Studies on the Endogenous Metabolism of *Escherichia coli*

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1. The endogenous metabolism of *Escherichia coli* has been studied by examining changes in cellular composition and of the suspending fluid during starvation of washed suspensions of the organism, in water or in phosphate buffer, at 37° under aerobic and anaerobic conditions. 2. When *E. coli* is grown in glucose-ammonium salts media the cells contain glycogen, which is utilized rapidly during subsequent starvation of the cells. 3. Ammonia is released by starved cells only after a lag period, which corresponds to the time taken for the cellular glycogen to be almost completely utilized. 4. If cells are grown under conditions that permit incorporation of ^{14}C into protein but not into glycogen and are then starved, release of $^{14}\text{CO}_2$ commences immediately and continues at a linear rate throughout the period of glycogen utilization; it is concluded that the presence of glycogen in the cell prevents the net degradation of nitrogenous materials but does not suppress protein turnover. 5. RNA is degraded by the cells immediately they are starved, ribose is oxidized and ultraviolet-absorbing materials are released to the suspending medium. 6. There is no significant utilization of lipid during the starvation of glucose-grown *E. coli*. 7. There is no loss of viability during the initial 12 hr. period of starvation under either aerobic or anaerobic conditions, but thereafter the cells die more rapidly under conditions of anaerobiosis. 8. These results are discussed in relation to the known patterns of endogenous metabolism and survival of other bacteria.

The utilization of intracellular materials by bacteria during starvation, the so-called endogenous metabolism, is probably a phenomenon of universal occurrence (for reviews see Dawes & Ribbons, 1962*a*, 1964). The energy derived from the oxidation of cellular constituents may be used to maintain the cell intact and to preserve viability until death eventually ensues. Experimental evidence in support of an energy of maintenance for *Escherichia coli* has been presented by McGrew & Mallette (1962) and by Marr, Nilson & Clark (1963), and current ideas on the energy of maintenance concept have been discussed by Dawes & Ribbons (1964).

Although *E. coli* has been extensively used in studies of bacterial metabolism, until recent years there has been little information on its metabolism when held in the absence of nutrients. Gronlund & Campbell (1961) detected formation of ammonia during endogenous respiration of the organism and postulated that internal nitrogenous reserves served as substrates for endogenous metabolism, although the observed ratios of oxygen consumed to ammonia produced indicated that some non-

nitrogenous source, such as lipid or carbohydrate, was also utilized.

Dagley & Dawes (1949) investigated factors influencing the carbohydrate content of *E. coli*, and the subsequent extensive researches of Holme & Palmstierna (1956*a,b*) on the glycogen content of the organism suggested that this storage product may serve as an endogenous substrate, although this possibility was not tested.

Ribbons & Dawes (1963) showed that the nature of the endogenous metabolism of *E. coli* depends on various environmental factors, and indicated a relationship to the nutritional status of the cells. We undertook the present work to attempt to resolve the role of carbohydrate and nitrogenous materials as endogenous substrates for *E. coli* and to study the survival pattern and changes in chemical composition that accompany starvation. Preliminary accounts have been presented to The Biochemical Society (Dawes & Ribbons, 1962*b,c*).

EXPERIMENTAL

Materials

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Organism. *Escherichia coli* N.C.T.C. 5928 was used throughout this work. The stock organism was maintained on nutrient-agar slants.

Medium. This study was devoted exclusively to cells grown on defined medium of the following composition (g./l.): KH_2PO_4 , 5.4; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4; $(\text{NH}_4)_2\text{SO}_4$, 1.2; glucose, 8.0; the pH was adjusted to 7.1 with 5N-NaOH. The glucose plus $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ was sterilized separately and the solutions were mixed with aseptic precautions when cool. In medium of this composition the precise factor limiting growth is not known, but it is not glucose, nitrogen, magnesium, phosphorus or sulphur.

Growth of the organism. *E. coli* was maintained in liquid medium of the above composition and subcultured every 2 weeks by inoculation of sterile medium (12.5 ml.) contained in conical flasks of 50 ml. capacity. Stationary-phase populations for inocula were obtained by overnight incubation (16 hr.) at 37° in a Gallenkamp reciprocating water bath (60 oscillations/min.; stroke 4 cm.). A freshly grown culture was always used to inoculate a second shaken culture (12.5 ml.), which reached the end of the exponential growth phase in 6–8 hr. A portion (1 ml.) of this culture was used to inoculate fresh medium (500 ml. or 1 l.) contained in 1 l. boiling flasks that were either aerated with sterile air or left static, according to the experimental requirements. Growth was followed turbidimetrically and cells for experimental work were harvested just after the onset of the stationary phase. Larger batches of cells were obtained with 5 or 10 l. of medium contained in boiling flasks (10 l.), the former being fitted with coarse sintered-glass aerators for aeration. Inocula for these large batches were 1% (50 or 100 ml. respectively) of a 15 hr. culture and the temperature of incubation was 37°. Bulk cultures were harvested with an air-turbine Sharples no. 1A laboratory super-centrifuge at 50 000 rev./min.; smaller volumes of culture were centrifuged at 15 000g for 3 min. in either an MSE Angle 13 centrifuge or a Servall SS-1 centrifuge without refrigeration.

The cells were generally washed twice with distilled water before resuspension to a desired cell density in phosphate buffer (0.67 M- KH_2PO_4 adjusted to pH 7.1 with 5N-NaOH). Deviations from this standard procedure, e.g. the use of ice-cold washes and overnight storage of cells as a pellet at 4°, are indicated in the individual Tables and Figures. Some effects of overnight storage have been recorded (Ribbons & Dawes, 1963).

