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ENDOGENOUS RESPIRATION OF PSEUDOMONAS AERUGINOSA DURING PERIODS OF PROLONGED STARVATION

ABSTRACT

During the investigation of the effect of age upon endogenous metabolism, advanced stationary phase cultures of Pseudomonas aeruginosa were found to be susceptible to cold-shock. The phenomenon was apparent through an increased oxygen uptake and an initial absence of extracellular ammonia during subsequent respiration at 30 C, which were shown to be due to the presence of an oxidizable substrate in the suspending fluid. Intracellular enzymes were released following the exposure of these cells to the cold, and a partial protection against damage was afforded by the addition of magnesium ions to the washing and suspending buffer.

The storage of a reserve material for utilization during endogenous metabolism could not be demonstrated in cells grown for various periods of time in a chemically-defined medium which contained glucose in excess of that required for growth. Further, when not previously exposed to the cold, an immediate evolution of ammonia was observed when this organism was respired at 30 C irrespective of the medium in which it was cultured or the age at which it was harvested.

The ribosome complement was seen to diminish during the prolonged incubation of cultures grown in the chemically-defined media, and was found to disappear almost completely when 48 hr cells, harvested from defined or complete media, were respired at 30 C for a further 48 hr. Chemical fractionation during the respiration period revealed an increase in the concentration of deoxyribonucleic acid and a decrease in the concentrations of ribonucleic acid and protein. Glucosamine was not found to be a major metabolite in the endogenous respiration of this organism.

Progressive starvation resulted in a reduction in the constituitive enzymes and/or cofactors involved in the oxidation of glucose, and an ability

to adapt to, and oxidize α- ketoglutarate was evident after a period of starvation which had reduced the ribosome complement to negligible proportions. Endogenously produced ammonia and acid-soluble UV-absorbing material were reincorporated upon the addition of an exogenous substrate following respiration for 48 hr, and a concurrent increase was recorded in the concentration of 50S ribosomes.

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by

ROBIN M. MacKELVIE
B. Sc., University of British Columbia, 1962

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
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We accept this thesis as conforming to the
required standard

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INTRODUCTION

Previous studies on the endogenous metabolism of Pseudomonas aeruginosa have revealed the nitrogenous nature of the substrates which are utilized by this microorganism during starvation (Warren, Ells and Campbell, 1960; Gronlund and Campbell, 1963). However, the nutritional conditions under which the cells were cultured for the experiments which led to this conclusion, were unfavourable for the formation of reserve materials such as glycogen or poly-β-hydroxybutyrate. Wilkinson (1959) has proposed that certain conditions should be met before a substance may be defined as an energy storage compound, and one of these conditions requires that such a compound should accumulate in the cell in the presence of a carbon-source which is in excess of that which is required for growth.

In the studies to be presented, P. aeruginosa was grown in a chemically-defined nitrogen-limiting medium in an endeavour to demonstrate the formation of a specific storage product. The effect of the age of the cells on endogenous metabolism was also investigated, and a comparative survey of the endogenous activity of 48 hour cells, which had been grown in a chemically-defined nitrogen-limiting or carbon-limiting medium or in a complete medium, was made during a protracted period of starvation.

REVIEW OF THE LITERATURE

The term "endogenous metabolism" could theoretically be applied equally well to that metabolism which is carried on in the absence of a utilizable extracellular substrate within an isolated "average" cell or, in contrast, that which is carried on within a given population of cells. Through technical necessity, only the second of the two alternatives can be considered, but the investigator is then faced with the fact that his observations embrace not only the strictly intracellular activities of the independent cells but also the results of possible intercellular reaction. Thus, only under conditions where the effect of the latter factor is negligible can a true portrayal of the metabolism of the "recognised" endogenous substrates be made. Such a study has been the main concern of many workers, and their endeavours have contributed to the extensive literature which has been well reviewed in recent years (Dawes and Ribbons, 1962a; Dawes and Ribbons, 1964; Gronlund, 1964).

The endogenous substrates fall into two broad classifications; firstly the energy storage compounds described by Wilkinson (1959), and secondly various constituitive cytoplasmic components. The energy storage compounds, consisting of polysaccharides, lipids and polyphosphate are specifically laid down under conditions where the carbon and energy source is in excess of that required for growth.

The known endogenous substrates of a constituitive nature are nitrogenous and include ribonucleic acid (RNA), protein, and free amino acid and peptide pools. Warren, Ells and Campbell (1960) reported that ammonia was released to the suspending fluid when resting cells of Pseudomonas aeruginosa were allowed to respire endogenously, and this was later shown to be a general phenomenon when a variety of bacterial species was similarly examined (Gronlund and Campbell, 1961). Subsequently, Gronlund and Campbell (1963) demonstrated that this organism oxidised RNA and protein during starvation. They noted that a considerable amount of ultraviolet (UV) absorbing material was released to the supernatant fluid, and an examination of this material revealed the presence of nucleosides, nucleotides and possibly free bases.

Neither membrane material nor cell wall material has been shown to serve as a substrate for endogenous metabolism, and this presumed immunity is possibly attributable to their essential function in the maintenance of the integrity of the cell. Except for the report by Harrison (1963), deoxyribonucleic acid (DNA) has not been found to be utilized during starvation, and on the contrary has been shown to increase in Aerobacter aerogenes (Strange, Wade and Ness, 1963) and in P. aeruginosa (Gronlund and Campbell, 1963).

The aspect of intercellular reactions in starving populations has been less extensively studied. Harrison

(1960) has reported that the survival of starving log phase A. aerogenes is dependent upon the density of the cell suspension, and that there is a critical density for optimal survival. He attributed this phenomenon to the presence of an effluent from dead and moribund cells which sustained those cells which remained viable. It was suggested that although the concentration of the leakage products was probably too low to support cell division, it was nevertheless sufficient to permit the survivors to adapt to a stationary phase physiology. Thus, with a reduced permeability, their own potential effluent would be conserved. Eventually, when they were no longer able to exercise sufficient control of their permeability mechanisms through debility, their effluent would contribute to the survival of more fortunately placed individuals. The association between permeability, cell effluent and survival has been shown by workers who have investigated the phenomenon of cold-shock.

The relative effect, on the viability of young and mature cells, of exposure to cold was first demonstrated by Sherman and Albus (1923). Using 4 hour and 12 day cultures of Escherichia coli grown on 1% peptone, they observed that whereas the viable count of mature cells remained virtually unaltered in the plate count diluent of 1% peptone when held at 2 C over a 3 hour period, the viability of the younger cells, similarly treated, decreased rapidly. When subsequently investigating lethal environmental factors within the natural

range of growth, Sherman and Cameron (1934) showed that logarithmic-phase E. coli grown at 45 C suffered a 95% loss in viability in 60 minutes upon inoculation into identical medium at 10 C. More pertinent, however, was their observation that although sudden changes within the temperature limits of growth may be lethal to young cells, gradual changes virtually abolished the loss in viability.

Hegarty and Weeks (1940) undertook the study of the resistance of E. coli to cold-shock during the entire growth range. Samples from a broth culture at 37 C were removed at frequent intervals for simultaneous plate counts and determinations of sensitivity to cold-shock by the method of Sherman and Albus (1923). They reported that the sensitivity to cold-shock extended throughout the entire logarithmic phase of growth, with cyclical fluctuations in susceptibility attributed to partial synchronization of division. Mature cells, referring to those of the 24 hour inoculum present in the early lag phase of the growing culture, and those present after 8 hours when the culture was entering the stationary phase, were unaffected.

Meynell (1958), in an attempt to modify the effect of cold-shock on nutrient broth grown E. coli by the use of various diluents, discovered that susceptible organisms were completely unharmed by sudden chilling to 4 C in 0.3M sucrose, and verified the earlier observation that gradual chilling to the same temperature in a potentially lethal diluent, such as

Ringer's solution, afforded complete immunity. On the basis of these observations and the knowledge that sucrose protected E. coli protoplasts against plasmoptysis and lysis (Lederberg, 1956), he suggested that an adaptive mechanism preventing the entry of (excess) water into the cell became inoperative at 4 C. If this were the case, slow cooling would allow adaptation to the new potentially lethal environment before the mechanism was inactivated. Meynell detected no morphological differences in formolised samples of unchilled cultures and formolised samples of cultures in which a majority of organisms had been killed by their addition to an equal volume of saline at -5 C. These studies involved the use of phase-contrast, darkground and electron microscopy. The total count, determined by phase-contrast microscopy using a Helber chamber, was essentially unchanged. Meynell also showed that organisms multiplying exponentially in glucose-salts medium at 37 C, with a doubling time of 1 hour, were far more resistant to sudden chilling than cells growing exponentially in nutrient broth with a doubling time of 20 minutes.

The comparative susceptibility to cold-shock of two strains of Pseudomonas pyocyanea, two strains of E. coli, Salmonella typhimurium and Staphylococcus aureus was investigated by Gorril and McNeil (1960). In general, there was no difference between strains where more than one was employed. While studying the effect of temperature on killing, they noted that the sudden transfer of the pseudomonad, whether in

the lag, log or stationary phases, from its growth temperature of 37 C to distilled water at temperatures ranging from 18 C to 37 C had no adverse effect on viability. However, the previously reported sensitivity of log phase cells to cold-shock was confirmed with the use of the diluent at 4 C where the survival at the period of maximal sensitivity was 0.12%, and this phenomenon was shown to be even more pronounced at 0 C where the survival was found to be 0.03%. Unlike E. coli, the overnight cultures of P. pyocyanea used as inocula were reported to be sensitive to cold water, for, in contrast to 100% survival in E. coli, only 15% of the P. pyocyanea survived when samples, taken from the culture medium immediately after inoculation, were exposed to 4 C for 1 hour. In another experiment, the temperature of the diluting water was standardised at 0 C and the effect of duration of exposure to this temperature on P. pyocyanea in various stages of the growth curve was studied. This revealed survivals of 1.6%, 0.1%, and 0.01% for "immediate", 30 minute and 60 minute exposures respectively, when the cells were in their maximally sensitive condition.

A series of tests by Gorril and McNeil on P. pyocyanea, using various diluents under standard conditions, revealed an increasing shocking activity in broth, 10% sucrose, buffer, Ringer's solution, 0.85% NaCl and distilled water. A half dilution of any of the salt diluents gave a marked

increase in the sensitivity of the cells to cold-shock, and they showed that, in general, divalent cations appeared to be more effective than univalent cations in decreasing the lethal effect of cold physiological saline. In the event that the maximal sensitivity of the microorganism to cold-shock, which appeared 3-4 hours after the lag phase in Gorril and McNeil's experiments, should be coincidental, conditions were altered to prolong the log phase. The results verified that P. pyocyanea remained highly sensitive to cold-shock as long as the cells remained in the log phase. A lack of apparent change in morphology and total count due to cold-shocking confirmed the earlier finding of Meynell (1958) with E. coli.

The comparative susceptibility to cold-shock of the bacteria tested showed that P. pyocyanea was more sensitive than either E. coli or S. typhimurium, and that S. aureus was remarkably resistant to exposure to ice cold water, saline or buffer for a period of 1 hour.

The effect of chilling on A. aerogenes in aqueous suspension was investigated by Strange and Dark (1962). Exponential-phase cultures, in dilute suspension and previously grown in a chemically-defined carbon-limiting medium, proved to be in the growth phase most susceptible to cold-shock as measured by loss in viability. Bacteria plated immediately after chilling showed no significant loss, but the extent of the loss thereafter increased with the duration of the exposure to cold. The inoculum used in these experiments was either freshly harvested stationary phase organisms

or washed suspensions of stationary phase cells which had been held in buffered saline for 20 hours at 37 C with aeration. They noted that whereas freshly harvested stationary phase organisms were relatively resistant to chilling, those which had been starved for 20 hours were almost as susceptible as those at the maximally sensitive point in the log phase. During the first half of the lag phase following inoculation of the chemically-defined medium with starved stationary phase cells, the sensitivity decreased until it approximated that of freshly harvested stationary phase cells, at which level the sensitivity was maintained for the remainder of the lag phase.

In experiments designed to assess the effect of various diluents on the cold-shocking of A. aerogenes, Strange and Dark reported that the lethal effect was less in distilled water than in buffered saline or Tris(hydroxymethyl)-aminomethane (Tris) buffer. The percentage viability of a suspension after 45 minutes at 0 C in the latter buffer was reduced to 1% compared with a 96% viability when held at 20 C. The corresponding percentages with water as diluent were variously 75%, 56%, 85% and 94% at 0 C, and 98%, 99%, 86% and 96% at 20 C. Suspecting that contaminating heavy metals were contributing to the lethal effect in Tris buffer, ethylenediaminetetraacetic acid (EDTA) was added, but the results were unaffected. A rapid rewarming of a suspension of washed exponential phase organisms to 18 C immediately following a rapid cooling to 0 C had no effect on viability,

but similar treatment after an exposure to 0 C for periods of 5 minutes or more produced a progressive loss in viability, at a slower rate, however, than that observed with the control organisms held at 0 C.

Strange and Dark found that the population density of the suspensions was a factor influencing survival in cold shock. The viability of suspensions with an initial count of 6×10^7, 1.2×10^8, 1.2×10^9, 2.4×10^9 and 1.2×10^{10} cells per ml was reduced to 32%, 45%, 80%, 95% and 95% respectively after 1 hour at 0 C. From these data, they postulated the presence of a protective principle in the suspending fluid of the more concentrated suspensions. By adding filtrates of concentrated, cold-shocked suspensions to less concentrated suspensions which were then held at 0 C for 45 minutes, a protective effect was demonstrated. A filtrate from shocked exponential cells afforded protection such that 98% of the cells remained viable, and in the presence of a similar filtrate from stationary phase cells 64% remained viable, while only 24% survived in the buffered saline control. Strange and Dark then embarked upon an investigation into the nature of the protective principle. They found that its protective activity was not affected by heating for 15 minutes at 100 C, and showed that its major stabilizing effect was exerted during chilling, although the filtrate was able to elevate the bacterial count when added after the chilling period. Examination of the filtrates of concentrated suspensions held

at 0 C revealed the presence of ninhydrin-reacting substances and adenosine triphosphate (ATP); both were in a higher concentration than in the control suspensions at 20 C. The ninhydrin-positive material was shown to be composed mainly of free amino acids and peptides of relatively small molecular weight. Neither acid-insoluble protein nor RNA were detected in these filtrates, however filtrates from both chilled and unchilled suspensions contained material with a maximum absorption at a wavelength near 260 mμ. This material was apparently greater in concentration than could be accounted for by the amount of ATP known to be present by the firefly luminescence technique and it accumulated initially in the chilled filtrate to a greater extent than in the unchilled filtrate. After 30 minutes at 0 C, however, there was no further excretion of the UV-absorbing material by the chilled cells, whereas the unchilled cells continued a steady release which was ascribed to the products of endogenous RNA degradation at 20 C. Neither magnesium nor calcium ions could be detected in the protective filtrates. Strange and Dark asserted that this rapid leakage of intracellular material in cold-shocked bacteria supported the suggestion of Meynell (1958) that the sudden chilling action adversely affects permeability control mechanisms.

The identified constituents of the filtrate as well as various substances not shown to be present in the filtrate, were tested for their ability to protect exponential phase A. aerogenes against cold-shock. Adenosine triphosphate,

at a concentration similar to that found in the filtrates, was completely without a protective effect. The addition of the ten identified amino acids produced a slower rate in the loss of viability in cells held at 0 C over a period of 1 hour, and the supplementation of this mixture with seven other amino acids afforded no further protection. A five-fold dilution of the 17 amino acid mixture did not reduce the protective effect, although with greater dilution it was lost. In no case did the fortifying of the reaction mixture with amino acids confer protection comparable to that of the complete filtrate.

In view of the known ability of spermine to stabilize spheroplasts of Pasteurella tularensis and E. coli (Mager, 1959) Strange and Dark tested its ability to protect cells against cold-shock. At 10^{-3}M spermine the loss of viability was enhanced, and at 10^{-5}M spermine protection was afforded only in the initial stages of the 0 C holding period. As recorded for E. coli (Meynell, 1958), 0.3M sucrose had a considerable protective effect against the cold-shocking of A. aerogenes. Erythritol, at the same concentration, was without benefit, but this compound, unlike sucrose, was known to penetrate A. aerogenes, which suggested to the authors that the protective influence of sucrose was probably related to its osmotic activity. The influence of various divalent metals was assessed in the knowledge that magnesium ions conferred stability on isolated protoplast membranes (Weibull, 1956) and spheroplasts (Lederberg, 1956;

McQuillen, 1958). Magnesium ions, calcium ions or manganese ions conferred substantial protection during chilling in either buffered saline or Tris buffer.

Strange and Ness (1963) examined the lethal effect of cold-shock in exponential phase cells of three Gram-negative organisms, E. coli strain Jepp, A. aerogenes and Serratia marcescens and showed that there was a correlation between the loss of viability and the liberation of cellular constituents. This strain of E. coli, when suspended at a density of 10^8 organisms per ml in phosphate-saline buffer and cooled rapidly to 0 C for 1 hour, exhibited no loss in viability as a result of this treatment when grown either on a complete medium or mannitol-ammonia-salts. S. marcescens grown in the complete medium was almost completely resistant. A. aerogenes, however, while showing more resistance when grown on the complete medium, was highly sensitive when grown in the chemically-defined medium. The viabilities of similar suspensions of these organisms at 20 C remain unchanged during 1 hour. Suspended at 4-5 x 10^9 organisms per ml, A. aerogenes grown on both media showed a 98-99% viability after the cold treatment, as did E. coli grown in defined medium. At intervals during the experimental period, filtrates of these more concentrated suspensions incubated at 0 C, as well as controls incubated at 20 C, were examined for the presence of materials absorbing at 260 m , ATP and ninhydrin-reacting substances. Analysis showed that, despite no loss in viability, the A. aerogenes grown in defined medium excreted

almost double the amount of UV-absorbing material compared with that excreted by the control, whereas the shocked cells grown on a complete medium released material at a rate entirely comparable with that of their control. Leakage from E. coli at 0 C was apparently less than at 20 C. Only the filtrates of the shocked suspensions of A. aerogenes grown on either medium contained any ATP and this disappeared during the second half of the experimental period. A slight, but steady increase of ninhydrin-reacting material occurred in all the filtrates examined, with the exception of that derived from cold-shocked, mannitol-grown A. aerogenes. With these cells the initial rate was approximately fifteen times greater than the 20 C control although this rate diminished thereafter. Thus, the data gleaned on the leakage of intracellular material by cold-shocked suspensions correlated with the viability loss when these same organisms were shocked in more dilute suspension. It was also evident that, with the viability maintained, the leakage was not a result of death, although it may have contributed to it ultimately. Strange and Ness presumed from their evidence that in dilute suspensions the concentration of the previously postulated protective material was too low to be of benefit.

As it seemed reasonable on the existing evidence to suppose that cold-shock, in some fashion, deranged permeability control mechanisms, Strange and Postgate (1964) essayed to discover whether material towards which intact A. aerogenes was known to be impermeable, would, in fact, penetrate

cold-shocked cells. In preliminary experiments to determine the effect on death-rate of various substances when present in a suspension of exponential phase cells chilled at 0 C for 1 hour, they found that trypsin or lysozyme were protective, pepsin or deoxyribonuclease (DNase) had no significant effect, but that low concentrations of acid or alkali were more lethal at 0 C than at 20 C. Similarly, the presence of ribonuclease (RNase) or anilo-naphthalene-8-sulphonate (ANS) was found considerably to enhance the lethality of cold-shock. The latter compound forms a fluorescent complex with some intracellular protein of A. aerogenes, and had previously been used to determine damage to the osmotic barrier in this organism (Postgate and Hunter, 1962). Washed cells were held in the presence and the absence of ANS at 0 C and 20 C for 1 hour, and determinations of viability and fluorescence were made at intervals. A slight, but progressive increase of up to 10% in 1 hour was noted in the fluorescence of the suspension held at 0 C which contained ANS. This was regarded as evidence for the penetration of ANS, the relatively small partner in the complex, into the chilled cells.

