 1940; van der Scheer,

Wyckoff & Clarke, 1941; Amoureux & Yeu, 1951). The molecular weight of a pseudoglobulin fraction from horse diphtheria antitoxin (probably consisting largely of β_2 -globulin) was found by Petermann & Pappenheimer (1941) to be 184 000 and that of pepsin-digested antitoxin to be 98 000. They suggested that the antitoxin molecule is split in a plane normal to the long axis. Petermann (1942*a*) found papain to have a similar action.

Most of this work was done with unfractionated sera containing various proportions of β - and γ -globulins, as was true also of the dialysis-precipitated and ammonium sulphate-precipitated fractions used by Harms (1948) and Anderson (1955) in their studies on the pepsin-refining of antitoxins. Although there are suggestions that γ -globulin gives a poor yield on pepsin treatment, possibly because it is precipitated during the heat treatment (Harms, 1948), and that most of the refined antitoxin comes from the β -globulin (Kekwick *et al.* 1941), no studies, except those of Petermann & Pappenheimer (1941) and Petermann (1942*b*), have been published on pepsin treatment of separated globulins. Recently devised chromatographic methods allow of the separation of large amounts, at any rate of γ -globulins, in a fairly pure state, and the present work is a study of the pepsin-refining of