Chemicals. Analytical-reagent-grade chemicals (Hopkin and Williams Ltd., Chadwell Heath, Essex; British Drug Houses Ltd., Poole, Dorset) were used wherever possible; other chemicals were of the best available purity from the same suppliers, and were recrystallized.

Radiochemicals. The ^{14}C -labelled algal-protein hydrolysate and $[\text{U-}^{14}\text{C}]\text{glucose}$ were obtained from The Radiochemical Centre, Amersham, Bucks.

Analytical methods

Bacterial densities and growth. Bacterial densities were determined turbidimetrically either in a Hilger Spekker absorptiometer with Chance glass filters H508 and OB2 or a Unicam SP. 600 spectrophotometer at 570 m μ ; extinctions were converted into bacterial densities from curves relating bacterial dry weight to extinction. Bacteria were washed and dried at 105° for 18 hr. Bacterial growth was followed turbidimetrically by the method of Dagley, Dawes & Morrison (1950).

Carbohydrate. Carbohydrate was determined by the

anthrone method of Trevelyan & Harrison (1952). Total cell carbohydrate hexose was measured by pipetting suitable aqueous suspensions of cells into the ice-cold anthrone reagent. The alkali-stable polyglucose (glycogen) of *E. coli* was extracted, washed and precipitated precisely as described by Palmstierna (1956). Purification of extracted glycogen was also carried out by the procedure of Cowgill & Pardee (1957). Reducing sugar was determined by the method of Nelson (1944).

Ribose. Free and purine-bound ribose was estimated by the orcinol method (Mejbaum, 1939) on cell-free supernatants, cell suspensions or the alkali-soluble nucleotides.

Amino acids and ammonia. Amino acids were estimated by the method of Yemm & Cocking (1955). Glycine and $(\text{NH}_4)_2\text{SO}_4$ were used as standards and values were corrected for the presence of ammonia, which was estimated independently by nesslerization (Paul, 1958). Ammonia was also determined by the more sensitive chloroindophenol method of Bolleter, Bushman & Tidwell (1961).

Peptides. Peptides were estimated by the method described by Dawes & Holms (1958a).

Free amino acid pools. Cell suspensions were immersed in boiling water for 10 min. and then centrifuged to obtain the clear supernatants containing free amino acids.

Protein. Protein was estimated by the methods of Lowry, Rosebrough, Farr & Randall (1951) and Stickland (1951), with bovine serum albumin, fraction V (Sigma Chemical

Fig. 1. Extraction and hydrolysis of RNA from *E. coli* by the procedure of Fleck & Munro (1962). Portions (2 ml.) of a suspension of *E. coli* (6 mg./ml.) were treated with 1 ml. of 2.1 N-perchloric acid at 0° for 10 min. The precipitates were washed twice with 0.7 N-perchloric acid at 0°. The extracted residues were then digested at 37° in 0.3 N-KOH (3 ml.) in a water bath for periods as indicated. At the end of each incubation the samples were neutralized and made 1 N with respect to perchloric acid, at 0°. The precipitate was washed twice with cold 0.5 N-perchloric acid and the supernatants were made up to 25 or 50 ml. as desired in 0.5 N-perchloric acid. The extinctions of the solutions were read at 260 m μ and their ribose contents estimated. Results are calculated as described in the Experimental section for ribose (Δ) and E_{260} (○).

Co., St. Louis, Mo., U.S.A.), as standard. These methods gave identical results with cells; the biuret method was not tested with supernatants.

RNA. RNA was extracted from the bacteria as ribonucleotides by alkaline hydrolysis (0.3N-KOH for 60 min. at 37°) of perchloric acid-washed cells. The procedure was that described by Fleck & Munro (1962); essentially all the RNA was hydrolysed within 60 min. at 37° (Fig. 1). The alkali-soluble material and washings of the residue were made up to 50 ml. in N-perchloric acid, and the ultraviolet-absorption spectra and the ribose content of these solutions determined. The values obtained were related to RNA content by using the base composition of *E. coli* reported by Roberts, Abelson, Cowie, Bolton & Britten (1957).

Spectra and ultraviolet measurements were carried out with either a Cary or a Beckman DB recording spectrophotometer.

Ethanol. Ethanol was determined on neutral steam-volatile distillates of suspending fluids by dichromate oxidation, followed by estimation of the residual dichromate with KI and Na₂S₂O₃ (Neish, 1952, 1957).

Steam-volatile acids. These were separated by steam-distillation in a Markham still after adjusting to pH 1 by the addition of H₂SO₄. Six volumes of distillate were collected and titrated with 0.02N-NaOH in a stream of CO₂-free air, with phenol red as indicator.

Manometric methods

The manometric methods used were as described by Ribbons & Dawes (1963). Anaerobic-starvation experiments were sometimes performed without KOH in the centre well of the flasks; as both hydrogen and carbon dioxide are evolved under these conditions by cells that contain glycogen, the endogenous fermentation rate is expressed as a $Q_{(\text{CO}_2 + \text{H}_2)}^{\text{N}}$ value.