The accentuating effect on lethality of RNase was most evident in logarithmic phase cells held at 0 C. Similar cells at 20 C were not immediately affected, and cold shock provoked no response from stationary phase cells either in the presence or absence of RNase. Sucrose conferred greater protection on the cells in the absence of RNase than in its presence, and both magnesium ions or the filtrate from a

chilled dense suspension considerably mitigated the action of RNase. During these experiments, an important observation was made with respect to the endogenous metabolism of RNA in cold-shocked cells, for it was found that the pre-shocking of a susceptible suspension elevated the rate at which RNA was metabolised when these organisms were subsequently aerated at 37 C. Further, an increase in endogenous RNA degradation at 37 C correlated with an increase in the duration of the chilling period. Thus, whereas the percentage of the initial content of RNA remaining at the end of 30 minutes aeration at 37 C was 98% with no prior chilling, the RNA content was found to be 64%, 43% and 28% at the end of the 37 C incubation period when the cells had been previously chilled for 30 minutes, 60 minutes and 120 minutes respectively. This marked accentuation of RNA degradation at 37 C following cold-shock masked any effect that Strange and Postgate might have hoped to see when RNase was present during the chilling period. However, if the incubation were carried out at 20 C or 25 C, the autodegradation of RNA was considerably less, and a consistently greater degradation was recorded for those suspensions which contained RNase during chilling. Exposure of similar cell suspensions to this enzyme at 20 C prior to incubation was without effect. The entry of RNase into chilled cells was further evidence for the hypothesis that cold-shock interfered with permeability control, but the fact that cold-shock also stimulated

endogenous RNA breakdown in this organism indicated it could have a more far-reaching effect.

The stabilizing effect of magnesium ions on ribosomes had been documented by McQuillen (1962), on spheroplasts by Lederberg (1956) and McQuillen (1958), and on isolated protoplast membranes by Weibull (1956). It was therefore of interest to investigate more fully the effect of added magnesium on chilled suspensions. Strange (1964) found that cells chilled for 2 hours at 0 C in the presence of 5mM magnesium sulphate, resuspended in fresh buffer lacking magnesium, and aerated at 37 C for 1 hour, lost 11% of their RNA in this latter period. In contrast, the RNA degraded in this period in suspensions chilled in the absence of magnesium was 40%. As an index of the effect of magnesium ions on permeability control, he elected to make use of the "optical effect" reported by Mager et al. (1956). This phenomenon, based on the observation that the optical density (OD) of suspensions of Gram-negative organisms changed with variations of the osmotic pressure of the suspending medium, was attributed to permeability control factors. The OD of suspensions of viable organisms in distilled water increased with increasing amounts of added electrolytes or nonelectrolytes until, near isotonicity, a maximum value was attained. The magnitude of this effect, called the optical effect (OE) was defined as a percentage ($OE = \frac{OD_S - OD_W}{OD_W} \times 100$, where OD_W represents the OD of the aqueous suspension, and OD_S the OD in the presence of solute)

and varied both with the solute and the organism.

Strange determined the optical effect of logarithmic-phase A. aerogenes in isotonic saline and recorded changes in this value when the cells were chilled. He found that small loss in optical effect resulted when stationary-phase cells were held either at 0 C or 20 C for 3 hours. Logarithmic phase cells held at 20 C for 3 hours likewise exhibited negligible loss, but the optical effect decreased to about 45% when these cells were exposed to the same duration of chilling. The rate of loss of optical effect exceeded the rate of loss in viability. Chilling these cells in 5mM magnesium sulphate afforded protection against loss of viability and optical effect, the latter being reduced to 80% at the end of the 3 hour chilling period. He concluded that this was further evidence for the hypothesis that the protection conferred by magnesium in cold-shock resulted from the stabilization of membranes which are concerned with permeability regulation.

From the literature, which was compiled by relatively few authors, the following salient features of cold-shock may be assembled. Sudden chilling and the subsequent duration of exposure to low temperatures is lethal in varying degrees to many bacteria. The degree of lethality is dependent upon the species of the microorganism, the age of the cells, the medium in which they were grown, the population density and the nature of the suspending fluid in which they were chilled. It is consistently reported that cells from

the exponential phase of growth are most susceptible, whereas those in the stationary phase are virtually insensitive. However, in most instances, observations on stationary phase cells were made within a few hours of cessation of growth, and the almost incidental allusion to the high level of sensitivity of very early lag phase A. aerogenes derived from an inoculum starved for 20 hours shows that the time elapsed since the onset of the stationary phase must be considered if cells from this phase are to be assessed for sensitivity to cold-shock. P. aeruginosa, at least the two strains tested by Gorril and McNeil (1960), is particularly sensitive to this treatment, and even early stationary phase cells responded to a minor degree by loss in viability.

Leakage products from dense, chilled suspensions were found to protect more dilute suspensions of the same cells from the effects of sudden chilling, as did a variety of other substances including magnesium ion. The traumatic effects of chilling in A. aerogenes have been demonstrated to be an accelerated endogenous degradation of RNA during subsequent aeration at 37 C, and a sufficient derangement of the cell membrane to allow the penetration of ANS and RNase. Larger molecules such as DNase, trypsin or pepsin were not found to penetrate under these conditions. Where record has been made, neither a change in total count nor in cell morphology was noted in suspensions wherein viability had been decreased due to cold-shock, however, as lysis must

be considered the ultimate in membrane damage, it is possible that more severe treatment may have revealed differences in both the morphology and the total count of cold-shocked cells.

MATERIALS AND METHODS

I. Organism, cultural conditions and preparation of suspensions

1. Organism

The organism used throughout these studies was Pseudomonas aeruginosa, ATCC 9027. Twenty-four hour stock cultures were grown in the chemically-defined nitrogen-limiting medium and stored at 5 C. Stock cultures were periodically tested for purity by subculturing on glycerol peptone agar on which medium this organism forms distinctive colonies and elaborates pyocyanine.

2. Media

i. Chemically-defined media

The nitrogen-limiting medium was composed of $NaH_2PO_4.H_2O$, 0.7%; $K_2HPO_4.3H_2O$, 0.52%; NH_4Cl, 0.04%; iron, 0.5 p.p.m. The pH was adjusted to 7.2 with 5N KOH, and after sterilizing at 15 lb for 20 min, separately sterilized glucose was added to a concentration of 0.7%, and sterile $MgSO_4.7H_2O$ to a concentration of 0.1%.

The carbon-limiting medium differed from the nitrogen-limiting medium only in that the concentrations of NH_4Cl and glucose were 0.14% and 0.2% respectively.

ii. Complete medium

This medium consisted of Tryptone (Difco), 1%; yeast extract (Difco), 0.5%; glucose, 1%; KH_2PO_4, 0.5%; iron, 0.5 p.p.m. Before sterilizing the pH was adjusted to 7.2 with 5N KOH, and when cool, sterile $MgSO_4.7H_2O$ was added to a concentration of 0.1%.

3. Culture of cells for experimentation

Roux flasks containing 100 ml of medium were inoculated with a 1% inoculum of a 24 hr culture grown in the same medium as that to be inoculated, and the cells were grown at 30 C for varying periods of time as required.

4. Preparation of washed cell suspensions

The cells were harvested and washed either at 5 C or at room temperature as indicated in the text. After centrifugation of the culture at 5000xg for 15 min, the cells were washed twice in the cold or at room temperature in 0.05M Tris buffer (pH 7.3), and resuspended in this buffer at a concentration approximately ten times that of the growth concentration for the cells grown in the defined media, or at twice the growth concentration for the cells grown in the complete medium.

II. Manometric Procedures

Respirometry was carried out in a Warburg respirometer at 30 C. The reaction mixture for the measurement of endogenous oxygen uptake was as follows, 1.0 ml of cell suspension (approx. 5-6 mg dry weight of cells), 2.0 ml of 0.05M Tris buffer (pH 7.3), and 0.15 ml of 20% KOH in the centre well.

The oxidation of exogenous substrates was examined by the substitution of 0.2 ml of substrate and 1.8 ml of buffer for the 2.0 ml of buffer in the endogenous reaction mixture. Each vessel contained 5 μm of glucose, or 7.5 μm of neutralised succinate or α-ketoglutarate, and sterilization was accomplished by suction through a 0.3μ Type PH Millipore disc (Millipore Filter Corp.).

The rate of oxygen uptake was expressed as μl of oxygen consumed in 60 min per mg dry weight of cells.

III. Chemical fractionation of whole cells

A modification of the procedure of Roberts *et al.* (1955) was employed in the chemical fractionation of the cells used in these studies. Approximately 20 mg dry weight of cells were resuspended in 6 ml of distilled water. Five ml of this suspension were added to 5 ml of cold 10% trichloracetic acid (TCA) in a Pyrex centrifuge tube and the reaction mixture was held on ice for 30 min. Following

centrifugation at 7,500xg for 15 min, the supernatant fluid (cold TCA-soluble fraction) was removed and the pellet was resuspended in 5 ml of 75% ethanol with the aid of a Vortex mixer. The extraction was carried out at 45 C for 15 min after which the ethanol-soluble fraction was recovered by centrifugation. Five ml of 5% TCA were added to the pellet, and after thorough mixing the covered tube was incubated at 90 C for 10 min. When cool, the tube was centrifuged and the supernatant fluid (hot TCA-soluble fraction) was removed. The residual pellet (hot TCA-insoluble fraction) was dissolved in 5 ml of 0.1N NaOH by heating at 50 C for 5 min.

IV. Preparation of cell-free extracts

1. Extracts for ribosome sedimentation patterns

Seventeen ml of cell suspension (approximately 28 mg dry weight of cells) were withdrawn from large Warburg vessels, or from erlenmeyer flasks (which were shaken in a water bath at 30 C), centrifuged at 5000xg for 15 min and the pellet resuspended in 1.5 ml of 0.05M Tris buffer (pH 7.4), containing 10^{-4}M Mg^{++}. One twentieth of a ml of DNase (1 mg per ml) was added before the suspension was transferred to the barrel of a cold French pressure cell (Milner, Lawrence and French, 1950). A pressure of 10,000 to 15,000 lb was applied to the pressure cell by a Carver hydraulic press during the dropwise expulsion of the crude extract. To ensure maximal breakage the crude extract was

re-passaged through the pressure cell prior to centrifugation at 3600xg for 7 min to sediment whole cells and the larger debris. The opalescent cell-free extract thus obtained was frozen until required.

2. Extracts for enzyme study

Cell-free extracts were prepared in a similar fashion to that previously described except that the cells were suspended in 0.1M Tris buffer (pH 7.4); the pH of the resultant extract was 6.5. Several catalase determinations made upon the cell-free supernatant fluids and the small residual pellets following centrifugation of the crude extracts, revealed that an 85% to 90% breakage had been obtained by these procedures.

V. Sucrose Gradients

Linear sucrose gradients were prepared in order to observe the ribosomal content of cells of various ages and after various periods of starvation. The method of preparing gradients was essentially that devised by Britten and Roberts (1960).

1. Preparation of gradients and centrifugation

One of the compartments of the tandem-chambered Plexiglas mixing apparatus was fitted with a fine-drawn bent glass tube which led from the bottom of the compartment. The

apparatus was mounted on a magnetic stirrer and a 5 ml Lusteroid centrifuge tube was positioned below this outlet in such a fashion that the sucrose solution would flow down the wall. The compartment fitted with the outlet was loaded with 2.5 ml of a 20% sucrose solution in 0.01M Tris buffer (pH 7.4), containing 10^{-4}M Mg^{++}, while into the adjacent compartment were placed 2.5 ml of a 5% solution of sucrose in similar concentrations of Tris and Mg^{++} (Gronlund and Campbell, 1965). The compartments were connected by an inverted U tube filled with 5% sucrose solution, and mixing was accomplished in the compartment bearing the outlet with the aid of the magnetic stirrer.

A 0.2 ml sample of cell-free extract was carefully layered on the surface of the gradient using a 1 ml tuberculin syringe fitted with a hooked No. 18 needle. The centrifuge tubes were then placed in the buckets of a SW 39 rotor, balanced, and centrifuged at 35,000 RPM for 2 hr in a Spinco model L preparative ultracentrifuge.

2. Collection of fractions and measurement of optical density

Following centrifugation a Lusteroid tube was placed in a specially constructed holder and a No. 22 hypodermic needle was inserted approximately 1 mm through the bottom of the tube. Fractions of approximately 0.2 ml were collected dropwise into 1 ml centrifuge tubes which were promptly corked to prevent evaporation. In the early trials,

the number of fractions was found to vary due to differences in the drop size which were occasioned by the formation of bubbles in the hypodermic needle. However, the application of a slight pressure to the surface of the gradient with the use of a Cautery bulb resulted in a more even flow, and in later attempts 24-25 fractions were regularly collected.

The fractions obtained from extracts of 24 hr cells were diluted 1:9 with distilled water, while fractions from extracts of older cells were diluted 3:17. The OD of these dilutions was measured at 260 mμ by means of a Beckman model DB spectrophotometer, and the values obtained with the older cells were corrected to make them equivalent in dilution to the values obtained for 24 hr cells. The OD values plotted in the Figures depicting ribosome sedimentation patterns are those recorded for a one tenth dilution of the collected fractions.

VI. Radioactivity measurements

Samples of the growth medium supernatant fluid, whole cells and cell fractions were plated, in duplicate, at infinite thinness on stainless steel planchets and dried under an infra-red lamp. Radioactivity was measured using a model 181 Nuclear-Chicago scaler equipped with an automatic gas-flow counter which had a thin end-window Geiger tube. Counts were corrected for background.

Radioactive areas on paper chromatograms or electrophoretograms were determined by running strips through a Nuclear-Chicago model C 100 B Actigraph II with a gas-flow counter, a model 1620 B Analytical Count Ratemeter, and a chart recorder.

VII. Chromatographic and electrophoretic techniques

1. Paper electrophoresis

Paper electrophoretograms of the growth medium supernatant fluid of the nitrogen-limiting medium were run in 0.05M NH_4HCO_3 (pH 7.8) for 2 hr using Whatman no. 1 paper (Tomlinson, 1964). A RSCo model 1911 power unit supplied 700 volts to the water-cooled electrophoresis apparatus, and picric acid was used as a marker.

2. Paper chromatography

i. 2,4-dinitrophenylhydrazones of keto-acids

For chromatography of the keto-acids in the growth medium supernatant fluid of the nitrogen-limiting medium, 2,4-dinitrophenylhydrazones were prepared by reaction of the samples with 2,4-dinitrophenylhydrazine in 2N HCl for 25 min at room temperature (Friedemann, 1957). Following extraction into ethyl acetate these derivatives, together with similarly prepared standards were concentrated and subjected to descending chromatography on Whatman no. 1 paper in n-butanol-ethanol-ammonia (0.5N),

(70-10-20 v/v) for 16 hr at 37 C (Smith, 1960).

ii. Sugars and related compounds

The supernatant fluids of the nitrogen-limiting medium and the complete medium, and the intracellular pool material of nitrogen-limited cells were examined for the presence of carbohydrate. To obtain intracellular pool material, 50 mg dry weight of cells were suspended in 5.0 ml of 0.7N perchloric acid (PCA) and held at 0 C for 30 min. (Hutchison, Downie and Munro, 1962). Following the adjustment of the pH to approximately 7.5 with KOH, the precipitate was removed by centrifugation, and the supernatant fluid (cold PCA-soluble fraction) was concentrated to 0.5 ml in a Craig flash evaporator.

Descending chromatography was carried out at room temperature for 14 hr on Whatman no. 1 paper using ethyl acetate-pyridine-aq. saturated boric acid (60-25-20 v/v) as the solvent (Grado and Ballou, 1961). This solvent effectively separates gluconic acid from 2-ketogluconic acid besides giving distinctive R_f values for glucose, pyruvate and α-ketoglutarate.

3. Thin-Layer chromatography

Thin layers of Silica Gel G (E. Merck AG. Darmstadt, Germany) were prepared on glass plates as described by Randerath (1963), and were used for the chromatography of amino acids in the supernatant fluid of the complete medium,

and in conjunction with the investigation into possible proteolytic activity in the suspending fluid of respiring cells. The chromatograms were run for 1.5 hr in chloroform-methanol-17% ammonium hydroxide (2-2-1 v/v) (Randerath, 1963).

4. Location reagents

For the detection of compounds resolved by electrophoresis, paper and thin-layer chromatography, the following reagents were used: glucose and related compounds, alkaline silver nitrate dip (Smith, 1960) followed by treatment with sodium thiosulphate to clear the background, or periodate-benzidine spray (Smith, 1960) or the ortho-phenylenediamine reagent of Lanning and Cohen (1951); amino acids, 0.2% ninhydrin in acetone (w/v) used in a spray (Smith, 1960). Neither gluconic acid nor α-ketoglutarate form coloured compounds with the orthophenylenediamine reagent although the latter is detectable with UV light.

VIII. Enzyme Assays

1. Catalase

The presence of the intracellular enzyme, catalase, in the suspending fluid of respiring cells was determined in order to evaluate the extent of cell lysis. A modification of the titrimetric method of Bonnischen, Chance and Theorell (1947) was employed.

i. Procedure

The reaction was carried out in a 50 ml beaker which was placed in a rubber ice-bucket mounted on a magnetic stirrer. Water at 23 C surrounded the reaction vessel at the commencement of the assay and the temperature was found to fall approximately one degree in 15 min. The 10 ml reaction mixture was composed of 0.05M Tris buffer (pH 7.3), enzyme at the appropriate concentration and H_2O_2 at 50 µm/ml. Aliquots of 1.0 ml were removed at one minute intervals from 2 min to 8 min after the addition of the substrate, and were pipetted into erlenmeyer flasks containing 2.0 ml of 2% H_2SO_4. Residual H_2O_2 in acid solution was titrated against 0.01N $KMnO_4$ over a magnetic stirrer. The enzyme concentration was selected to yield a linear degradation of H_2O_2 of between 15-25 µm/ml during the 8 min reaction period.

ii. Preliminary information

Preliminary tests revealed that the activity recorded for whole cells was entirely comparable to that of cell-free extracts, and thus assays were performed on Warburg cup supernatant fluids and whole cell suspensions. Supernatant fluids were filtered through a 0.3µ Type PH Millipore filter to ensure the absence of particulate matter, particularly in those samples which were cloudy due to excessive lysis. Assay, refiltration and re-assay of a filtrate showed that catalase was not retained by these filters. The assays were usually carried out on the same

day on which the samples were taken, however the enzyme was found to be stable when stored on ice for periods of up to one week.

Enzyme units were defined as μm H_2O_2 degraded per min per 1.0 ml of suspension (or its filtrate) at a concentration of approximately 0.5 mg dry weight of cells per ml.