Protein-turnover experiments

To obtain cells that have their proteins uniformly labelled with ¹⁴C but their glycogen unlabelled, medium of the following composition was prepared (g./l.): glucose, 8.0; MgSO₄·7H₂O, 0.4; casein hydrolysate (Bacto casamino acids; Difco Laboratories, Detroit, Mich., U.S.A.), 0.01; KH₂PO₄, 5.4; (NH₄)₂SO₄, 0.8; pH adjusted to 7.1 with 5N-NaOH. The glucose plus MgSO₄·7H₂O was sterilized separately and added aseptically when cool. Sterile ¹⁴C-labelled algal-protein hydrolysate was then added (10 μC, 77 μg.). The medium was prepared in 1 l. boiling flasks, filled to the neck to secure semi-anaerobic conditions, and each flask given a light inoculum (0.1 ml.) of a stationary-phase glucose-ammonium salts culture. Nitrogen exhaustion limits growth in this medium, and after growth had ceased the cells were harvested, washed three times on the centrifuge, initially with 0.67M-phosphate buffer, pH 7.1, finally with distilled water, and then used for starvation experiments. The resulting cell suspension (approx. 6 mg./ml.) was distributed in portions (2.6 ml.) in a series of Warburg flasks that contained 0.2 ml. of 5N-NaOH in the centre well and 0.2 ml. of 2N-H₂SO₄ in the side bulb. After temperature equilibration, acid was tipped from the side arms of two flasks (zero-time samples) and these were then used for analysis. Oxygen consumption was followed with the remaining flasks and at suitable intervals further

pairs of flasks were removed after acid had been tipped from the side bulbs.

Alkali and filter papers were transferred quantitatively from the centre wells to 10 ml. measuring cylinders and the solution was made up to a known volume with CO₂-free water. Cell suspensions were transferred to sample bottles before subsequent analysis. The total carbohydrate content and radioactivity of the cells and the ammonia content of the suspending fluid were determined. The radioactivity of the respiratory CO₂ trapped in the alkali was measured by precipitation of replicate portions as BaCO₃, by using the methods described by Sakami (1955). The radioactivity of the cells was determined by spreading portions (0.1 and 0.2 ml.) of suitably diluted suspensions on stainless-steel planchets having an effective raised area of 1.5 cm². (Dawes & Holms, 1958b).

To confirm that the glycogen of cells grown under these conditions was not significantly labelled, glycogen was isolated from the cells, purified by the technique described by Cowgill & Pardee (1957) and its radioactivity determined. Less than 1% of the total radioactivity of the cells was present in the glycogen.

Isotopic counting procedures. All estimations were made at or corrected to infinite thinness by using an end-window Geiger-Müller tube and an Ekco automatic scaler type N 530G. Counts were collected for a period sufficient to ensure that the counting error was less than ± 2%. Corrections were applied for background and coincidence.

Cell fractionation

To ascertain the fate of the lipid content of *E. coli* during starvation, cells were uniformly labelled by two sub-cultures and by growth in [U-¹⁴C]glucose medium of the same composition [standard glucose-ammonium salts medium containing [U-¹⁴C]glucose (50 μC, 8.0 g. of glucose/l)] and then, after being harvested and washed, subjected to the fractionation procedures described by Roberts *et al.* (1957). Usually about 10 mg. wet wt. of cells was taken for analysis. Variation of the lipid extraction solvents was tried in different experiments (Table 6).

Starvation procedure

Cells were starved either by aerating with water-saturated sterile air or by shaking washed suspensions of the organism at 37° as described by Ribbons & Dawes (1963). In some experiments, e.g. those designed to measure possible protein turnover, the starvation was carried out in Warburg flasks.

Viability estimations

To determine the survival characteristics of *E. coli* under the conditions of starvation used, a washed suspension was prepared, with precautions to prevent contamination, from cells freshly harvested from the standard glucose-ammonium salts medium. Portions (10 ml.) of the suspension were distributed in a series of sterile 50 ml. conical flasks. For anaerobic-starvation experiments the flasks were flushed with nitrogen and sealed, whereas for aerobic experiments the flasks were sealed retaining an atmosphere of air. The flasks were incubated at 37° on Gallenkamp reciprocating water baths (shaking rate 60 oscillations/min.; stroke

4 cm.) and at appropriate times pairs of flasks were removed. Samples (1 ml.) were removed with aseptic precautions and subjected to serial dilution with 1 ml. transfers to 100 ml. volumetric flasks, containing sterile phosphate buffer (0.67 M-KH₂PO₄ adjusted to pH 7.1 with 5N-NaOH), to secure a final population of approx. 10² cells/ml. Portions (1 ml.) of the final dilution were added to replicate test tubes containing 9 ml. of sterile nutrient-agar, which were held at 45° to keep the agar molten, mixed thoroughly and poured into sterile Petri dishes. Colonies were counted after incubation for 24 and 36 hr.

The remainder of the cells from each incubation flask was used for chemical analyses.

RESULTS

Exponential-phase cells. Aeration of washed suspensions of aerobically grown *E. coli* that have been harvested during the exponential phase of growth causes a rapid decrease in the carbohydrate content and Q_{O_2} (endogenous) value of the cells (Fig. 2). After approx. 60 min. there is no further

utilization of carbohydrate and the Q_{O_2} levels out at a small but finite value, suggesting that material other than carbohydrate is now being oxidized. Ammonia is released from the cells, but only after a short lag period, which corresponds to the time during which carbohydrate is being metabolized. Although this lag period is characterized by only two experimental points in Fig. 2, it has been thoroughly documented elsewhere (Tables 1, 2 and 4, and Ribbons & Dawes, 1963).

The free amino acid pool of the cells was examined to determine whether it was the source of the ammonia (Fig. 3). There is little ammonia in the hot-water-extractable pool and it does not increase during starvation. The concentration of the ninhydrin-positive substances is undiminished and, in fact, increases slightly, apparently to attain a threshold value, after which amino acids are released into the suspending fluid, together with the ammonia. Individual amino acids were not determined. Similar results were obtained with exponential-phase cells that had been grown without aeration.

Extraction and determination of cellular glycogen revealed that this was the rapidly utilized carbohydrate, and that when it was exhausted the

Fig. 2. Carbohydrate content, endogenous respiration rate and ammonia release during starvation of a washed suspension of aerobically grown *E. coli* harvested during exponential growth. A cell suspension (100 ml.; 13.6 mg./ml.) in 0.67 M-phosphate buffer, pH 6.8, was aerated with water-saturated air at 37°, and at intervals samples (10 ml.) were withdrawn into 25 ml. of ice-cold water and centrifuged. At the same time samples (5 ml.) were taken and used immediately for Warburg determinations. The cell pellets were resuspended in ice-cold water and samples taken for cell density measurements and chemical analysis. ●, Total cellular carbohydrate; ○, Q_{O_2} (endogenous); Δ, ammonia released from cells.

Fig. 3. Ammonia and amino acid release during starvation of a washed suspension of aerobically grown *E. coli* harvested during exponential growth. Details were as given for Fig. 2. ▲, Ammonia in hot-water-extractable pool; Δ, ammonia released from cells; □, total amino acids in pool; ■, total amino acids released from cells.

carbohydrate content of the cells remained constant, usually at about 3% of the dry weight.

Stationary-phase cells. One of the factors that determines the nature of the endogenous metabolism is the phase of growth from which the cells are harvested (Ribbons & Dawes, 1963). The observed differences between exponential-phase and stationary-phase cells are quantitative ones. Stationary-phase cells respire endogenously at higher rates and contain large reserves of glycogen, which is the initial substrate oxidized. Starvation experiments with stationary-phase cells have generally been more extensive in both the period of starvation and the analyses of the cells and their products. Table 1 records the changes that occur during the aerobic starvation of anaerobically grown cells. The fall in dry weight, Q_{O_2} and $Q_{(CO_2+H_2)}$ values is paralleled by the loss of carbohydrate, which occurs mainly from the glycogen fraction of the cells. The glycogen is apparently oxidized since it does not appear as carbohydrate in the suspending fluid; the concentration of the non-reducing extracellular carbohydrate in the supernatant remained unchanged. Release of ammonia and amino acids occurs only after at least 90% of the glycogen has been utilized. The lower respiratory quotient observed at this stage could not be confirmed in other experiments.

Anaerobic starvation. Washed suspensions of cells grown anaerobically under an atmosphere of nitrogen were starved under nitrogen at 37° in a series of 50 ml. conical flasks. The cells and suspending fluid were analysed (Table 2).

The glycogen fraction is again metabolized very rapidly; neutral and acidic volatile products are formed and released to the suspending fluid. The yield of these fermentation products (approx. 7.5 μ equiv. of ethanol/ml. and approx. 7.1 μ equiv. of steam-volatile acid/ml. from 7.7 μ equiv. of hexose residues in glycogen) indicates that the endogenous fermentation involves only a thio-elastic cleavage of pyruvate. (These cells have an active formate-hydrogenlyase system and acetate is the principal steam-volatile acid.) The small amount of extracellular carbohydrate again undergoes no change.

The endogenous Q_{O_2} values of the anaerobically starved cells does not fall at an appreciable rate (cf. aerobic starvation in Table 1), which supports our previous conclusion (Ribbons & Dawes, 1963) that this 'residual' oxygen consumption is due to the unfermented glycogen fraction. In this experiment the cells had been washed free of the products of endogenous fermentation before oxygen consumption was measured.

Substrates of endogenous metabolism. The experiments so far described do not indicate the source of the ammonia. However, cell materials other than

Table 1. *Changes in the chemical composition of E. coli and the suspending fluid during starvation of anaerobically grown cells under aerobic conditions*

Time (min.)	Bacterial dry wt. (mg./ml.)	Carbohydrate content (μ g./ml.)									
		Cellular			Supernatant						
		Total	Glycogen	Water-soluble material	Anthrone-positive material	Reducing sugar	Ammonia (μ equiv. of NH_3 /ml.)		Free amino acids (μ equiv. of $-NH_2$ /ml.)		Peptide (μ equiv. of $-NH_2$ /ml.)
							Cells	Supernatant	Cells	Supernatant	Cells
0	14.9	32.0	1755	107	355	6	0.05	0.46	1.25	0.05	0.012
30	14.1	34.5	1500	89	403	0	0.02	0.32	1.22	0.15	0.008
70	13.7	32.0	1020	79	405	3	0	0.50	1.12	0	0.011
140	13.1	13.3	171	72	409	1	0	0.47	1.18	0.45	0.013
210	13.7	6.9	39	78	402	1	0	1.07	1.36	0.67	0.014
320	13.4	4.2	0	76	399	2	0.14	2.42	1.53	1.19	0.014
365	13.5	—	0	79	401	0	0.10	3.18	1.40	1.42	0.016

Anaerobically grown stationary-phase cells from 101. of medium were washed twice and resuspended in phosphate buffer, pH 7.1 (final vol. 150 ml.), and aerated at 37° (approx. 800 ml. of water-saturated air/min.). At suitable intervals samples (10 ml.) were pipetted into ice-cold water (25 ml.) and immediately centrifuged; the cells were resuspended (vol. 10 ml.) and supernatants were kept at -14° until required for chemical analysis. Further samples (10 ml.) were taken for dry weight determination (3 ml.), Q_{O_2} determination (2 ml. duplicate), and respiratory quotient (R.Q.) determination (2 ml.). $Q_{(CO_2+H_2)}$ determination (1 ml.) and turbidity measurement (1 ml.).

Table 2. *Changes in the chemical composition of E. coli and the suspending fluid during starvation of anaerobically grown cells under anaerobic conditions*

Anaerobically grown stationary-phase cells were harvested from 201. of medium, and stored overnight as a paste in the Sharples rotor at 4°. The cells were washed twice with ice-cold freshly distilled water and suspended in freshly boiled and cooled phosphate buffer, pH 7.1 (final vol. 200 ml.). Portions (20 ml.) were dispensed into each of ten 100 ml. conical flasks and gassed with O₂-free N₂, stoppered and incubated at 37° in the metabolic shaking bath. Samples (19 ml.) were pipetted into ice-cold water (21 ml.) and centrifuged. The supernatants were stored at -14° and the cells immediately suspended with distilled water to give a final volume of 25 ml. Samples (1 ml.) of the resuspended cells were dispensed immediately into Warburg flasks for determination of Q_{O₂}.