2. Glucose-6-phosphate dehydrogenase

Determinations of extracellular glucose-6-phosphate dehydrogenase were made as a confirmatory measure of cell lysis. The reaction was carried out in 1.0 ml volumes and the mixture was composed of 0.2 ml of 0.2M Tris buffer (pH 7.4), 0.1 μm of triphosphopyridine nucleotide (TPN), 2.5 μm of glucose-6-phosphate, cell-free extract or Warburg cup supernatant fluid and distilled water. The reagents were mixed in a quartz cuvette and the increase in OD at 340 mμ on the Beckman model DB spectrophotometer was recorded.

Units of activity were defined as ΔOD at 340 mμ per 0.5 min per 1.0 ml of cell-free extract (or Warburg cup supernatant fluid) at a concentration equivalent to approximately 5.0 mg dry weight of cells per ml.

3. Proteinases

Proteolytic activity was quantitatively sought in the supernatant fluids of cells after prolonged starvation using a modification of the method of Weil and Kocholaty

(1937). A 0.5 ml sample of the supernatant fluid and a control of 0.5 ml of 0.05M Tris buffer (pH 7.3) were incubated with 0.1 ml of a 6.6% solution of gelatin for 3 hr at 37 C. Five µl amounts of the reaction mixture and glycine markers were spotted on thin-layer chromatograms before and after the incubation period. The chromatograms were subsequently examined for the presence of ninhydrin-positive material.

IX. Analytical methods

1. Dry weights of cell suspensions

Duplicate or triplicate 5 ml samples of the cell suspensions in previously weighed aluminum foil containers were heated at 100 C until the moisture had evaporated and then were transferred to individual screw-cap jars containing a layer of activated silica gel dessicant (Davidson Chemical Company). In this manner they were dried to constant weight, and the results obtained were corrected for the presence of buffer.

2. Isolation of poly-β-hydroxybutyrate

Thirty ml samples of washed suspensions of *P. aeruginosa*, equivalent to approximately 150 mg dry weight of cells, were incubated overnight at 37 C with 40 ml of the alkaline hypochlorite reagent described by Williamson

and Wilkinson (1958). Following centrifugation the pellet was washed three times with distilled water, extracted twice with ethyl-ether, once with acetone and twice with chloroform (Doudoroff and Stanier, 1959). The chloroform extracts were transferred to previously weighed aluminum foil cups, and the chloroform evaporated under an airstream. Following dessication at 37 C over P_2O_5 and wax chips the foil cups were reweighed.

3. Isolation of polyphosphate

The procedure outlined by Harold (1963) was followed in an endeavour to isolate polyphosphate. By virtue of the high chain length of bacterial polyphosphate, it is virtually insoluble in solutions of high ionic strength, and it is thus to be found in the precipitate following the digestion of cells with the alkaline hypochlorite reagent described by Williamson and Wilkinson (1958). A washed cell pellet of approximately 500 mg dry weight of cells was suspended in 25 ml of the alkaline hypochlorite solution and digested for 45 min at 37 C. Following centrifugation, the pellet was washed twice with 5 ml amounts of 1.5M NaCl containing 10^{-3}M EDTA and then extracted twice with 2.5 ml of distilled water. Polyphosphate is precipitable from the aqueous extracts by the addition of solid NaCl or KCl to 1.5M, and ethanol.

4. Deoxyribonucleic acid

The method described by Schneider (1957) employing diphenylamine, which reacts with purine-bound deoxyribose, was used for the determination of DNA. Standard curves were prepared from purified calf thymus DNA (Nutritional Biochemicals Corp.) in the range of concentration of 10-80 μg/ml in the reaction mixture.

5. Ribonucleic acid

Ribonucleic acid was determined with the use of a modification of the orcinol procedure of Schneider (1957), which measures purine-bound pentose. The reagent was prepared by adding 1.0 gm of orcinol in 10 ml of 95% ethanol to 100 ml of concentrated HCl containing 0.2 gm of anhydrous $FeCl_3$ immediately prior to use. Standard or test samples contained in 1.5 ml were added to 1.5 ml of the reagent; the tubes were covered and placed in a boiling water bath for 45 min, and after cooling, the OD at 660 mμ was measured on a Beckman model B spectrophotometer. Standard curves were prepared with yeast RNA (Nutritional Biochemicals Corp.) using concentrations between 7-27 μg/ml in the reaction mixture.

Because DNA reacts significantly with the orcinol reagent, standard curves using DNA were made with each assay. Thus, with prior knowledge of the DNA concentration in a sample, a correction could be made for the contribution made by DNA to the OD in the orcinol reaction.

6. Protein

i. Samples

The concentrations of protein were determined in the hot TCA-insoluble fractions obtained by the fractionation procedure already described (section III), and on the Warburg cup supernatant fluids. Three ml aliquots of the supernatant fluids were deproteinised by the addition of 1 ml of 2.8N PCA, the reaction mixture subsequently being held at 0 C for 30 min (Hutchison, Downey and Munro, 1962). Following centrifugation at 7,500xg for 15 min, the cold PCA-soluble material was removed, and the residue was resuspended in 1.5 ml of 0.125N NaOH.

ii. Method

The method of Lowry et al. (1951) was employed to determine protein concentrations. Crystalline egg albumin in concentrations ranging from 8-46 µg/ml in the reaction mixture was used in the preparation of standard curves.

7. Ammonia

Ammonia concentrations in the supernatant fluids of the chemically-defined media and in Warburg cup supernatant fluids were determined by the microdiffusion method of Conway (1950). Values between 0-0.75 µm of ammonia could be measured under the conditions employed.

8. Keto acids

The growth medium supernatant fluid of the carbon-limiting medium was investigated for the presence of pyruvate and ∝-ketoglutarate during the growth period. The method employed was that of Friedemann (1957) for the determination of total keto acids, except that Vortex mixing was substituted for nitrogen bubbling in the extraction procedure. The method is based on the solubility of keto acid 2,4-dinitrophenylhydrazones in, or their extraction from organic solvents by, Na_2CO_3 solution, and the subsequent colour reaction on the addition of strong alkali. A standard curve was prepared with ∝-ketoglutarate ranging in concentration in the reaction mixture between 3-13 µg/ml. The OD at 435 mµ was recorded on a Beckman model B spectrophotometer.

9. Glucose

The concentrations of glucose were determined in the supernatant fluids of the nitrogen-limiting and carbon-limiting media by the "glucostat" method of Worthington Biochemical Corp. Hydrogen peroxide is produced by the enzymatic reaction of glucose oxidase and is measured spectrophotometrically in the presence of peroxidase and ortho-dianisidine. Glucose in concentrations between 3-36 µg/ml in the reaction mixture was used to prepare standard curves.

10. Total carbohydrate

Washed cell suspensions of variously aged nitrogen-limited cells were assayed for total carbohydrate according to the microdetermination technique of Trevelyan and Harrison (1952). The assay is based upon the reaction of anthrone with polysaccharides or sugars in strong acid. Standard curves were prepared with the use of glucose ranging in concentration from 4-21 μg/ml in the reaction mixture.

11. Glucosamine

i. Hydrolysis of whole cell suspensions

Two ml of cell suspension (approx. 3-4 mg dry weight of cells) were hydrolysed in 4N HCl in sealed glass tubes at 100 C for 15 hr. The hydrolysates were evaporated at 40 C under an airstream and the residue was resuspended in 3 ml of distilled water prior to re-evaporation. The washing procedure was repeated once more before quantitative transfer of the residue to a 2 ml volumetric tube where the solution was neutralized with dilute NaOH while held over a Vortex miser. The volume of the hydrolysate was made up to 2 ml with distilled water and the black particulate matter was removed by centrifugation.

ii. Method

Glucosamine concentrations were determined according

to the procedure described by Kabat and Mayer (1961), which is based on modifications of the original Elson-Morgan method. These modifications were designed to reduce interference from sugars and amino acids, and an increased sensitivity was obtained by careful attention to the pH of the reaction mixture and the use of a critical concentration of acetylacetone (Belcher et al., 1954). The reaction involves condensation of the amino sugar with alkaline acetylacetone to form a pyrrole type compound, which is then reacted with Ehrlich's reagent, p-dimethylaminobenzaldehyde, to produce a pink colour. Tris buffer was found to be unreactive with alkaline acetylacetone. Standard curves were prepared using glucosamine hydrochloride in final concentrations between 1-10 μg/ml in the reaction mixture.

12. Measurement of ultraviolet absorbance

Material which absorbed UV at 260 mμ was determined in the cold PCA-soluble fraction of Warburg vessel supernatant fluids with either a Beckman model DB spectrophotometer or a Bausch and Lomb double beam recording spectrophotometer. The procedure used for deproteinisation of the supernatant fluids was that described in section IX 6 i.

13. Viable cell counts

Estimations of viable cells in various suspensions were made using the plate count procedure. Serial dilutions were made in room temperature 0.033M phosphate buffer (pH 7.2)

prior to plating in Plate Count Agar. Six to eight plates were made for any given determination, and the counts of those plates which fell within plus or minus 4% of the mean were averaged.

14. Total cell counts and size distribution

Total cell counts of Warburg cup suspensions were made with the use of a model B Coulter Counter (Coulter Electronics Inc.). Samples were appropriately diluted in 0.9% formolized saline[1] and counted at a lower threshold setting of 1. The instrument had not been calibrated for absolute size values, but this setting was found to give an acceptable value for the background count of the saline, and for which corrections were made. The reciprocals of the amplification and aperture current were 1.0 and 0.707 respectively, and the counter was found to be sensitive to external electrical interference.

The lower threshold dictates the minimum size of the particles which are counted. By raising the lower threshold, the smaller cells in the suspension pass uncounted, and thus, through a controlled manipulation of the lower threshold, the distribution of cell size may be ascertained in the sample.

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[1]Filtered saline was supplied by the Royal Jubilee Hospital, Victoria, B. C.

EXPERIMENTAL RESULTS AND DISCUSSION

PART I. The effect of the cultural conditions and the age of the cells upon endogenous metabolism

It has been shown that 20 hr cells of Pseudomonas aeruginosa, grown in a carbon-limiting glucose-ammonium salts medium, liberate ammonia when respiring endogenously in a Warburg respirometer. The ratio between the ammonia evolved and the oxygen consumed suggested that amino acids and protein constituted the endogenous substrates of these cells (Warren, Ells and Campbell, 1960). Subsequently, the oxidation of protein was confirmed by measuring the $C^{14}O_2$ evolution of cells specifically labeled with proline-U-C^{14}, and the utilization of RNA was also implicated during the endogenous respiration of this organism when it was shown that cells, specifically labeled in the nucleic acid fraction, evolved $C^{14}O_2$ which originated primarily from the ribosomes (Gronlund and Campbell, 1963). However, the conditions of growth employed in the culturing of the cells for these experiments did not comply with the first of the three criteria deemed necessary by Wilkinson (1959) if the presence of a specific storage product, such as glycogen or poly-β-hydroxybutyrate, were to be demonstrated. This criterion requires that such a product should

accumulate under conditions where the exogenously-supplied energy source is in excess of that required for growth. Consequently, for the present studies, the synthetic medium employed in this laboratory (Warren et al., 1960) was modified so that the nitrogen source became exhausted between 15-18 hr, and glucose, determined by the glucostat method, at approximately 30 hr after inoculation. The buffering components were doubled to accommodate an anticipated reduction in pH due to the metabolism of the excess glucose.

The oxygen uptake and the release of ammonia during 2 hr of respiration of 14 hr cells (approx. 4-5 mg dry weight per cup) grown in the nitrogen-limiting medium are shown in Figure 1. These cells were harvested in the cold at a time when they were just entering the stationary phase. The oxygen to ammonia ratios of 5.4, 4.7 and 4.9 for three consecutive 40 min intervals during respiration differ little from the value of 4.5 which is the calculated ratio for the oxidation of glutamic acid. These ratios indicate that, with respect to the substrates of endogenous respiration, these cells were essentially the same as those harvested from the original carbon-limiting medium.

The maximum concentration of poly-β-hydroxybutyrate in Bacillus megaterium, grown in a medium favourable to its production, was found to occur at a time when the cells were entering the stationary phase (Slepecky and Law, 1961).

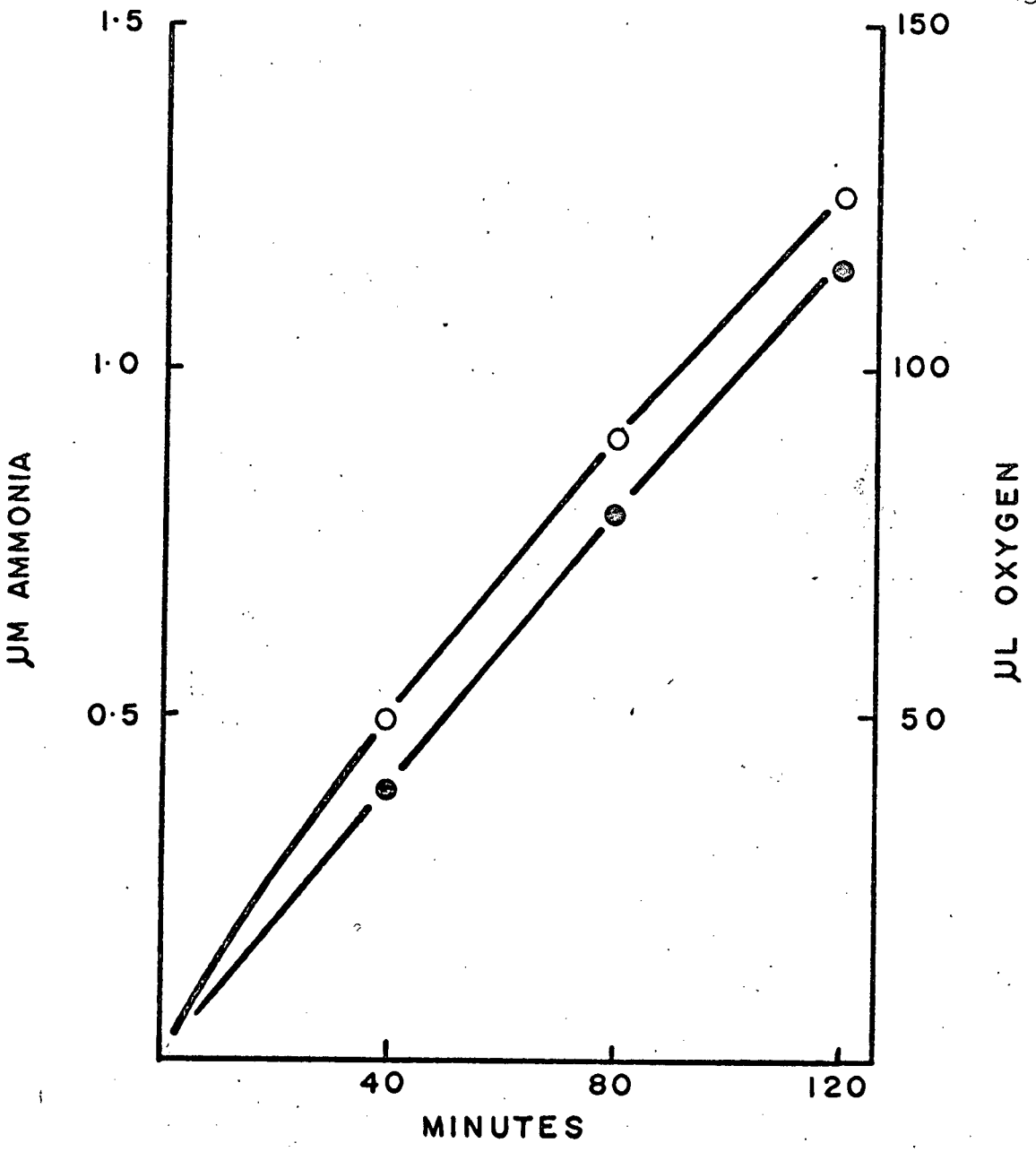

Figure 1. Oxygen uptake and release of ammonia by 14 hr nitrogen-limited cells (approx. 4-5 mg dry weight of cells).

Symbols: open, oxygen uptake; closed, ammonia release.

However, Phelps (1963) has shown that 40% of the dry weight of Pseudomonas solanacearum was attributable to poly-β-hydroxybutyrate after 64 hr of incubation at 30 C in the presence of excess sucrose, and consequently the effect of prolonging the incubation period of P. aeruginosa in the nitrogen-limiting medium was examined. The oxygen uptake and the release of ammonia of 12, 24 and 48 hr cells, harvested in the cold and corrected to 5 mg dry weight per Warburg cup are shown in Figure 2. A very noticeable increase in the O_2:NH_3 ratios was evident with the progressive aging of the cells, and the fact that no ammonia was liberated to the suspending fluid during the first 90 min of respiration of the 48 hr cells strongly suggested that an endogenous carbonaceous substrate was responsible for the reincorporation of ammonia during this period. The provision of an exogenous substrate will bring about the reincorporation of endogenously-produced ammonia in several microorganisms including P. aeruginosa (Gronlund and Campbell, 1961), and recently Dawes and Ribbons (1965) have demonstrated that the catabolism of accumulated glycogen in resting E. coli also serves to reincorporate the ammonia which is released as a result of the degradation of nitrogenous materials. The addition of ammonium ions to resting E. coli, in which there was an accumulation of glycogen, resulted in a small amount of protein synthesis at the expense of glycogen (Holme and Palmstierna, 1956), and similarly, Schlegel,

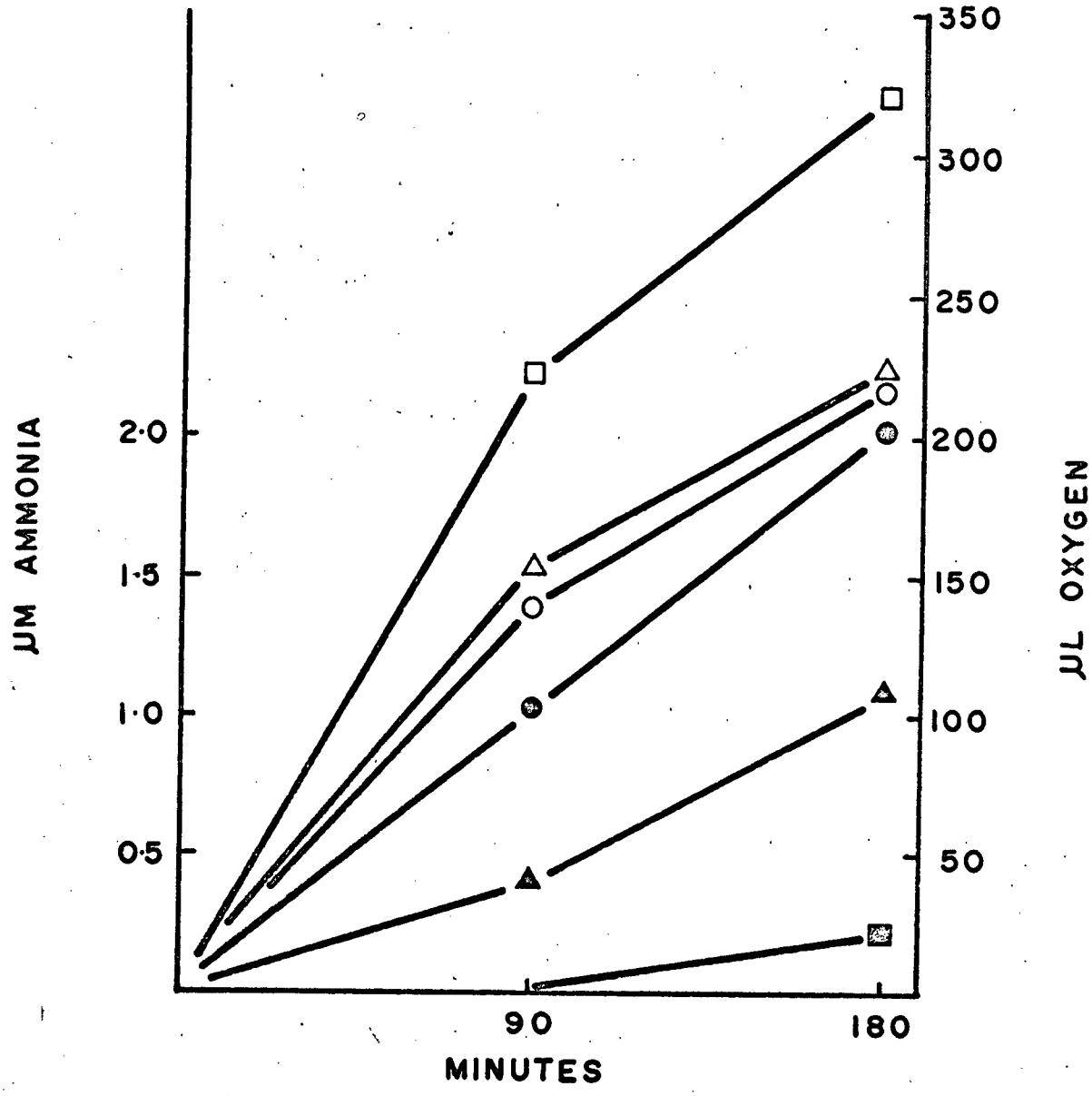

Figure 2. Oxygen uptake and release of ammonia by 5 mg dry weight of variously aged nitrogen-limited cells.