Time (min.)	Bacterial dry wt. (mg./ml.)	Q _{O₂}	Carbohydrate content (μg./ml.)									
			Cellular			Ammonia (μequiv. of NH ₃ /ml.)		Free amino acids (μequiv. of -NH ₂ /ml.)		Supernatant		Steam-volatile acid content (μequiv./ml.)
			Total	Acid- soluble		Supernatant		Cells		Supernatant		
				Glycogen	soluble	Cells	Supernatant	Cells	Supernatant	Ethanol content (μequiv./ml.)	pH	
0	16.1	35.0	2455	1920	88	0.15	0.39	0.15	2.40	0.31	6.99	2.42
40	14.1	35.4	1515	1162	46	0.23	0.46	0.23	2.12	0.53	6.64	9.90
80	14.1	33.4	1240	890	52	0.18	0.40	0.18	2.07	0.47	6.44	9.40
120	14.4	30.0	1125	762	47	0.15	0.62	0.15	2.19	0.85	6.43	8.45
200	14.5	32.5	1070	689	52	0.23	1.01	0.23	2.10	0.84	6.41	7.10
325	14.5	29.6	1149	669	65	0.16	0.81	0.16	1.92	1.66	6.31	8.60
1410	14.1	23.0	855	358	71	0.14	1.47	0.14	1.94	2.70	5.98	9.93
1650	13.35	—	855	367	70	0.12	1.66	0.12	1.99	2.96	5.96	9.63

Table 3. *Oxygen consumption and carbohydrate utilization during aerobic starvation of anaerobically grown E. coli*

Each Warburg flask contained 2.6 ml. of a washed suspension (2.87 mg./ml.) of stationary-phase cells in 0.67 M-phosphate buffer, pH 7.1, and 2 N-H₂SO₄ (0.2 ml.) in the side arm. The centre well contained 0.2 ml. of 5 N-KOH. The carbohydrate content of each flask was 1367 μg. and the ribose content 370 μg. at zero time. Incubation was at 37° in an atmosphere of air. Zero time is the period when measurements of gaseous exchange commenced, and samples were taken for analysis after stopping the reactions with the 2 N-H₂SO₄.

Time (min.)	Carbohydrate utilized		(a) O ₂ consumed		(b) O ₂ required for hexose combustion (μmoles/flask)	(c) Difference (a-b)		(d) Ribose utilized (μmole/flask)	(e) O ₂ required for ribose combustion (μmoles/flask)	Difference (c-e)			
	(μg./flask)	(μmoles/flask)	(μl.)	(μmoles)		(μmoles)	(%)			(μmoles)	(μmoles)	(μmoles)	(%)
30	214	1.32	139	6.18	7.92	-1.74	-22.0	—	—	—	—		
60	338	2.08	281	12.53	12.48	0.05	0.4	—	—	—	—		
135	659	4.06	557	24.90	24.36	0.54	2.2	0.15	0.75	-0.21	0.2		
300	911	5.62	898	40.10	33.72	6.38	16.0	0.33	1.65	4.73	11.7		

Table 4. *Respiratory quotient, oxygen consumption and carbohydrate utilization during aerobic starvation of anaerobically grown E. coli*

Each Warburg flask contained 2.6 ml. of a washed suspension (25.8 mg./ml.) of stationary-phase cells in 0.67 M-phosphate buffer, pH 7.1. The centre well contained 0.2 ml. of 5 N-KOH or, for respiratory quotient (R.Q.) determinations, 0.2 ml. of water. The carbohydrate content of each flask was 930 µg. and the ribose content 230 µg. at zero time. Incubation was at 37° in an atmosphere of air. Ammonia could not be detected in these flasks after 6 hr. starvation. Some of the original suspension was starved on the metabolic shaker bath for comparison; this revealed that carbohydrate utilization was faster and more complete than in the Warburg apparatus. However, even in this case, an increase in ammonia concentration could not be detected after 6 hr.

Time (min.)	R.Q.	Carbohydrate utilized		(a) O ₂ consumed (µl.)	(b) O ₂ required for hexose combustion (µmoles/flask)	(c) Difference (a-b)		(d) Ribose utilized (µmole)	(e) O ₂ required for ribose combustion (µmoles)	Difference (c-e)	
		(µg./flask)	(µmoles/flask)			(µmoles)	(%)			(µmoles)	(%)
60	1.01	330	2.04	255	12.19	-0.81	-6.6	0.146	0.73	-1.54	-13.5
120	1.03	481	2.97	423	18.89	1.09	6.1	0.406	2.03	-0.94	-5.0
180	1.01	526	3.25	520	23.20	3.70	19.0	0.390	1.85	1.85	8.0
270	1.01	555	3.42	597	26.60	6.10	29.7	0.593	2.87	3.23	12.1
377	1.01	569	3.51	633	28.25	7.15	33.9	0.593	2.87	4.28	15.1

glycogen are degraded and oxidized, since amino acids and ultraviolet-absorbing materials are released from the cells and, after the initial 60 min. of starvation, the consumption of oxygen is in excess of that required for the complete combustion of glycogen alone. Tables 3 and 4 record the total oxygen consumption during starvation, and the extent to which it is in excess of the requirement for complete oxidation of glycogen. Some of this excess can be accounted for by the oxidation of ribose (RNA, nucleotide, nucleoside and free). The other products of RNA degradation were not identified, although ultraviolet-absorbing materials are released into the suspending buffer (Fig. 4), and this occurs with cells starved under both aerobic and anaerobic conditions.