Symbols: open, oxygen uptake; closed, ammonia release; circles, 12 hr; triangles, 24 hr; squares, 48 hr.

Gottschalk and von Bartha (1961) demonstrated that the addition of ammonium ions to resting suspensions of Azotobacter chroococcum caused an accelerated degradation of accumulated poly-β-hydroxybutyrate concurrent with the uptake of the ammonium ions.

In an attempt to confirm the presumed presence of a carbonaceous reserve material, 1.0 μm of ammonium ions was added to a respiring suspension of 48 hr nitrogen-limited cells (approximately 5 mg dry weight) which had been harvested and washed in the cold. The added ammonia was rapidly incorporated with a concomitant elevation in oxygen consumption (Table 1). Considerable variation was noted in oxygen uptake, endogenous ammonia release and the incorporation of the exogenously-supplied ammonia in several experiments; however, the process was essentially one of oxidative assimilation and it is assumed that the incorporated ammonia was ultimately embodied in newly synthesized protein. This assumption is based upon the report by Duncan and Campbell (1962) that the major percentage of the radioactivity incorporated into the cells was found in the hot TCA-insoluble fraction when endogenously-produced (or exogenously-supplied) ammonia was taken up in the presence of C^{14}-labeled glucose.

All attempts to isolate and identify the suspected carbon and/or energy storage compounds met with failure. The ratios between total carbohydrate, in μg/ml,

Table 1. Oxygen consumption and the concentration of ammonia in the supernatant fluid of respiring 48 hr cells (approx. 5 mg dry weight) in the presence and absence of added ammonia

Time	μl O_2		μm NH_3	
min	endogenous	plus NH_3	endogenous	plus NH_3
0	0	0	0	0.97
15	40	45	0	0.65
30	79	100	0	0.27
45	112	150	0	0
60	141	189	0	0
80	172	216	0	0
100	194	235	0	0
120	220	255	0	0

and protein, in mg/ml, were 5.84 and 5.92 for 24 hr and 48 hr cells respectively, at which times no change in the protein concentration was evident. Thus a further quest for the accumulation of a carbohydrate material in cells grown in the nitrogen-limiting medium was not undertaken.

The procedure of Williamson and Wilkinson (1958) was followed in an endeavour to isolate poly-β-hydroxybutyrate from approximately 150 mg dry weight of 48 hr cells, but its presence could not be demonstrated, which confirmed an earlier conclusion that this organism does not synthesize this polymer (Duncan and Campbell, 1962). The absence of this compound was incidentally confirmed when an attempt was made to isolate polyphosphate according to the method described by Harold (1963). No polyphosphate could be detected in

approximately 500 mg dry weight of 48 hr cells.

Ethanol-extractable lipid was not found to accumulate during the 72 hr incubation of a nitrogen-limited culture. Cold TCA precipitates of cells were twice extracted with 75% ethanol and once with 95% ethanol and these extracts were then pooled before drying and weighing. Extractions were performed in duplicate on cells incubated for 24, 48 and 72 hr, and the ethanol-soluble material was found to account for 27%, 23% and 25% of the dry weights respectively.

In view of the complete failure to demonstrate the presence of any of the possible storage compounds in cells which were grown in a medium considered favourable for their formation, it became of interest to determine the fate of the glucose which had been shown to disappear from the medium at approximately 30 hr after inoculation. An early experiment, using cells which had been grown for 72 hr, had shown that no ammonia was released to the suspending fluid during 8 hr of endogenous respiration, and hence cells of this age were considered suitable subjects for experimentation. Glucose-U-C^{14} (approx. 0.5 μc/ml) was added to a 23.5 hr culture and the cells were harvested and washed in the cold at 72 hr, then chemically fractionated according to the modified procedure of Roberts et al. (1955). Upon harvesting, the distribution of radioactivity was found to be 76% in the supernatant fluid of the growth medium, 4% in the cells, and 20% unaccounted for and presumably lost as $C^{14}O_2$ during the incubation period.

The nature of the material accounting for the high percentage of the label remaining in the supernatant fluid of the growth medium was investigated. Substances which were capable of forming 2,4-dinitrophenylhydrazones were extracted into ethyl acetate and accounted for 15% of the radioactivity of the supernatant fluid, and an examination of these derivatives by Actigraph scanner on a paper chromatogram revealed only the presence of ∝-ketoglutarate and pyruvate in approximately a 2:1 ratio. The materials remaining in aqueous solution after the removal of ∝-ketoglutarate and pyruvate were subjected to electrophoretic separation and a single radioactive peak appeared at the same distance from the origin as the 2-ketogluconate and gluconate standards. No other peak was evident, thus confirming the absence of glucose and the complete removal of ∝-ketoglutarate and pyruvate. Resolution of the single radioactive area on the electrophoretogram was accomplished by paper chromatography and the label was found almost exclusively to reside in 2-ketogluconate. It was therefore apparent that although the glucose had disappeared at approximately 30 hr after inoculation, there was, nevertheless, an abundance of available carbon still present in the medium at 72 hr.

The distribution of radioactivity in the chemical fractions of the cells of the 4% of the label which was initially added to the culture is shown in Table 2. For purposes of comparison, data on the distribution of C^{14} in 20 hr

carbon-limited cells grown on glucose-U-C^{14}, and reproduced from Duncan and Campbell (1962), have been included.

Table 2. Percentage distribution of C^{14} in the chemical fractions of nitrogen-limited and carbon-limited cells

	% of total C^{14} in cell fractions	
Fraction	72 hr nitrogen-limited cells	20 hr carbon-limited cells
Cold TCA-soluble	5.0	6.0
Ethanol-soluble	19.0	19.0
Hot TCA-soluble	8.5	16.0
Residue	67.5	59.0

A difference in the disposition of the label in the hot TCA-soluble and insoluble fractions is displayed between the 72 hr nitrogen-limited and 20 hr carbon-limited cells. It will subsequently be shown that during prolonged incubation, a net degradation of ribosomal material occurs in the growth flask whether the cells are nitrogen-limited or carbon-limited. Glycogen or poly-β-hydroxybutyrate would be expected to be found in the residual fraction; however, in view of the demonstrated absence of these compounds in 72 hr nitrogen-limited cells, the increased percentage of label in the residual fraction is considered to be an arithmetical compensation for the reduced percentage in the hot TCA-soluble fraction.

Some reason, other than the presence of a non-nitrogenous reserve material, was therefore sought to account for the finding that the O_2:NH_3 ratios of respiring nitrogen-limited cells increased with the age of the culture from which they were harvested. It was found that this phenomenon was a function of the age of the cells, rather than of the medium in which they were grown, for progressively aged cells grown in carbon-limiting medium were shown to behave in a similar fashion to the nitrogen-limited cells during respiration (Figure 3). Harrison (1960) had suggested that an effluent from dead and moribund cells contributed to the survival of a starving population of A. aerogenes, and it therefore seemed reasonable to suppose that a similar situation might prevail in P. aeruginosa, particularly with cells which were well advanced in the stationary-phase of growth. Accordingly, 72 hr nitrogen-limited cells were harvested and washed in the cold, and the cells, resuspended to a concentration ten times that of growth were incubated at 30 C for 30 min in a sealed tube to prevent the oxidation of any leakage products which might accumulate. After chilling, the suspension was centrifuged at 5000xg for 15 min and the supernatant fluid was filtered through a 0.3μ Type PH Millipore filter disc. In a Warburg experiment, this filtrate was added to 14 hr nitrogen-limited cells (approximately 4.5 mg dry weight) which had been harvested and washed in the cold. The effects on oxygen consumption and the release of ammonia during the experimental period may be seen in Figure 4.

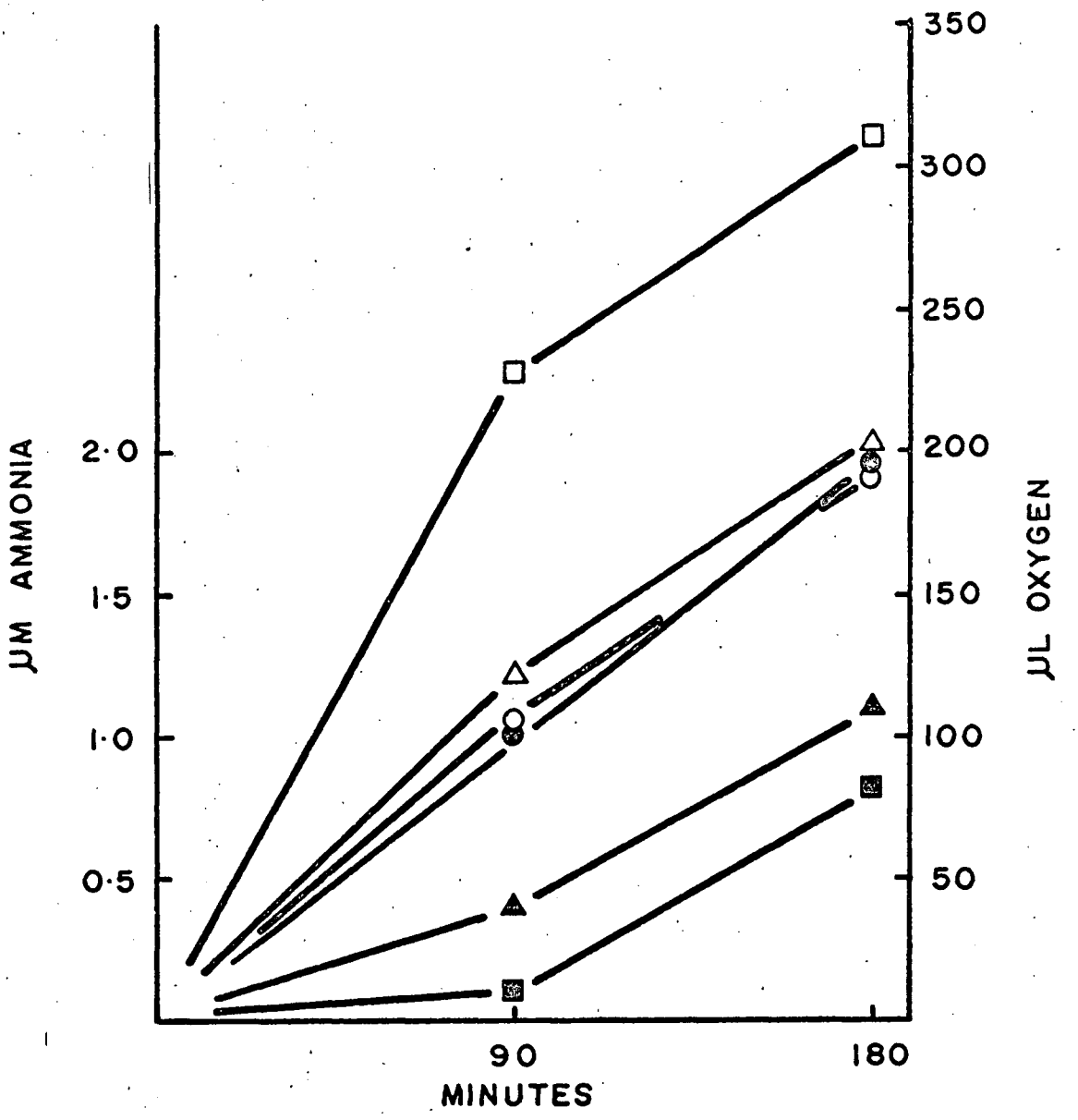

Figure 3. Oxygen uptake and release of ammonia by 5 mg dry weight of variously aged carbon-limited cells.

Symbols: open, oxygen uptake; closed, ammonia release; circles, 12 hr; triangles, 24 hr; squares, 48 hr.

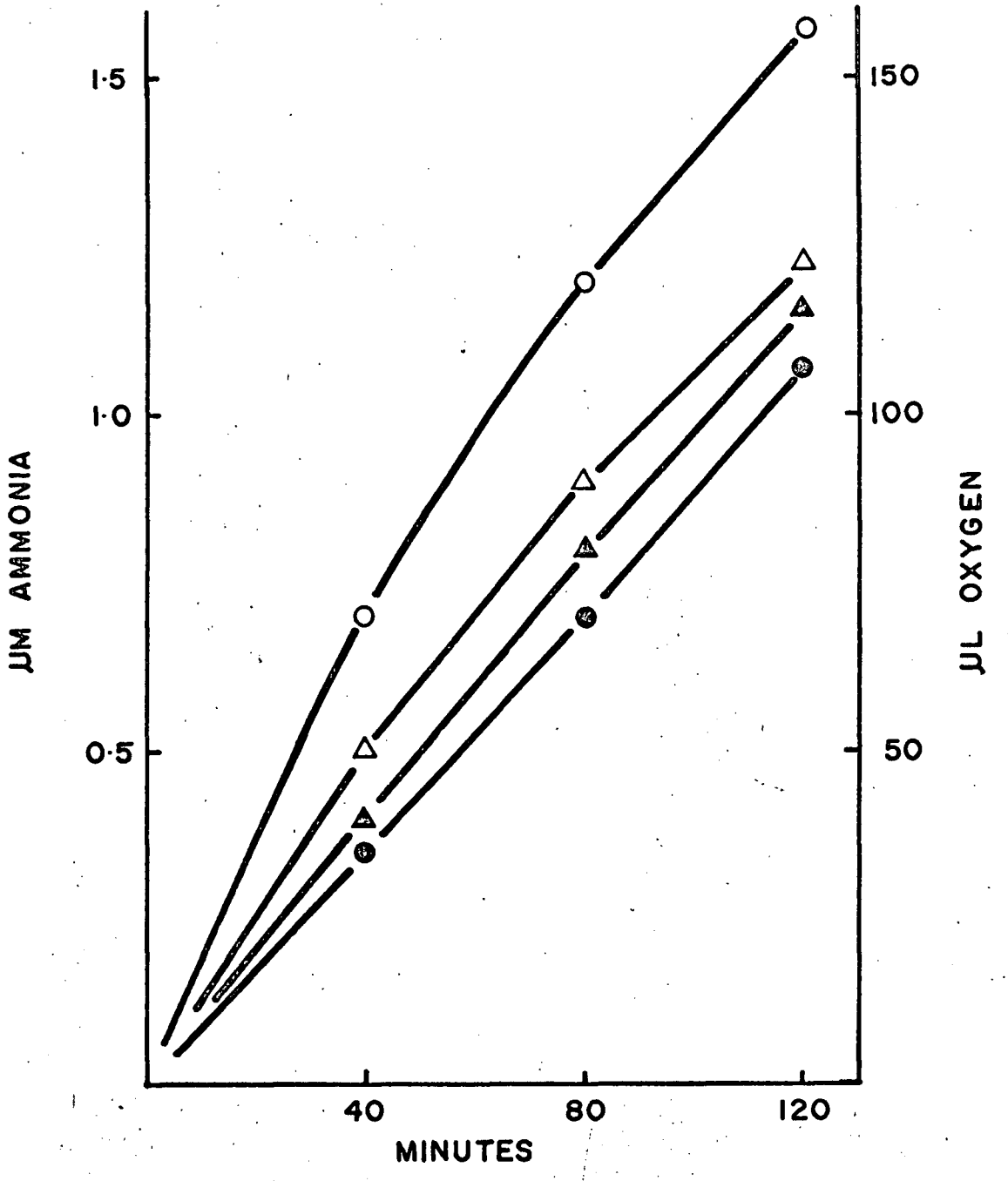

Figure 4. Oxygen uptake and release of ammonia by 14 hr nitrogen-limited cells (approx. 4-5 mg dry weight of cells) in the presence and absence of the filtrate of 72 hr cells.

Symbols: open, oxygen uptake; closed, ammonia release; circles, plus filtrate; triangles, minus filtrate.

Although the depression in the release of ammonia in the presence of the filtrate was slight, it should be pointed out that each point was determined by closely-approximating duplicate ammonia determinations on the supernatant fluids of different Warburg cups, and hence the depression is not considered to be fortuitous. The nature of the oxidizable material in the filtrate is open to wide speculation; however, it would appear to have a high carbon to nitrogen ratio, and possibly includes nucleosides and amino acids.

In view of the implication of leakage products as a contributing factor to the high O_2:NH_3 ratios observed during the starvation of older cells, the acid-soluble UV-absorbing material present in the supernatant fluids was examined. The results obtained from cold-harvested cells grown in the nitrogen-limiting medium, and which are corrected to a basis of 5 mg dry weight of cells, are shown in Table 3.

Table 3. Acid-soluble UV-absorbing material in the supernatant fluids of variously aged nitrogen-limited cells during respiration. Values are corrected to 5 mg dry weight of cells

	OD at 260 mμ		
Age	Respiration time (hr)		
hr	0	3	Δ
12	.252	.860	.608
24	.244	.850	.606
36	.326	1.53	1.20
48	.292	1.48	1.19

Although the initial level of the UV-absorbing material appears to be similar regardless of the age of the cell, there is obviously a much higher concentration of this material in the supernatant fluid of the older cells at the end of the 3 hr respiration period.

The viability of 48 hr cold-harvested cells grown in the nitrogen-limiting medium, preparatory to, and during a 3 hr Warburg experiment is shown in Table 4. Washed cell suspensions were routinely made up at ten times the growth concentration and were subsequently diluted one third on being dispensed to Warburg cups. This accounts for the title

Table 4. Viability of 48 hr nitrogen-limited cells preparatory to, and during a 3 hr Warburg experiment

Sample	Viable cells x 10^{-8} per ml
Initial suspension	120
Theoretical 0 hr	40
0 hr	27
1 hr	25
2 hr	27
3 hr	28

"Theoretical 0 hr" which is listed in the table. It was calculated that a 33% loss in viability occurred following the preparation of the suspension and before the zero hour of the respiration period, an interval during which the cold cells were dispensed to Warburg cups and equilibrated to 30 C. No change in cell number was evident, however, during the

ensuing starvation period.