Experiments were undertaken to demonstrate the endogenous utilization of RNA with the results shown in Table 5. Ribose is lost from the cells immediately they are starved. Further, after its release from the RNA, ribose is either oxidized or fermented, according to the conditions of aerobiosis.

We could not demonstrate any significant utilization of fat reserves during starvation of glucose-grown *E. coli*. Lipid utilization had seemed unlikely in view of the report of Dagley & Johnson (1953) that large amounts of fat are deposited during growth on acetate as the sole carbon and energy source but not when glucose serves in this

Fig. 4. Release of ultraviolet-absorbing material during aerobic and anaerobic starvation of *E. coli*. A washed suspension (9.5 mg./ml.) of anaerobically grown stationary-phase cells in 0.67 M-phosphate buffer, pH 7.1, was dispensed into 50 ml. conical flasks and sealed under air or N₂. Samples taken were diluted with an equal volume of ice-cold distilled water and the cells removed by centrifugation. The supernatants were diluted tenfold for determination of their E_{260} values. ○ Aerobic; △, anaerobic.

Table 5. *Endogenous utilization and fate of RNA*

Washed suspensions (9.5 mg./ml. in 0.67 M-potassium-sodium phosphate buffer, pH 7.1) of anaerobically grown stationary-phase cells were starved at 37° by shaking in air or under N₂. Samples (5 ml.) were taken as indicated, centrifuged and resuspended to 5 ml. with water. They were diluted appropriately for analysis of ribose (total) expressed as $\mu\text{g.}$ of orcinol-positive material/ml., and this was converted into RNA (column 4), whereas column 5 records $\mu\text{g.}$ of RNA/ml. as determined by the Fleck & Munro (1962) procedure. Supernatants were analysed for ribose and ultraviolet-absorbing materials.

Expt. no.	Time (hr.)	Ribose (total) content of cells		RNA content of cells ($\mu\text{g.}/\text{ml.}$)	Ribose in supernatant fluids ($\mu\text{g.}/\text{ml.}$)	Release of <i>E</i> ₂₆₀ materials (<i>E</i> ₂₆₀ /ml.)*
		As ribose ($\mu\text{g.}/\text{ml.}$)	As RNA ($\mu\text{g.}/\text{ml.}$)			
1						
(Air)	0	283	1060	800	15.0	—
	3	218	825	—	18.5	0.45
	6	215	810	650	16.0	0.52
	10.5	219	820	720	13.9	0.61
	19.5	204	765	625	11.3	0.83
	27	162	610	465	7.3	1.31
2						
(Nitrogen)	0	290	1080	800	15.1	—
	3	243	915	645	20.0	0.41
	6	226	845	630	16.5	0.45
	10.5	217	805	655	13.0	0.49
	19.5	175	660	500	10.0	0.73
	27	133	495	375	9.2	0.99

* Readings for tenfold dilution of supernatants.

Table 6. *Stability of the lipid fractions of E. coli during starvation under aerobic conditions*

Cells grown anaerobically in a medium containing [U-¹⁴C]glucose (see the Experimental section) were harvested and washed three times with ice-cold distilled water, and a suspension (4.15 mg./ml.) in 0.67 M-phosphate buffer, pH 7.1, was starved at 37° by shaking in air. Samples were fractionated by the procedures of Roberts *et al.* (1957) after various periods of starvation. Results of Expt. 1 are expressed as total counts/min./ml. of the starving suspension. Expts. 2 and 3 record different fractionations of the same starving suspension and are expressed as a percentage of the total counts/min./ml. of the cell suspension at zero time; the recorded counts at zero time were 165 200 counts/min./ml. of cell suspension. The cell density at zero time was 6.5 mg./ml.

Total radioactivity of starving cell suspension (counts/min./ml.)				
Expt. no.	Starvation period (min.)...			
		0	140	290
1	Intact cells	53 600	53 600	44 800
	Cold-trichloroacetic acid fraction	2120	2000	2120
	75% Ethanol fraction	17 760	18 200	18 000
	Hot-trichloroacetic acid fraction	9 520	9 240	6160
				5 840
Percentage of total radioactivity in cell fractions				
	Starvation period (min.)...			
		0	120	340
2				1350
	75% Ethanol fraction	20.7	21.5	19.5
	Ethanol-ether fraction	1.9	1.7	2.4
	Hot-trichloroacetic acid fraction	25.8	24.5	23.8
3				
	Cold-trichloroacetic acid fraction	4.5		4.7
	Chloroform-methanol fraction	39.9		39.9
	75% Ethanol fraction	2.0		—
	Hot-trichloroacetic acid fraction	15.4		11.7
	Residue	31.4		25.3

capacity. Our experiments with cells uniformly labelled by growth on $[U-^{14}C]$ glucose as the sole source of carbon and of energy are reported in Table 6. The relative constancy of the radioactivity in the fractions extracted by the different lipid solvents employed illustrates the general stability of the lipid fraction of these cells during starvation.

Protein turnover and degradation. The results shown in Figs. 2 and 3 and Tables 1 and 2, as well as those obtained previously with cells grown in complex media (Ribbons & Dawes, 1963), indicate that net degradation of protein does not occur until virtually all of the glycogen in the cells has been utilized. Cells grown on tryptone, which do not contain glycogen, degrade nitrogenous materials with release of ammonia immediately they are starved (Ribbons & Dawes, 1963). The question posed is whether the presence of glycogen in the cells completely suppresses the metabolism of protein or whether turnover occurs during the period of glycogen utilization. To investigate this problem cells were grown anaerobically in an ammonium salt medium containing an excess of glucose as the sole carbon and energy source, with ^{14}C -labelled protein hydrolysate and carrier amino acids added; nitrogen exhaustion limited growth in this medium (see the Experimental section).