The stabilizing effect of magnesium ions on ribosomes has been documented by McQuillen (1962), on spheroplasts by Lederberg (1956) and McQuillen (1958), and on isolated protoplast membranes by Weibull (1956). Owing to the presence of leakage products which was demonstrated in the suspending fluid of 72 hr cells, magnesium ions at 10^{-2}M concentration were added to the cold buffer used in the washing procedure, to the buffer used in making up the suspension and to the buffer in which the cells were diluted upon being dispensed to the Warburg cups. Figure 5 shows the effect on oxygen uptake and concentration of ammonia in the supernatant fluid during the respiration of 48 hr cells (approx. 5 mg dry weight) grown in the nitrogen-limiting medium. Control cells were both washed in the cold and respired in the absence of magnesium ions. Although not shown in previous figures for purposes of clarity, a low concentration of ammonia was usually found in the 0 hr supernatant fluids which was reabsorbed during the initial part of the respiration period. This finding is, however, illustrated in Figure 5, and it will also be seen that a radical change in the O_2:NH_3 ratios was brought about by the presence of magnesium ions. Gronlund and Campbell (1963) have reported a depression of oxygen uptake and ammonia production by 20 hr carbon-limited cells in the presence of magnesium ions during 3 hr of starvation, and have attributed

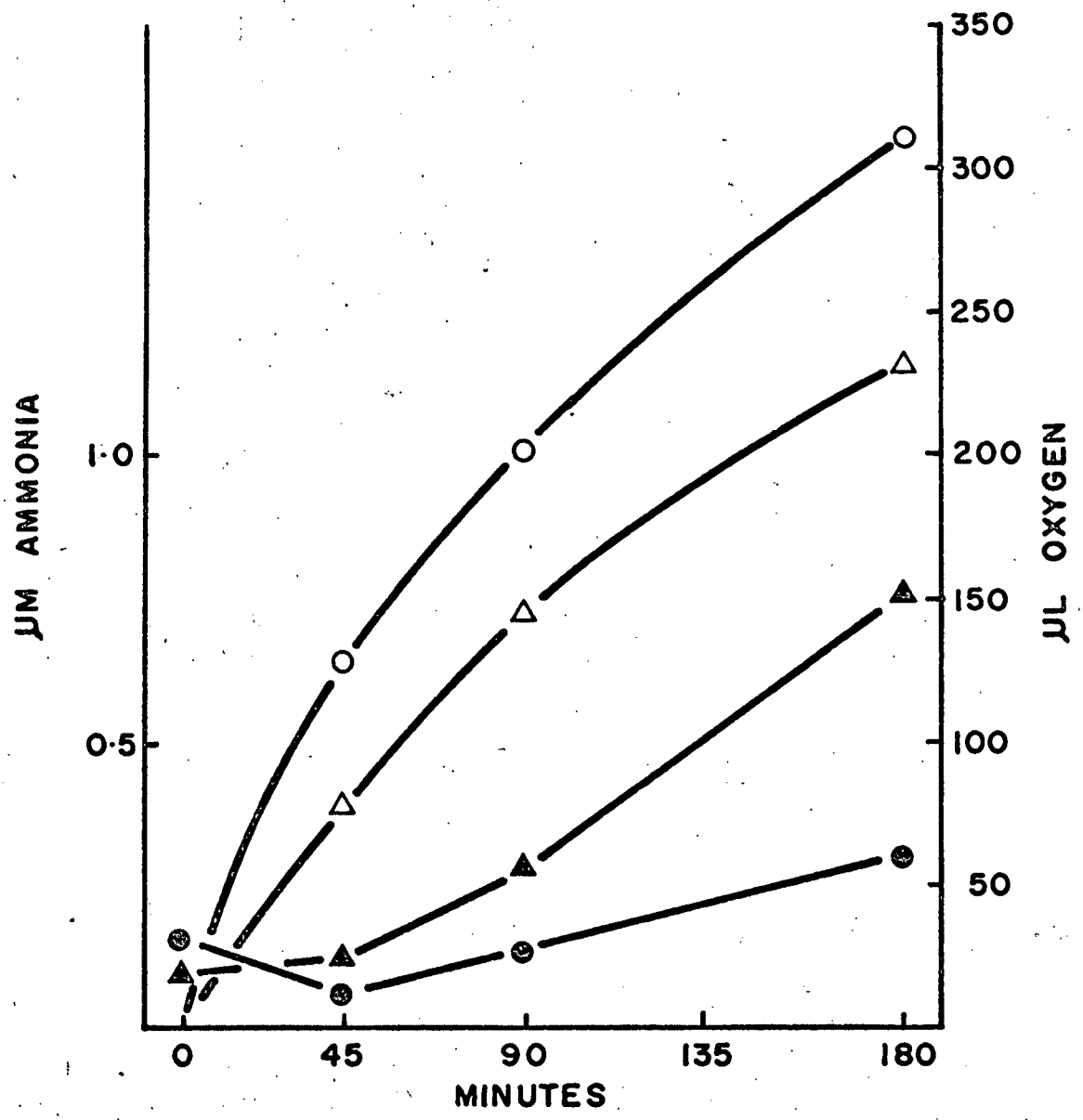

Figure 5. The effect, on oxygen uptake and the concentration of ammonia, of the addition of 10^{-2}M Mg^{++} to the washing and suspending buffer of 48 hr nitrogen-limited cells (approx. 5 mg dry weight of cells).

Symbols: open, oxygen uptake; closed, ammonia per cup; circles, minus Mg^{++}; triangles, plus Mg^{++}.

this observation to the stabilization of the ribosomes. Figure 5 indicates that while magnesium ions depressed the uptake of oxygen, they nevertheless elevated the ammonia production. Viability data obtained preparatory to, and during the starvation of these 48 hr cells which were cold-harvested and respired either in the presence or absence of magnesium ions are presented in Table 5. The figure shown opposite the title "Theoretical initial suspension" is derived from a plate count made on the culture prior to harvesting.

Table 5. Viability of 48 hr nitrogen-limited cells harvested and respired in the presence and the absence of magnesium ions

Sample	Viable cells x 10^{-8} per ml	
	Minus Mg^{++}	Plus Mg^{++}
Theoretical initial suspension	420	420
Initial suspension	280	380
Theoretical 0 min	93	126
0 min	84	135
90 min	98	145
180 min	109	133

Some loss of cells during the harvesting and washing procedures may be expected but it appears that the cells from which magnesium ions were withheld suffered a greater loss in viability during this period. During subsequent respiration, there was no change in the viability of the cells which had been washed in, and were respiring in the

presence of magnesium ions, whereas the control cells appeared to show evidence of renewed growth.

The difference in effect between a permeability malfunction which allows the diffusion of small molecules from the cells to the suspending fluid and lysis, is one of degree. In order to assess the extent of suspected membrane impairment in older cells, an assay for the presence of the intracellular enzyme, catalase (Campbell, Hogg and Strasdine, 1962), was made upon the suspending buffer of variously-aged cells which were harvested and washed in the cold from the nitrogen-limiting medium. Table 6 shows the percentages of the total catalase which were found in the supernatant fluids of the initial suspension immediately upon its preparation, at 0 hr of the Warburg experiment which was approximately 20-30 min later, and after 1 hr and 3 hr of starvation.

Table 6. The percentage of the total catalase present in the suspending fluid of variously-aged cells grown in nitrogen-limiting medium

	% of total catalase in suspending fluid			
Sample	Age of the cells (hr)			
	14	20	24	48
Initial suspension	0.6	1.1	1.5	3.2
0 hr	3.5	4.3	5.8	11.9
1 hr	10.8	12.5	-	-
3 hr	13.0	14.9	32.0	50.3

A significant amount of extracellular catalase is in evidence at the termination of the experimental period, even in the comparatively young cells, and this finding gave rise to a reconsideration of the methods employed in the handling of these cells prior to their use in respirometry.

There is a legitimate reluctance to harvest cells for studies on endogenous respiration under conditions other than in the cold. In order to study the physiological composition or the metabolic competence of cells of a given age, a period of starvation is inevitable during the preparation of the cells for experimentation unless the biological activities during this period can be greatly curtailed. This is conveniently done by harvesting and washing the cells in the cold, and subsequently maintaining the washed suspension on ice until it is required for use. However, as may be seen in Table 7, harvesting, washing and maintaining 48 hr cells at room temperature prior to the commencement of the experimental period considerably diminished the release of catalase to the suspending fluid prior to, and during a Warburg experiment. A second intracellular enzyme, glucose-6-phosphate dehydrogenase (Campbell, Hogg and Strasdine, 1962), was assayed concurrently with catalase in the control cells which were harvested and washed in the cold. In general the immunity to cold-shock of microorganisms in the stationary phase has been emphasized, however, only the early stage of this phase of growth was considered (Hegarty and Weeks, 1940; Meynell, 1958; Strange

Table 7. The percentage of total catalase or glucose-6-phosphate dehydrogenase in the suspending fluid of 48 hr nitrogen-limited cells harvested and washed in the cold or at room temperature

	% of total catalase in suspending fluid		
	Cells harvested and washed		
	in the cold		at room temperature
Sample	Enzyme		
	Glucose-6-phosphate dehydrogenase	Catalase	Catalase
Initial suspension	11.0	5.0	0.8
0 hr	30.5	15.2	1.7
1 hr	-	-	2.9
3 hr	60.0	50.2	3.2

and Dark, 1962).

The comparative effect on the concentrations of acid-soluble UV-absorbing material, of harvesting and washing 48 hr cells grown in the nitrogen-limiting medium and in the carbon-limiting medium either at room temperature or in the cold is seen in Table 8. The figures are corrected to a 5 mg dry weight of cells basis and show the concentrations which prevailed at the beginning and the end of a 3 hr Warburg experiment. Harvesting and washing at room temperature caused a considerable reduction in the release of acid-soluble UV-absorbing material in the Warburg cup supernatant fluids of cells which had been grown

in either medium. However, even when harvested and washed at room temperature, the carbon-limited cells exhibited a greater release of this material than the nitrogen-limited cells.

Table 8. Optical density of acid-soluble material in the suspending fluids of respiring 48 hr nitrogen-limited and carbon-limited cells (corrected to 5 mg dry weight of cells) harvested and washed in the cold or at room temperature

OD at 260 mμ				
Medium in which cells were grown				
Nitrogen-limiting			Carbon-limiting	
Cells harvested and washed				
hr	in the cold	at room temperature	in the cold	at room temperature
0	.200	.190	.260	.198
3	.620	.265	2.41	.660
Δ	.420	.075	2.15	.462

The values for the dry weights of cells harvested and washed at room temperature or in the cold differed by under 3% which was within the limits of experimental error; viability figures however are not available for comparison.

In contrast to the 48 hr cells grown in the nitrogen-limiting medium, similarly aged cells grown in the carbon-limiting medium invariably appeared slimy in the culture medium before harvesting. This has been reported

to be a DNA-containing slime (Hogg, unpublished data). It is conceivable that this material might have contributed to the higher values obtained for the acid-soluble UV-absorption noted in the supernatant fluids of the carbon-limited cells. In the absence of viability data it is not known whether this slime represents an excretion from living or dead cells; nor is it known to what extent, if any, the DNA which this slime is reported to contain may be fragmented. In view of the stability of DNA, physical fragmentation of the polymer during a Warburg experiment is deemed improbable. The increase in acid-soluble UV-absorbing material could, however, be attributed to the disentanglement from the slime of suitably sized fragments which were formed prior to excretion, or it is possible that a DNase released by lysing cells during the Warburg run may have been responsible for the degradation of polymeric DNA to components of acid-soluble size. However, the greater release of acid-soluble UV-absorbing material by the carbon-limited cells during starvation may also represent a fundamental difference in the endogenous metabolism of these cells compared with that of cells grown in the nitrogen-limiting medium.

The relative effect, of room temperature harvesting versus cold harvesting of 48 hr nitrogen-limited cells, on oxygen uptake, viability and the concentration of ammonia in the Warburg cup supernatant fluids during a 3 hr Warburg experiment was determined. Protein analyses of the cell pellets were carried out after removal of the supernatant

fluids for ammonia determinations primarily as an index of lysis. The oxygen uptake values and the concentrations of ammonia in the supernatant fluids are shown in Figure 6. The harvesting and washing of these cells at room temperature markedly influenced the O_2:NH_3 ratios, and the immediate release of ammonia, evident with the room temperature-harvested cells, confirmed the absence of a carbonaceous reserve material. However, it is evident that ammonia production was slightly depressed until 90 min. Once linearity of oxygen uptake had been obtained by the room temperature-harvested cells, the O_2:NH_3 ratio was 6.45, and this ratio, in a confirmatory experiment, was found to persist until the tenth hour of starvation.

Harvesting and washing in the cold presented the familiar pattern of oxygen uptake and ammonia release, but these cells too, in a separate experiment, were found to attain a steady O_2:NH_3 ratio (at 4.5 hr) which persisted until the tenth hour of respiration.

Protein was determined in the cell pellets of centrifuged cup contents during the starvation period and the results are shown in Table 9. In view of the lack of a release of ammonia to the supernatant fluids for the major part of the experimental period, the loss in the cellular protein of the cold-harvested cells probably indicated lysis, although an excretion of amino acids to the supernatant fluids cannot be excluded.

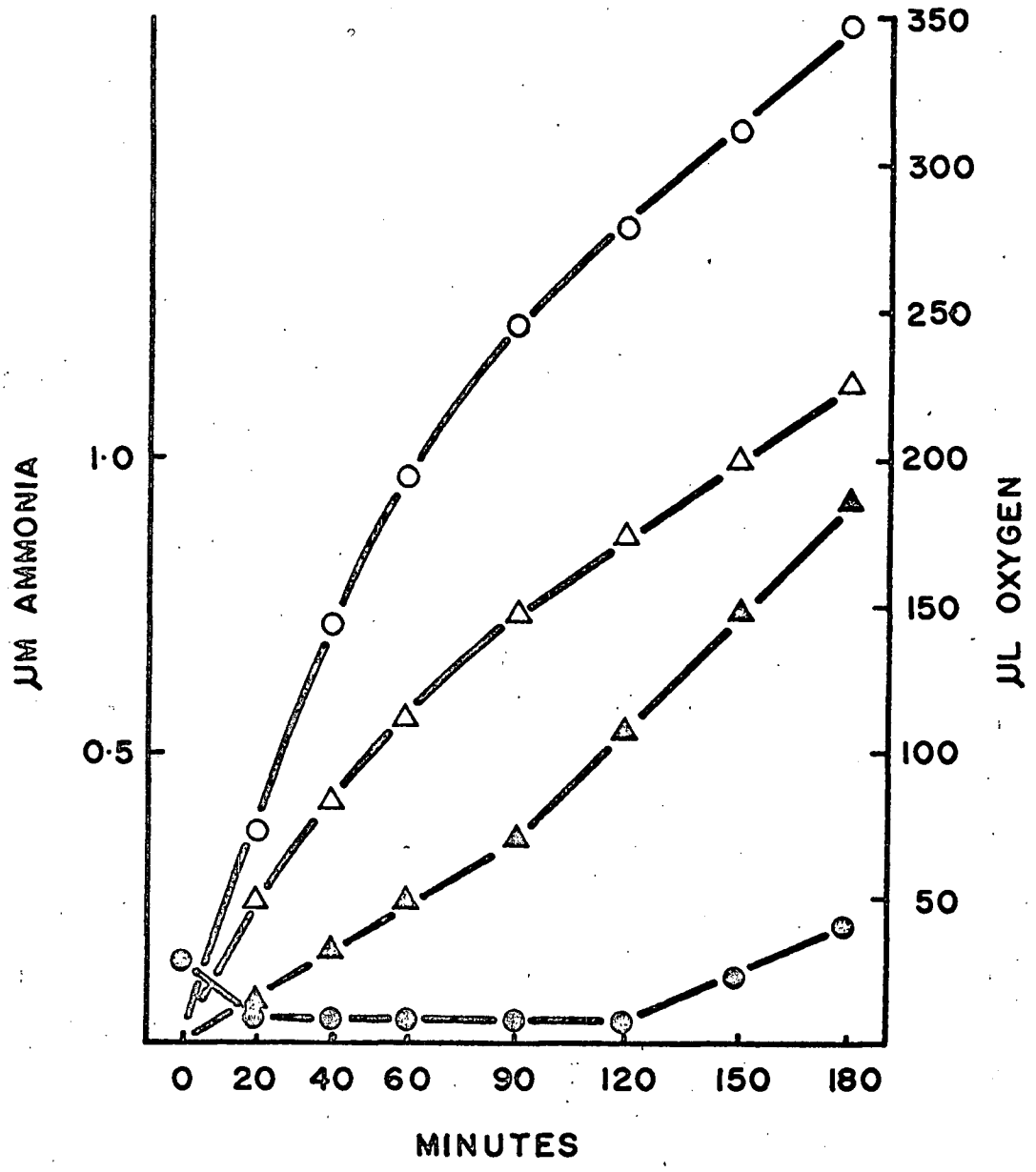

Figure 6. The effect, on oxygen uptake and the concentration of ammonia, of harvesting and washing approx. 5 mg dry weight of 48 hr nitrogen-limited cells in the cold and at room temperature.

Symbols: open, oxygen uptake; closed, ammonia per cup; circles, cold harvest; triangles, room temperature harvest.

The viable counts prior to, and during the starvation of these 48 hr nitrogen-limited cells harvested at room temperature and in the cold are shown in Table 10.

Table 9. Weight of protein in approximately 5 mg dry weight of respiring 48 hr nitrogen-limited cells harvested and washed in the cold or at room temperature

	mg Protein	
Time	Cells harvested and washed	
min	in the cold	at room temperature
0	3.46	3.75
60	3.26	3.82
120	3.10	3.72
180	3.02	3.74

Table 10. Viability of 48 hr nitrogen-limited cells preparatory to and during 3 hr respiration when harvested and washed in the cold or at room temperature

	Viable cells x 10^{-8} per ml	
	Cells harvested and washed	
	in the cold	at room temperature
Theoretical initial suspension	430	430
Initial suspension	280	380
Theoretical 0 hr	93	126
0 hr	86	122
1 hr	83	117
2 hr	92	126
3 hr	103	123

These figures bear a remarkable resemblance to those obtained when 48 hr nitrogen-limited cells were harvested in the cold in the presence and the absence of magnesium ions (Table 5).