Under these conditions it was found, as expected, that the cells contained a substantial amount of glycogen that was essentially unlabelled, whereas the proteins of the cell were heavily labelled. After

Fig. 5. Release, during starvation at 37° , of $^{14}CO_2$ from ^{14}C -labelled protein of *E. coli* containing unlabelled glycogen. Details are given in the Experimental section. Each Warburg flask contained 17.9 mg. dry wt. of cells (total radioactivity 468 000 counts/ksec.). $\square$, Total carbohydrate content of cells; $\circ$, $^{14}CO_2$ released.

being harvested and washed, cells were starved in Warburg flasks as described in the Experimental section. Radioactivity appeared in the respiratory carbon dioxide at a linear rate immediately the cells were starved and throughout the period that glycogen was present in the cells (Fig. 5). It is concluded that glycogen does not suppress the turnover of protein during starvation.

It was observed throughout that when starvation experiments were conducted in Warburg flasks the rate of glycogen utilization was generally rather less than that in corresponding experiments with conical flasks in a water-bath shaker.

Survival of *E. coli* during starvation. The survival pattern of cells starved under the conditions used in the present work was investigated (Fig. 6). No significant death of cells occurs during the initial 12 hr. period under either aerobic or anaerobic conditions; but thereafter the cells die rather more rapidly under anaerobiosis, e.g. at 24 hr. there were 76 and 58% survivors under aerobic and anaerobic

Fig. 6. Survival of *E. coli* starved under aerobic and anaerobic conditions at 37° . The cells were grown anaerobically and harvested 1–2 hr. after cessation of growth; the initial dry wt. of starving suspension was 9.5 mg./ml. Details are given in the Experimental section. $\bullet$, Aerobic starvation: total cell counts at 0, 27 and 72 hr. were 3.8×10^{10} , 3.77×10^{10} and 3.68×10^{10} cells/ml. respectively. $\circ$, Anaerobic starvation: total cell counts at 0, 27 and 72 hr. were 3.78×10^{10} , 3.59×10^{10} and 3.83×10^{10} cells/ml. respectively.

Table 7. *Changes in various cellular constituents and the suspending medium during long-term starvation*

Time (hr.)	Cells										Supernatants			
	Turbidity (Spekter reading)		Carbohydrate content (μ g./ml.)		RNA content (μ g./ml.)		Protein content (mg./ml.)		pH		NH ₃ release (μ equiv./ml.)		Protein release (μ g./ml.)	
	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
0	0.290	0.282	697	671	741	—	5.68	5.83	7.14	7.14	0.22	0.25	76	21
3	0.253	0.268	244	377	—	535	5.79	5.80	7.12	6.95	0.51	0.56	260	211
6	0.253	0.255	238	361	511	456	5.64	5.57	6.83	6.89	0.81	0.76	274	300
10.5	0.245	0.252	259	327	589	514	5.72	5.61	6.98	6.87	1.36	1.00	392	340
19.5	0.210	0.210	246	306	501	513	5.77	5.86	7.32	6.74	1.96	2.13	457	452
27	0.195	0.195	224	265	345	383	5.61	5.42	7.35	6.74	3.06	3.23	645	570
44	0.177	0.167	217	206	127	269	5.77	5.45	7.36	6.68	5.46	5.39	831	773
59.5	0.176	0.167	194	207	127	195	—	5.41	7.37	6.74	6.17	7.59	918	455
72.5	0.153	0.144	216	173	102	165	5.77	5.19	7.42	6.69	6.80	11.60	1090	420

Results were obtained from the survival experiment depicted in Fig. 4. The initial washed suspension in phosphate buffer, pH 7.1, contained 9.5 mg. dry wt. of cells/ml. (about 4×10^{10} cells/ml.). Portions (20 ml.) were distributed in 50 ml. sterile conical flasks for starvation under air or N₂. At each sampling time a flask was taken from the shaking bath, 1 ml. was removed aseptically for viability and turbidity determinations, and 9 ml. was centrifuged, the pellet being resuspended (vol. 10 ml.); the supernatant and resuspended cells were stored at -14°. The remainder of the flask contents was stored at -14° until analysed for RNA and total ribose. The carbohydrate and protein contents of the cells were determined on the resuspended fractions.

conditions respectively. Some other parameters of these experiments are shown in Table 7. These cells, although harvested from anaerobic cultures in the stationary phase, actually contained much less carbohydrate (7.35%) than was expected. The lower pH values of the supernatants from the anaerobic flasks may account for the differences in viability that are observed. The protein content of the cells was also measured, but no clear-cut demonstration of protein degradation in the aerobic series was possible, even though more protein, as revealed by the method of determination (Lowry *et al.* 1951) used, was released from the cells under aerobic conditions. Certainly some protein is degraded under anaerobic conditions. There is a fall in turbidity of the cell suspensions during starvation, a phenomenon that we previously correlated with the decrease in dry weight primarily associated with the loss of glycogen from the cells (Ribbons & Dawes, 1963).

DISCUSSION

The existence of an endogenous metabolism in micro-organisms need not necessarily imply the existence of specialized reserves of either carbon or energy within the cell and, in the absence of nutrients, general cellular materials may be degraded. For example, *Pseudomonas aeruginosa*, an organism that when grown on glucose-ammonium salts media does not possess reserves of either carbohydrate or lipid, degrades RNA and protein when it is deprived of nutrients (Campbell, Gronlund & Duncan, 1963; Gronlund & Campbell, 1961, 1963). In organisms that do possess reserves, e.g. of carbohydrate, lipid (including poly- β -hydroxybutyrate) and possibly polyphosphate, the problem is raised of the relative importance of the reserves and of the cellular protein and RNA to endogenous metabolism. Does the possession of a reserve material exert a sparing action on the degradation of such 'basal' cell constituents as protein and RNA?