The difference in the endogenous behaviour of 48 hr nitrogen-limited cells when harvested and washed at room temperature or when harvested and washed in the cold is obvious, and it is apparent that exposure to cold is to be strictly avoided if a legitimate study of endogenous metabolism is to be made in cells which are well advanced in the stationary phase of growth. Accordingly, in a final experiment to assess the effect of the age of the cells on their endogenous metabolism, suspensions of 12, 24 and 48 hr nitrogen-limited and carbon-limited cells were prepared at room temperature. The oxygen uptake values and the concentrations of ammonia in the suspending buffer are shown in Figures 7 and 8 for carbon-limited and nitrogen-limited cells respectively. All figures have been corrected to a basis of 5 mg dry weight of cells per Warburg cup. A decrease in the concentration of ammonia in the supernatant fluids at 0 hr was related to advancing cell age, but nevertheless, the variously aged cells, grown on either medium, readily evolved ammonia. However, the amount of ammonia produced decreased with the increased age of the cells; thus the 24 hr and 48 hr carbon-limited cells produced 85% and 53% of the ammonia evolved by the 12 hr carbon-limited cells respectively, and the corresponding

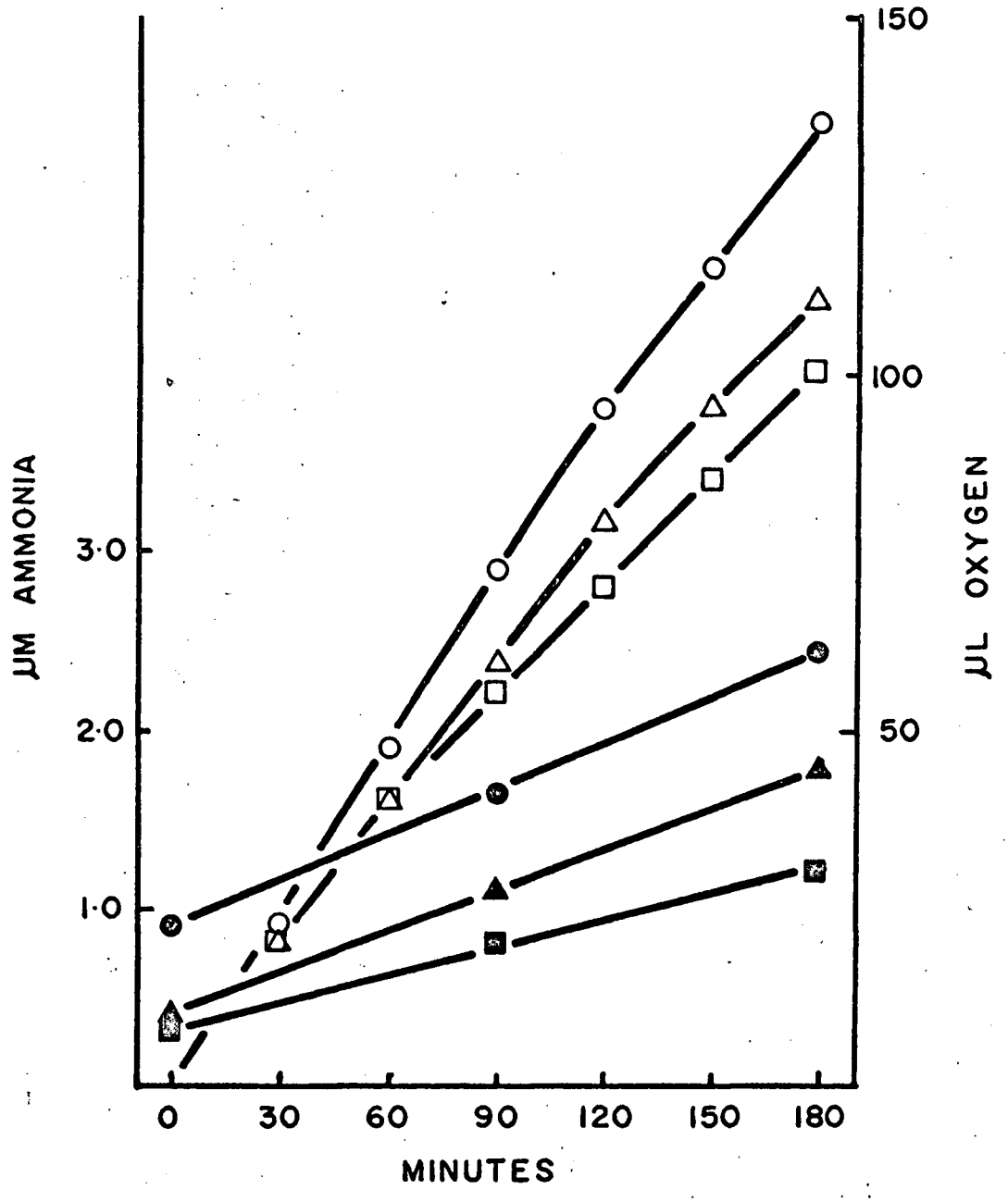

Figure 7. Oxygen uptake, and the concentration of ammonia in the supernatant fluids of variously aged carbon-limited cells (5 mg dry weight of cells).

Symbols: open, oxygen uptake; closed, ammonia per cup; circles, 12 hr; triangles, 24 hr; squares, 48 hr.

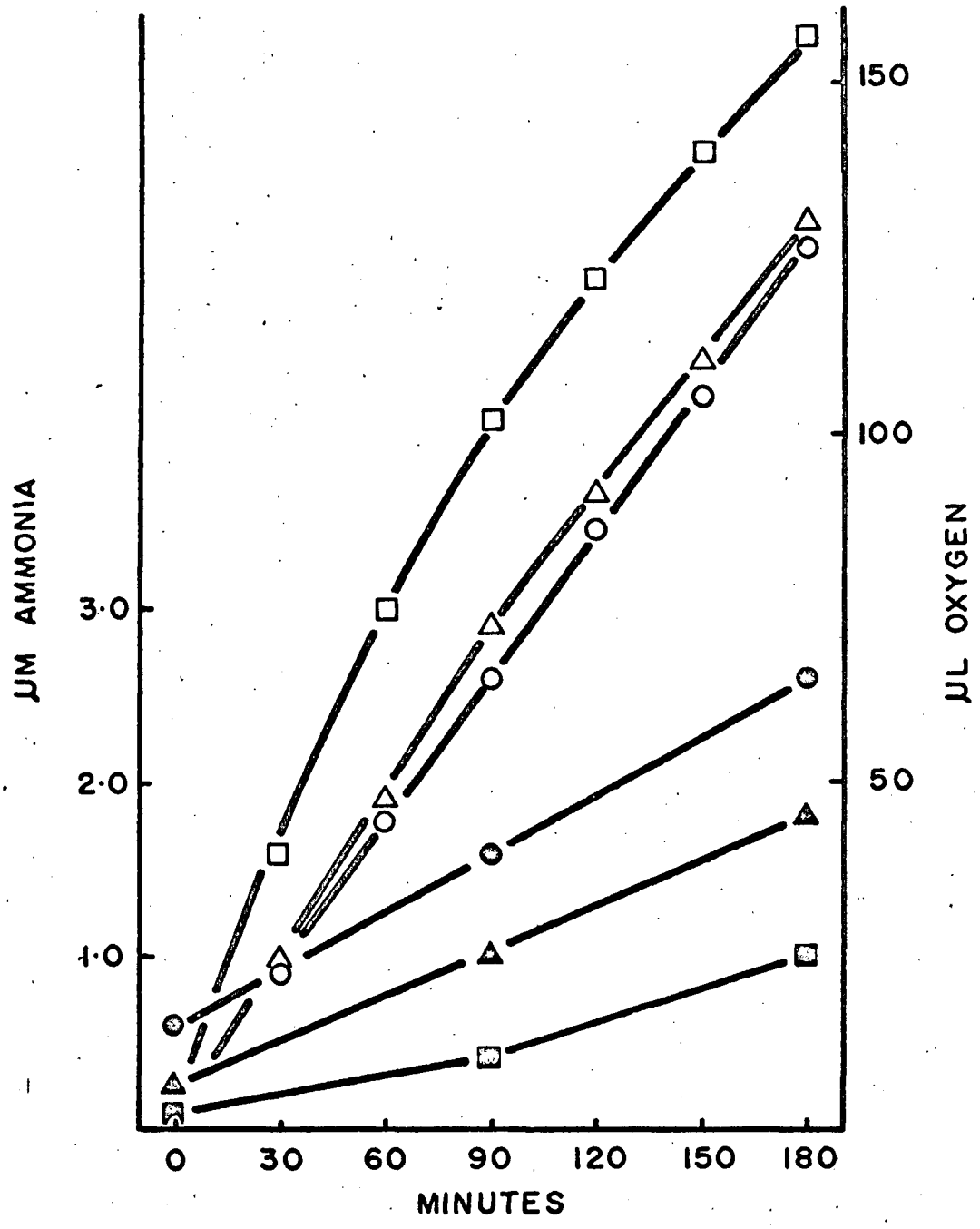

Figure 8. Oxygen uptake, and the concentraion of ammonia in the supernatant fluids of variously aged nitrogen-limited cells (5 mg dry weight of cells).

Symbols: open, oxygen uptake; closed, ammonia per cup; circles, 12 hr; triangles 24 hr; squares, 48 hr.

percentages were 72 and 45 respectively for the 24 hr and 48 hr nitrogen-limited cells. The molar O_2:NH_3 ratios are given in Table 11 where it can be seen that except for the first 90 min of starvation of the 48 hr nitrogen-limited cells, a low ratio prevailed; however in all cases a lower ratio was evident in the second of the two intervals.

Table 11. O_2:NH_3 ratios of variously aged carbon-limited and nitrogen-limited cells during 90 min intervals of 3 hr respiration

Growth medium	Age (hr)	O_2:NH_3 ratio	
		Respiration interval (min)	
		0-90	90-180
Nitrogen-limiting	12	4.2	3.7
	24	3.8	3.5
	48	5.2	4.8
Carbon-limiting	12	3.0	2.7
	24	4.7	2.9
	48	14.2	4.5

The carbon-limited cells demonstrated a decreased endogenous activity with increased age, and, because the values for oxygen and ammonia have been corrected to a common dry weight of cells, this finding may be due to the presence of dead, though not completely disintegrated, cells in the older cultures prior to harvesting.

If this were the case, the presence of dead cells might be correlated with the presence of slime in 48 hr cultures of carbon-limited cells. However, it is also possible that a decreased endogenous activity may be an intrinsic characteristic of cells when more advanced in age.

The 48 hr cells grown in the nitrogen-limiting medium deviate from the general pattern. As noted earlier (Figure 6) there is a slight depression in the evolution of ammonia in the early part of the respiration period. Examination of a concentrated cold PCA extract of 50 mg of these cells showed that the elevated O_2:NH_3 ratio could not be attributed to the presence of carbohydrate in the pool, and the possibility of a fundamental difference in physiology of 48 hr nitrogen-limited cells, when compared with similarly aged carbon-limited cells, cannot be excluded.

PART II. The effects of prolonged starvation on P. aeruginosa

With the problems associated with harvesting older cells largely surmounted, an investigation was undertaken into the changes which occurred in 48 hr cells and their supernatant fluid during a protracted respiration period. This investigation was prompted by the previous observation that 48 hr cells grown on either nitrogen-limiting medium or carbon-limiting medium attained a linear oxygen uptake after 4-5 hours, and that this steady uptake continued for a further 6 hours. The comparative behaviour of 48 hr cells grown in the defined nitrogen-limiting or carbon-limiting or in a complete medium was studied, and in order to ascertain more detailed information about these cells and their growth conditions, some preliminary experiments were carried out. This was considered advisable, for as Ribbons and Dawes (1963) have pointed out, the prior history of cells can markedly influence the character of their subsequent endogenous metabolic activities.

1. Preliminary Experiments

i. Nitrogen-limiting, glucose-ammonium salts medium

The disappearance of ammonium ions at 15-18 hr and the disappearance of glucose, as determined by the glucostat method, at about 30 hr were confirmed. A previous experiment with radioactively-labeled glucose had shown that a considerable amount of 2-ketogluconate was present

in the growth medium supernatant fluid at 72 hr, as well as lesser amounts of α-ketoglutarate and pyruvate in a 2:1 ratio, and so it was not deemed necessary to re-assay for these compounds at 48 hr. The pH at 48 hr was 6.5, showing the adequacy of the buffering system in the presence of the keto acids. The cells (about 5 mg per ml dry weight when suspended at 10 times growth concentration) appeared relatively free of extracellular slime, but an increasing froth was noted in the suspending buffer during the washing procedure. Ribosome patterns were obtained from freshly harvested cells grown for 24, 48 and 72 hr, and they showed that the ribosomal concentration decreased during prolonged incubation; however, the details and a discussion on the implications of this finding are deferred.

ii. Carbon-limiting, glucose-ammonium salts medium

This medium differed from the nitrogen-limiting medium only in the concentrations of glucose and ammonium ions. The former had completely disappeared by 18 hr and no α-ketoglutarate or pyruvate were detectable in the supernatant fluid of the medium during this period of growth. The concentration of residual ammonia at the termination of growth was found to be 16 μm per ml. The dry weight of washed cells resuspended at 10 times growth concentration was approximately 5.5 mg per ml and a considerable amount of extracellular slime was evident. However, unlike the

nitrogen-limited cells, there was negligible frothing in the suspending buffer used during the washing of the cells. Ribosome sedimentation patterns were obtained for cells grown for various ages in this medium and the findings are also deferred for later discussion.

iii. Complete medium

Although Dawes and Ribbons (1962b) had shown that the endogenous metabolism of E. coli grown on tryptone-glucose medium differed negligibly from that of cells grown on glucose-ammonium salts medium, a complete medium supplemented with 1% glucose, basically that of Gronlund and Campbell (1961), was included in this survey in order that a variety of building blocks for possible storage compounds should be available. Neither carbon nor nitrogen was limiting in this medium at 48 hr, for chromatography of the growth medium supernatant fluid, at the time of harvesting, revealed the presence of ninhydrin-positive material, 2-ketogluconate, and a trace of ∝-ketoglutarate but no glucose. However the cell density was approximately 7 times as great as that derived with the chemically-defined media and overcrowding may have retarded growth. The cells, when harvested from this medium at 48 hr (approx. 7 mg per ml dry weight when suspended at twice the growth concentration) were, like those derived from the carbon-limiting defined medium, slimy in appearance and difficult to resuspend evenly.

2. Experimental conditions

Forty eight hour cells were harvested and washed aseptically at room temperature and dispensed to sterile Warburg flasks. Aseptic precautions were also taken when previously sterilized substrates were added to the sidearms of certain designated flasks. No contaminant growth was evident from stained smears or streaked plates of glycerol-peptone agar made at intervals during the Warburg experiments. *P. aeruginosa* forms distinctive colonies on this medium and also elaborates its characteristic pigment, pyocyanine. That such a rigorous attention to asepsis was not perhaps necessary was demonstrated when certain cells, handled with no similar precaution, were allowed to respire for six days, at the end of which time no contamination could be detected. The presence of pyocyanine was not evident in the supernatant fluids of respiring cells grown in the chemically-defined media, and it is considered possible that the wide range and high rate of metabolic activity of a scavenger organism such as *P. aeruginosa* prevented potential contaminants from becoming established.

3. Results

i. Oxygen uptake and release of ammonia

The oxygen uptake and the ammonia released to the Warburg cup supernatant fluids by 5 mg of cells grown for 48 hr in the three media are shown in Figure 9. It will be seen

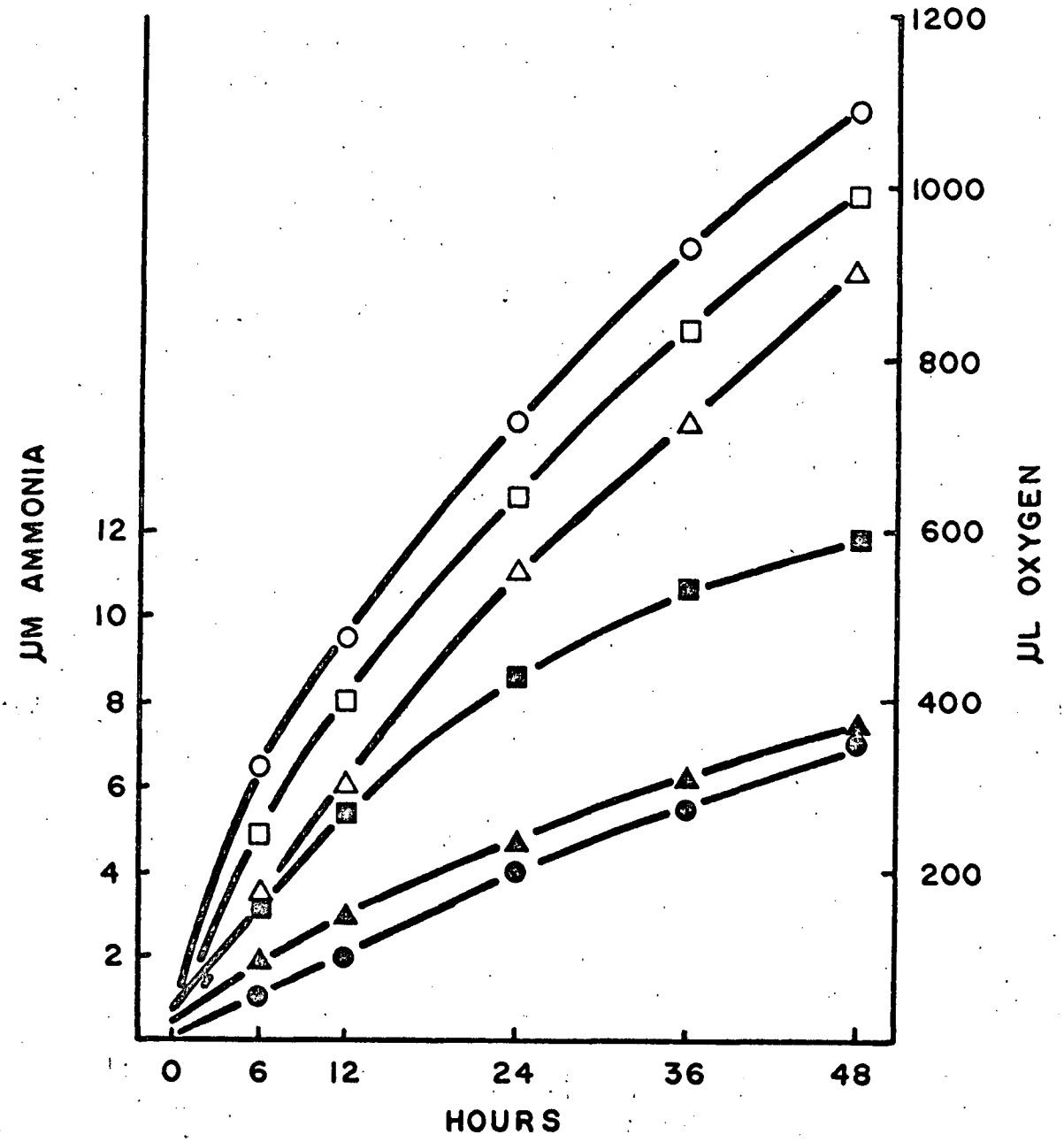

Figure 9. Oxygen uptake, and the concentration of ammonia in the supernatant fluids of 5 mg dry weight of 48 hr cells grown in various media.

Symbols: open, oxygen uptake; closed, ammonia per cup; circles, nitrogen-limiting; triangles, carbon-limiting; squares, complete medium.

that ammonia was released by all types of cell immediately, and that the zero time concentrations varied with the different cells. That the cells grown on the complete medium should demonstrate no initial lag in ammonia release shows that the provision of an array of possible building blocks for the formation of a carbonaceous reserve material was of no avail. In fact the relatively low molar O_2:NH_3 ratios for the cells grown in this medium, which are seen in Table 12, would indicate that an abundance of nitrogenous material was available and that this was, profligately, not oxidised to completion; an alternative, although less probable explanation might be that basic amino acids or amino acids with a low molecular weight were preferentially utilised. The high ratio exhibited in the initial stage of respiration by the nitrogen-limited cells confirmed earlier observations made in this respect.