We have previously shown that *E. coli* grown on a glucose-ammonium salts medium always contains glycogen, the precise amount being a function both of the phase of growth from which the cells are harvested and of the conditions of aerobiosis employed (Ribbons & Dawes, 1963). On subsequent starvation this glycogen is rapidly metabolized and, under the conditions employed in our experiments, is usually completely utilized in approx. 60 min. and the oxygen consumed corresponds closely to that required for the complete oxidation of the glycogen. During the period of glycogen utilization the cells contain little ammonia and none is released from the cells; simultaneously with the disappearance of the glycogen ammonia release commences.

Clearly glycogen is a preferentially utilized reserve and its possession by the cells prevents the net degradation of nitrogenous materials. The experiment with cells containing labelled protein reveals, however, that the mechanism is not one of complete suppression of protein metabolism since turnover is occurring, even during the period when the cells contain glycogen. The glycogen apparently serves as a source of both energy and of carbon to permit reincorporation of the ammonia released by protein breakdown. This situation may be compared with that observed by Duncan & Campbell (1962) with *P. aeruginosa*, which releases ammonia immediately it is starved. The ammonia is reincorporated, however, if exogenous glucose is added to the cell suspension, and thus the endogenous glycogen of *E. coli* appears to fulfil a role similar to that of exogenous glucose in *P. aeruginosa*. The provision of an exogenous source of glucose for washed suspensions of *E. coli* further increases the lag period during which ammonia is not released (Ribbons & Dawes, 1963). Tryptone-grown cells of *E. coli*, which are always devoid of glycogen, release ammonia immediately they are starved (Ribbons & Dawes, 1963), thus emphasizing the role of glycogen in preventing the net degradation of nitrogenous materials under conditions of complete starvation. Holme & Palmstierna (1956b) had previously demonstrated that glycogen could serve as a source of carbon for the synthesis of nitrogenous materials when *E. coli* was incubated with a nitrogen source in the absence of a carbon source.

The rate of release of $^{14}\text{CO}_2$ from labelled cellular protein during the utilization of endogenous glycogen corresponded to approx. 0.6% of the initial cellular radioactivity/hr. This is considerably less than the 4–5% rate of protein turnover reported by Mandelstam (1958) and Mandelstam & Halvorson (1960) for *E. coli* mutants starved of one essential amino acid in an otherwise adequate medium, or in the presence of an exogenous carbon and energy source. The difference may reflect a greater capacity for turnover in a medium that provides everything save a single nutrient, or furnishes an excess of a carbon and energy source, as opposed to starvation in a simple salt solution.

Cellular lipid was not significantly utilized by glucose-grown cells, but RNA degradation, as measured by ribose utilization, commenced immediately the organism was starved. It is known that the nucleic acids of exponentially growing *E. coli* are stable, whereas in non-growing cells, irrespective of the factor causing cessation of growth, only DNA is stable and RNA undergoes appreciable degradation (e.g., see Mandelstam, 1960). With *Aerobacter aerogenes* the RNA of cells harvested from a glucose-tryptone medium

is much more stable than that of cells from defined or tryptic meat broth media (Strange, Dark & Ness, 1961). The importance of Mg^{2+} as a stabilizer of ribonucleoprotein has been established (Bowen, Dagley & Sykes, 1959), and Wade (1961) has demonstrated two routes of RNA breakdown, one of which is dependent on Mg^{2+} . We have not been concerned with the fate of the nucleosides produced and released in RNA degradation, but net deamination clearly does not occur during the period of glycogen utilization.

A comparison of our observations with *E. coli* with the extensive findings of the Porton workers (Strange *et al.* 1961; Strange, Wade & Ness, 1963; Postgate & Hunter, 1962, 1963) on the starvation and survival of *A. aerogenes* is pertinent. They showed that washed suspensions of cells harvested from carbon-limiting mannitol-ammonium salts medium contained only small amounts of carbohydrate (5–8%) and degraded their protein and RNA with little change of carbohydrate content, whereas cells from a complex tryptone-glucose medium had a high carbohydrate content (15–20%) and glycogen was depleted during the initial 25 hr. of starvation with a small change in protein and no change in RNA content. The rate of loss of glycogen at 37° was much less than we have observed with *E. coli*; indeed, one of the striking features of our findings has been the rapidity with which cellular glycogen is metabolized by starving cells.

Strange *et al.* (1961) concluded that the presence of glycogen in the cell favours survival of *A. aerogenes*, and showed that when it is not available protein and RNA may yield readily metabolized sources of carbon as amino acids and ribose, the nitrogen appearing as ammonia and free bases, although ammonia had not been detected by Gronlund & Campbell (1961). On the other hand, the addition of exogenous glucose to the suspensions greatly accelerated death. In contrast with *E. coli* the endogenous amino acid concentration of *A. aerogenes* decreased during starvation (Strange *et al.* 1963); our measurements, however, were made over much shorter periods (see, e.g., Fig. 3). The degradation of ribosomal RNA did not increase the RNA in the suspending fluid, but degradation products, mainly deaminated bases, were released.

Comparison with the observations of Strange *et al.* (1961) suggests that *A. aerogenes* harvested from a defined medium survives better than does *E. coli* under aerobic conditions at 37°. After 12 hr. starvation without significant loss of viability, *E. coli* dies more rapidly under anaerobic than aerobic conditions, probably because of accumulation of the products of glycogen fermentation.

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