Table 12. The O_2:NH_3 ratios during intervals of the respiration period

	O_2:NH_3 ratios of the intervals				
Medium	0hr-3hr	3hr-12hr	12hr-24hr	24hr-36hr	36hr-48hr
Nitrogen-limiting	29.0	6.8	5.3	5.6	3.9
Carbon-limiting	7.8	4.3	5.9	6.2	6.9
Complete	4.0	3.5	3.7	4.4	4.6

The 48 hr Warburg experiment using the nitrogen-limited cells was extended to six days and a remarkable capacity to survive was noted in this microorganism (Figure 10). The Q_{O_2} during the last day was calculated to be 11% of that of the first day, while, by the end of the sixth day, the viability was 7% of the initial value, or 9×10^8 viable cells per ml (Table 13). Thus the suspension was far from sterile and it is thought not improbable, in view of data concerning the loss of ribosomal material between 0 hr and 48 hr, yet to be presented, that survival during the latter part of this six day starvation period may have resulted in large measure from the utilization, by survivors, of cell substance released to the suspending fluid through the lysis of weaker cells.

ii. Viability, total count and size distribution

The concentrations of viable cells in the Warburg flasks at various times throughout the respiration period are indicated in Table 13. The values have been corrected to 5 mg dry weight of cells. The loss of viability in cells grown on both carbon-limiting and nitrogen-limiting media was approximately equal and represents about a 25% loss by the end of the 48 hr experimental period. The cells grown on the complete medium, however, showed a 57% increase in the early part of the respiration period, and this is possibly related to the previous suggestion that these cells carried a heavy pool of nitrogenous material,

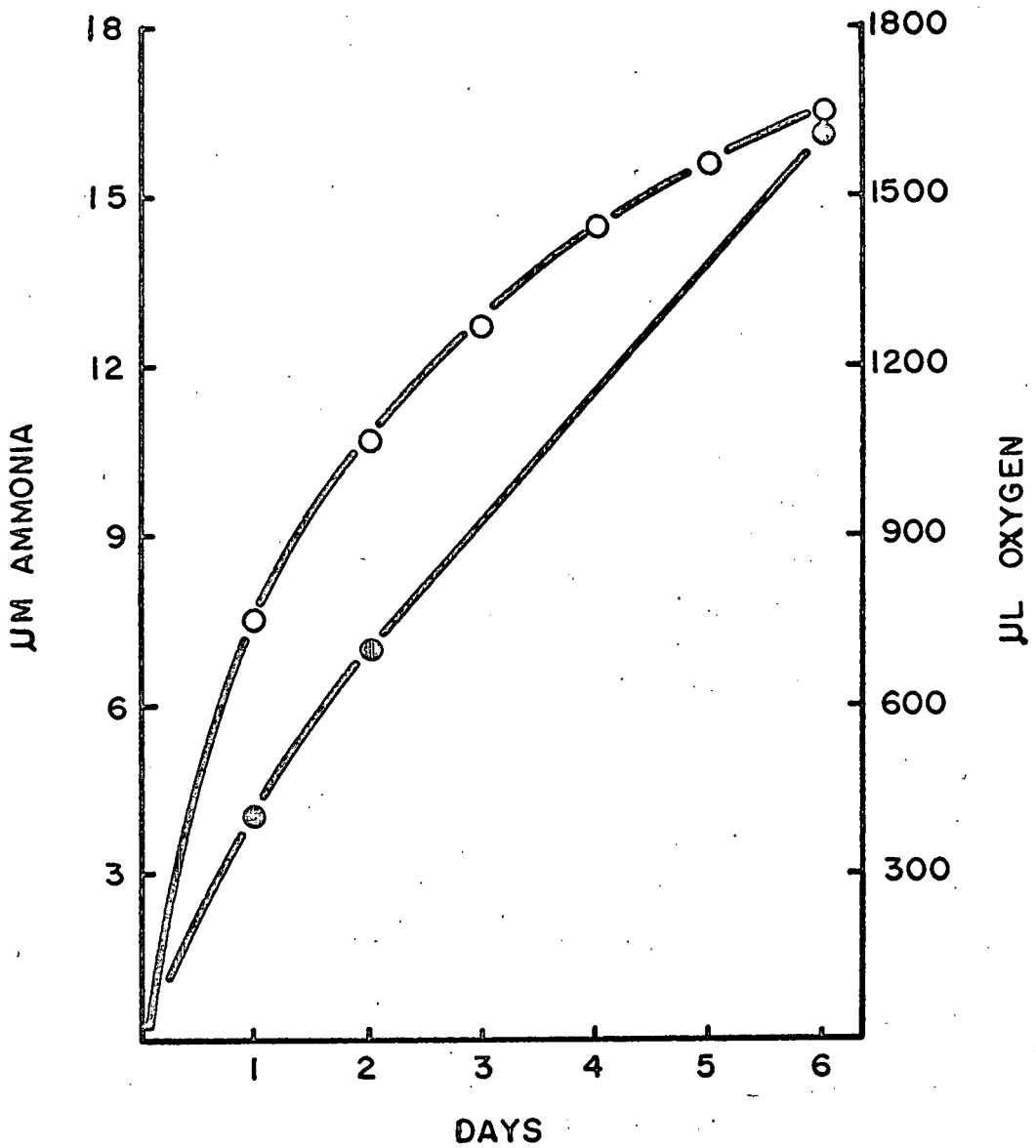

Figure 10. Oxygen uptake and release of ammonia by 5 mg dry weight of 48 hr nitrogen-limited cells during 6 days respiration.

Symbols: open, oxygen uptake; closed, ammonia evolution.

Table 13. Concentrations of viable cells during the respiration period

Time	Medium in which cells were grown		
	Nitrogen-limiting	Carbon-limiting	Complete
hr	viable cells x 10^{-8} per ml		
0	124	120	63
3	126	113	78
12	114	115	99
24	107	106	95
36	72	98	97
48	88	90	100
144	9	-	-

and which also relates to the fact that these cells were considerably larger and probably ready to divide. As shown in Figure 11 there was a marked change in the size distribution in these cells between 0 hr and 48 hr. In contrast, the size distribution of carbon-limited cells (Figure 12) indicates that they were initially small, and that there was no subsequent change during the experimental period. The size distributions at 0 hr and 48 hr for the nitrogen-limited cells were almost identical to those of the carbon-limited cells. A comparison between the size distributions at 48 hr of cells grown in the chemically-defined media and the complete medium reveals that the latter cells were the smaller. For instance, when the lower threshold of the Coulter counter was set at 1.5, 66% of the total count was countable for the carbon-limited cells versus 46% for the

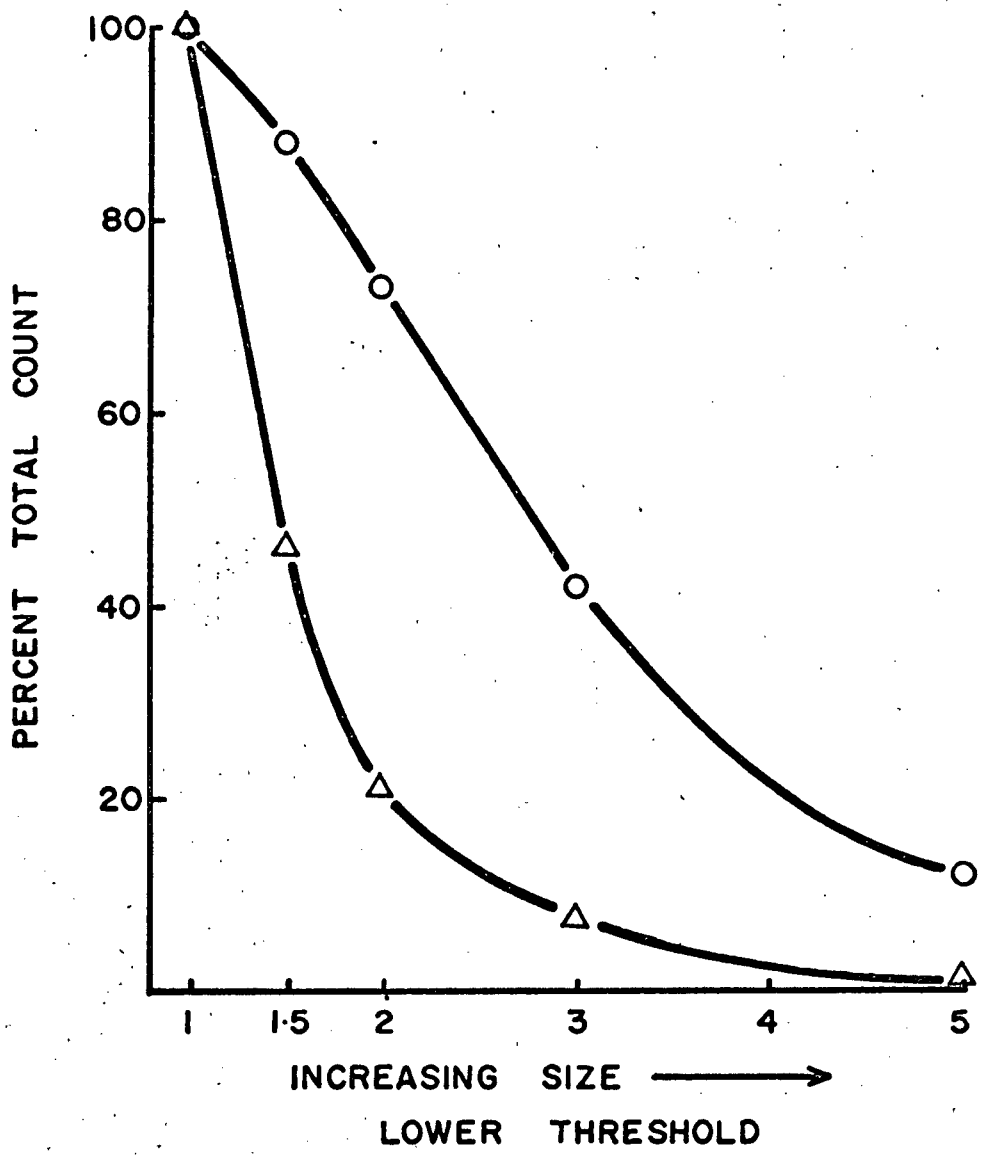

Figure 11. The size distribution of cells grown in the complete medium at 0 hr and after 48 hr respiration.

Symbols: circles, 0 hr; triangles, 48 hr.

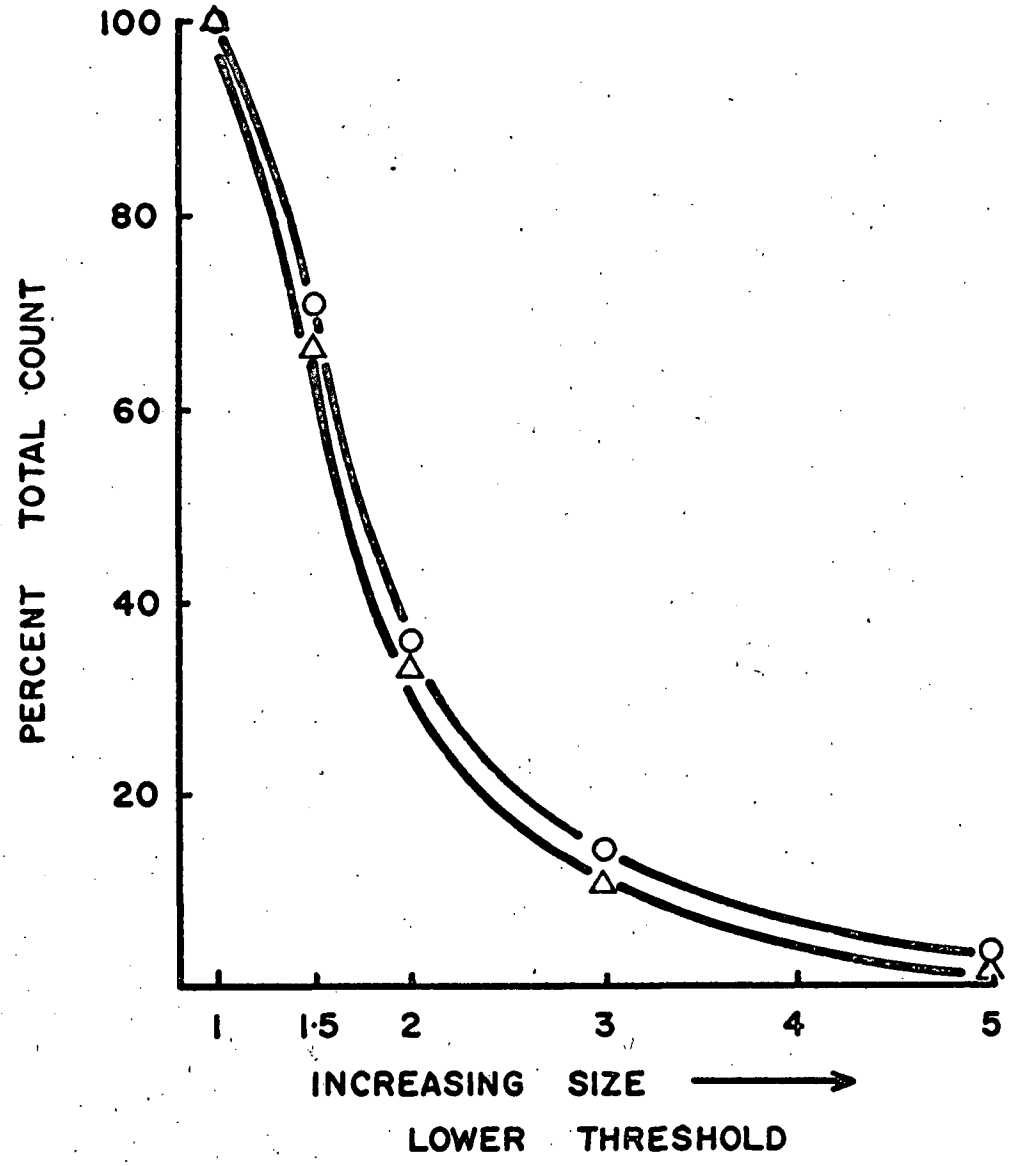

Figure 12. The size distribution of carbon-limited cells at 0 hr and after 48 hr respiration.

Symbols: circles, 0 hr; triangles, 48 hr.

cells grown in the complete medium, or, with the lower threshold set at 2, the percentages of the total count which were recorded, were 33 and 21 for the two types of cell respectively. An indication of the size of the cells may also be gained from a comparison of viable and total counts which is to be found in Table 14.

Table 14. Viable and total numbers of cells at the beginning and the end of the respiration period

	Medium in which cells were grown					
	Nitrogen-limiting		Carbon-limiting		Complete	
Time	Counts x 10^{-8} cells per ml					
hr	viable	total	viable	total	viable	total
0	124	106	120	100	63	85
48	88	-	90	88	100	94

A higher total than viable count would be expected, and this is seen to be the case with the 0 hr cells grown on the complete medium which were known to be larger. However it is apparent that many of the cells grown in the chemically-defined medium, and also those grown in the complete medium and respired for 48 hr were too small to be recorded at the lowest practical threshold which could be employed with the electronic counter. The

value for the total number of nitrogen-limited cells present at the end of the experimental period has been omitted from Table 14 because it was obviously incorrect.

iii. Ribosomes

Electron micrographs of thin sections of bacteria have revealed that the cytoplasm is filled with particles about 200 A° in diameter (Ryter, Kellenberger, Birch-Anderson and Maaløe, 1958; Chapman, 1959), and these particles, or ribosomes, were found in the six bacterial species which were examined by Schachman, Pardee and Stanier (1952). Much experimental evidence points to the fact that the ribosomes are the principal sites of protein synthesis (Borsook et al., 1950; Littlefield et al., 1955; McQuillen et al., 1959), and, like the amount of RNA (Schaechter, Maaløe and Kjeldgaard, 1958), the number of particles appears to vary inversely with the generation time (Tissières, Watson, Schlessinger and Hollingworth, 1959; Mendelsohn and Tissières, 1959), and may amount to as much as 30% of the dry weight in E. coli when the generation time is 20 minutes (Tissières et al., 1959).

Bacterial ribosomes, in vitro, exist in varying degrees of aggregation, the main controlling factor being the concentration of magnesium ions in the suspending fluid. Thus, Tissières et al. (1959) found that ribosomes obtained from cell-free extracts of E. coli were of four kinds which were characterised by their sedimentation

constants of 30S, 50S, 70S and 100S. At 10^{-4}M Mg^{++} only the 50S and 30S particles were present. On raising the concentration of Mg^{++} to 10^{-2}M one 30S was found to combine with one 50S to form a 70S particle; and at still higher magnesium concentrations, two 70S particles aggregated in a 100S particle. The four kinds of ribosomes in E. coli appeared to have approximately the same density and the same composition, namely 63% RNA and 37% protein. Electron micrographs (Hall and Slayter, 1959; Huxley and Zubay, 1960) showed that the 50S and 30S particles of E. coli differed morphologically, and further, that the association between them was specific. Cohen and Lichstein (1960) noted that Mg^{++} and spermidine exerted a stabilizing effect on the ribosomes of E. coli, and it appeared that both Mg^{++} and spermidine functioned together, since a greater aggregation of 50S and 30S particles was achieved when they were allowed to act in concert than when either substance acted alone. They suggested that besides neutralising cations, Mg^{++} and spermidine possibly had a role in linking smaller ribosomal units into larger polymeric units.

Early preparations of ribosomes were found to possess various enzymatic activities, but with improved preparative procedures many of these were no longer apparent. However, Elson (1958, 1959a, 1959b) found that essentially all of the RNase in E. coli was present in ribonucleoprotein preparations, and that the enzyme was inactive so long as the nucleoprotein was intact. Dissociation of the 70S

particle to 50S and 30S particles activated the enzyme, and it was found to be located exclusively in the 30S ribosomes (Elson and Tal, 1959). Wade and Robinson (1963) detected polynucleotide phosphorylase, but not RNase, in the ribosome fraction of P. fluorescens. The former enzyme had also been shown earlier to be largely associated with the ribosome fraction in P. aeruginosa (Strasdine, Hogg and Campbell, 1962). A further study of the polynucleotide phosphorylase of P. aeruginosa revealed that it was present in a latent form in the 70S ribosomes and that a reduction of the Mg^{++} concentration from 10^{-2}M to 10^{-4}M, which allowed the 70S ribosome to dissociate to 50S and 30S particles, activated the enzyme. The enzyme was found to be securely bound to both the lesser particles following the dissociation of the 70S ribosome (Gronlund and Campbell, 1965).

The utilization of RNA during starvation has been demonstrated in A. aerogenes (Strange, Dark and Ness, 1961), Sarcina lutea (Burleigh, Dawes and Ribbons, 1963), P. aeruginosa (Gronlund and Campbell, 1963) and in E. coli (Dawes and Ribbons, 1962c; Dawes and Ribbons, 1963). In the latter organism, ribosomal RNA was reported to account for 80% of the total RNA (Tissières, Watson, Schlessinger and Hollingworth, 1959) so the inference of the ribosomal origin of the RNA metabolised during starvation is clear. However, the ribosomes of P. aeruginosa have more definitely

been implicated as a substrate for endogenous metabolism. Cells grown in the presence of uracil-2-C^{14}, which was found to label the nucleic acid fraction selectively, evolved $C^{14}O_2$ during subsequent starvation. The $C^{14}O_2$ was derived solely from the RNA, and physical fractionation showed that there was a significant decrease of label in the ribosome fraction. Cells grown in the presence of proline-U-C^{14} demonstrated endogenous evolution of $C^{14}O_2$ from the hot TCA-insoluble fraction, and again a significant loss of label was noted from the ribosomes (Gronlund and Campbell, 1963). The participation of the ribosomes, particularly the 50S particle, in the endogenous metabolism of 20 hr P. aeruginosa grown in a carbon-limiting medium has recently been confirmed (Gronlund and Campbell, 1965).

Caldwell, Mackor and Hinshelwood (1950) found that the RNA content of bacteria was approximately proportional to the rate at which they grew; and both the ribosome content and the state of association of ribosomal particles were found to vary with the medium in which the cells were grown and the position of the culture in the growth cycle, thus reflecting a dependence upon the growth rate (Dagley and Sykes, 1958; Bowen, Dagley and Sykes, 1959; McCarthy, 1960). It was, therefore, of considerable interest to investigate the status of the ribosomes of P. aeruginosa when grown for protracted periods in both nitrogen-limiting and carbon-limiting media, and to follow their fate in 48 hr cells

during a subsequent 48 hr respiration period. The ribosome sedimentation patterns obtained from cell-free extracts in 10^{-4}M Mg^{++} of 24, 48 and 72 hr cells immediately upon harvesting from nitrogen-limiting and carbon-limiting defined media are shown in Figures 13 and 14 respectively. The gradient patterns for cells grown on either medium were remarkably similar, and in neither case was the 30S particle detected at 72 hr. The percentage remaining at 48 hr and 72 hr with respect to the 24 hr concentration of 50S particles was calculated from the total optical density of the 50S peaks. The results appear in Table 15.

Table 15. Optical densities of 50S ribosome peaks at 260 mμ

Medium in which cells were grown				
	Nitrogen-limiting		Carbon-limiting	
Age of culture	Optical density at 260 mμ of 50S ribosomes			
hr	OD at 260 mμ	% of 24 hr present	OD at 260 mμ	% of 24 hr present
24	2.98	100	3.38	100
48	2.30	76	2.46	73
72	1.16	39	1.26	37

This dissipation of ribosomal material, as measured by UV-absorption, would appear to be in accord with the findings of Mandelstam and Halvorson (1960) who studied the turnover of protein and RNA in E. coli. They showed that

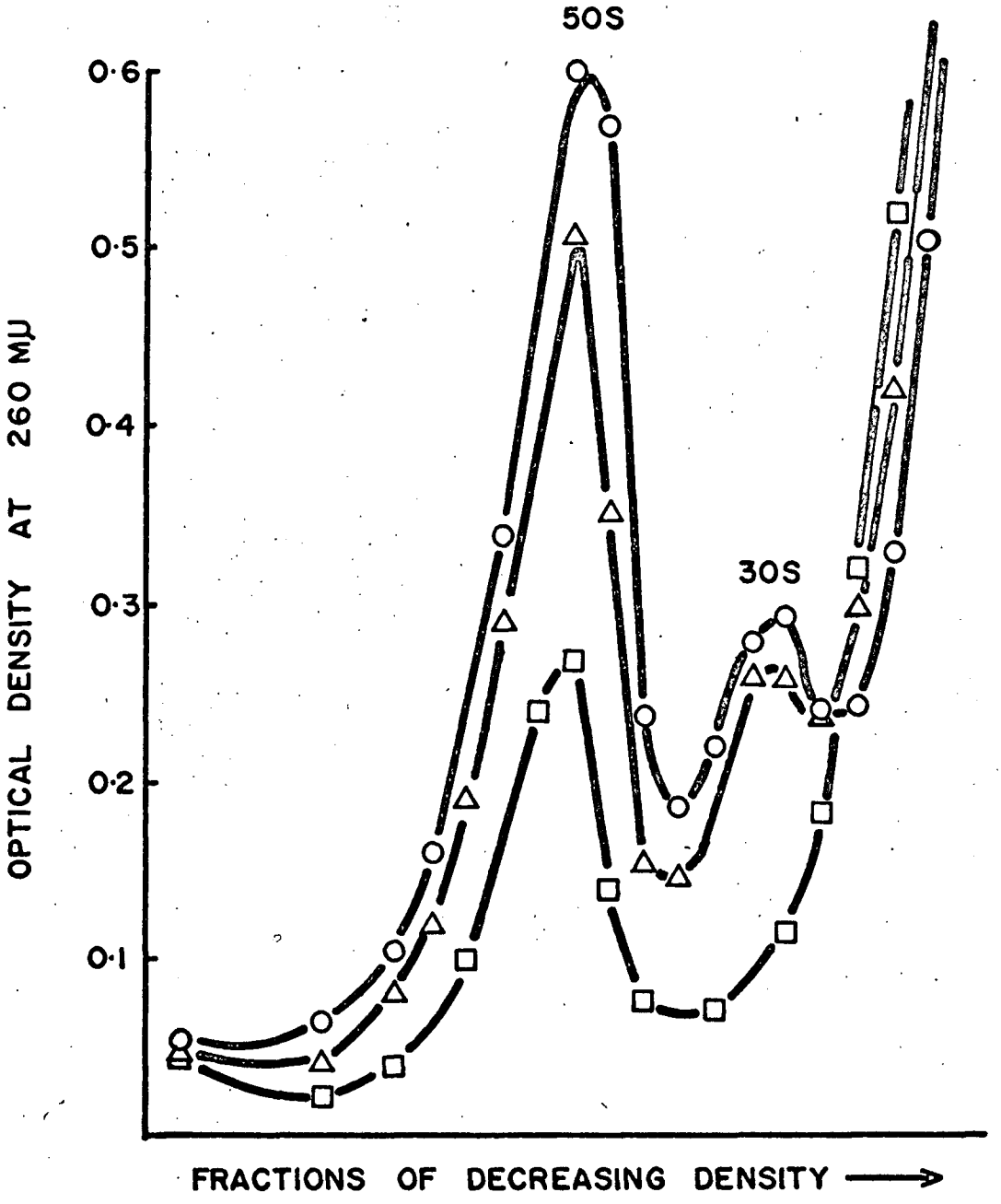

Figure 13. Ribosome sedimentation patterns of extracts of variously aged nitrogen-limited cells made immediately upon harvesting. Gradients contained 10^{-4}M Mg^{++}.

Symbols: circles, 24 hr; triangles, 48 hr; squares, 72 hr.

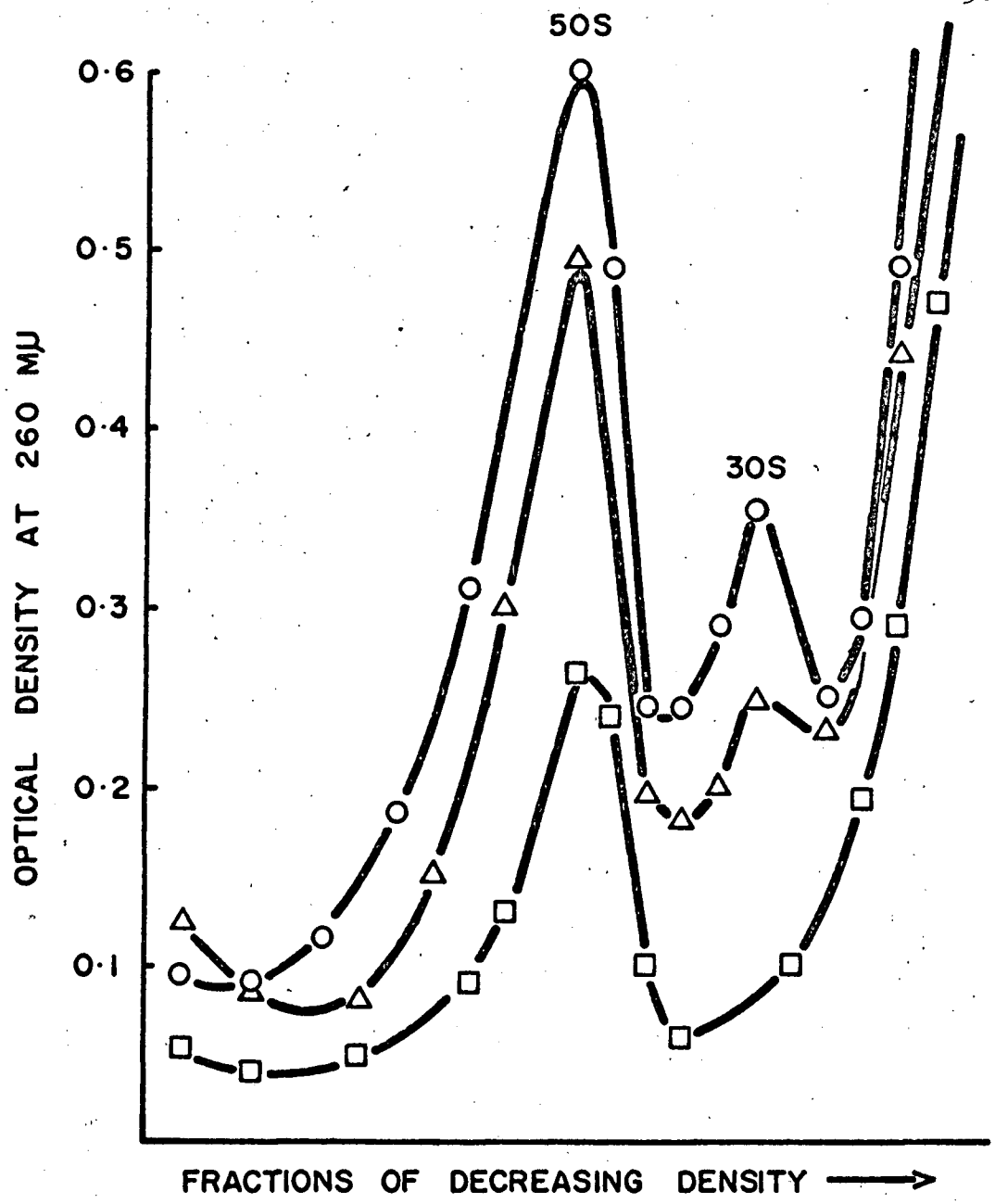

Figure 14. Ribosome sedimentation patterns of extracts of variously aged carbon-limited cells made immediately upon harvesting. Gradients contained 10^{-4}M Mg^{++}.

Symbols: circles, 24 hr; triangles, 48 hr; squares, 72 hr.

under conditions of nitrogen starvation, the RNA of the ribosomes was degraded at about 5% per hour, whereas it was resynthesized at only 1.5% per hour. They also reported that much of the free amino acids and nearly all of the ribonucleotides passing through the free pool during starvation were of ribosomal origin. The conditions under which E. coli was allowed to starve in the experiments of Mandelstam and Halvorson are essentially similar to those prevailing when P. aeruginosa was grown for extended periods in the nitrogen-limiting medium. However, when the latter organism is similarly grown in the carbon-limiting medium it is reasonable to suppose that some of the ribosomal degradation products entering the free pool are channeled off into energy-yielding pathways.

Figures 15, 16 and 17 illustrate the ribosome sedimentation patterns obtained during the 48 hr respiration period from nitrogen-limited and carbon-limited cells and those grown in the complete medium respectively. The patterns, prepared from cell-free extracts in 10^{-4}M Mg^{++}, reveal that the 30S particles were either absent or in very low concentration. The calculated content of 50S ribosomes is shown in Table 16, where it is seen that the rate of degradation of these particles diminishes with time. The relatively high concentration of 50S ribosomes at 0 hr in the cells grown in the complete medium would indicate that the cells were not as physiologically old as those grown in the

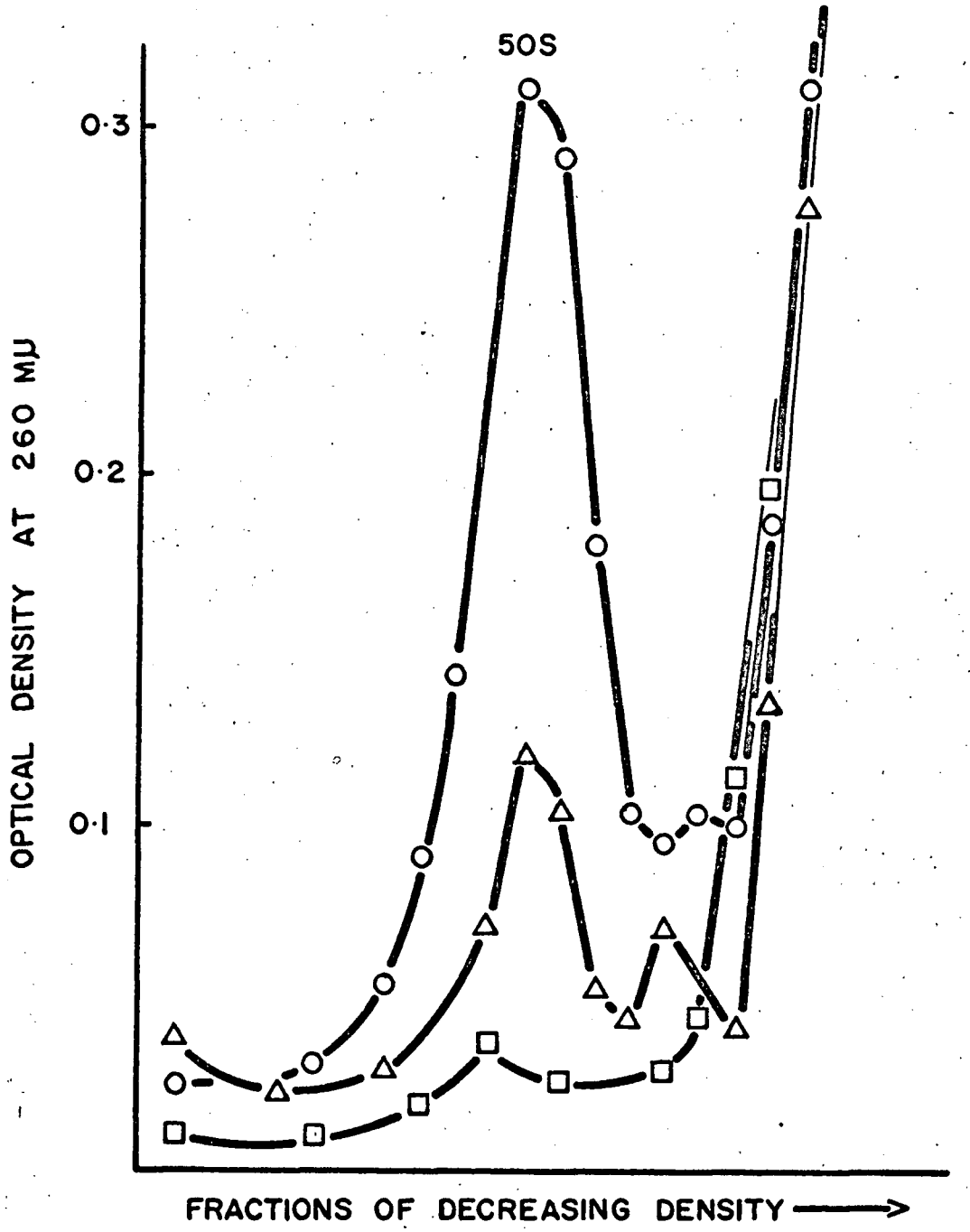

Figure 15. Ribosome sedimentation patterns of extracts of nitrogen-limited cells during 48 hr respiration. Gradients contained 10^{-4}M Mg^{++}.

Symbols: circles, 0 hr; triangles, 24 hr; squares, 48 hr.

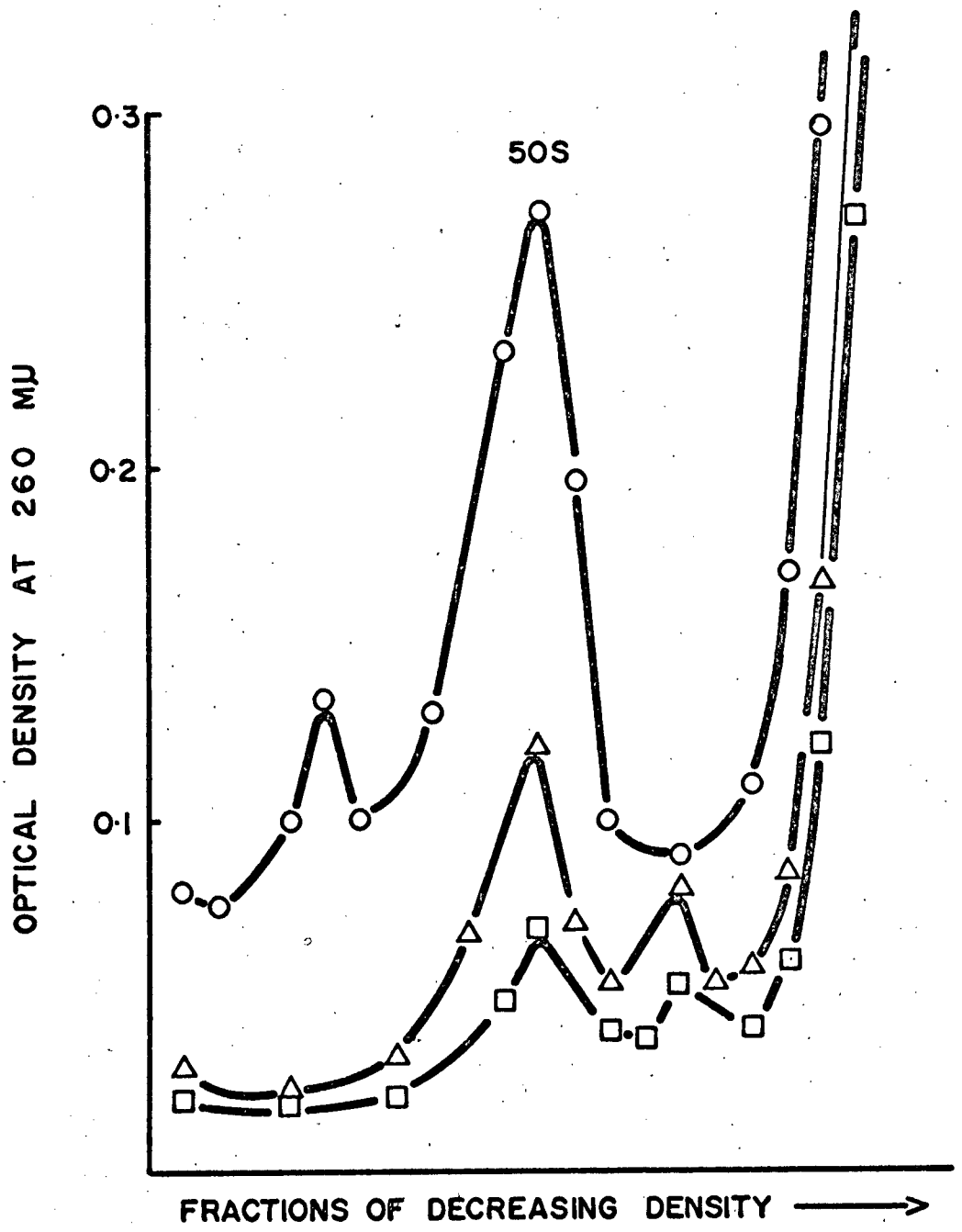

Figure 16. Ribosome sedimentation patterns of extracts of carbon-limited cells during 48 hr respiration. Gradients contained 10^{-4}M Mg^{++}.

Symbols: circles, 0 hr; triangles, 24 hr; squares, 48 hr.

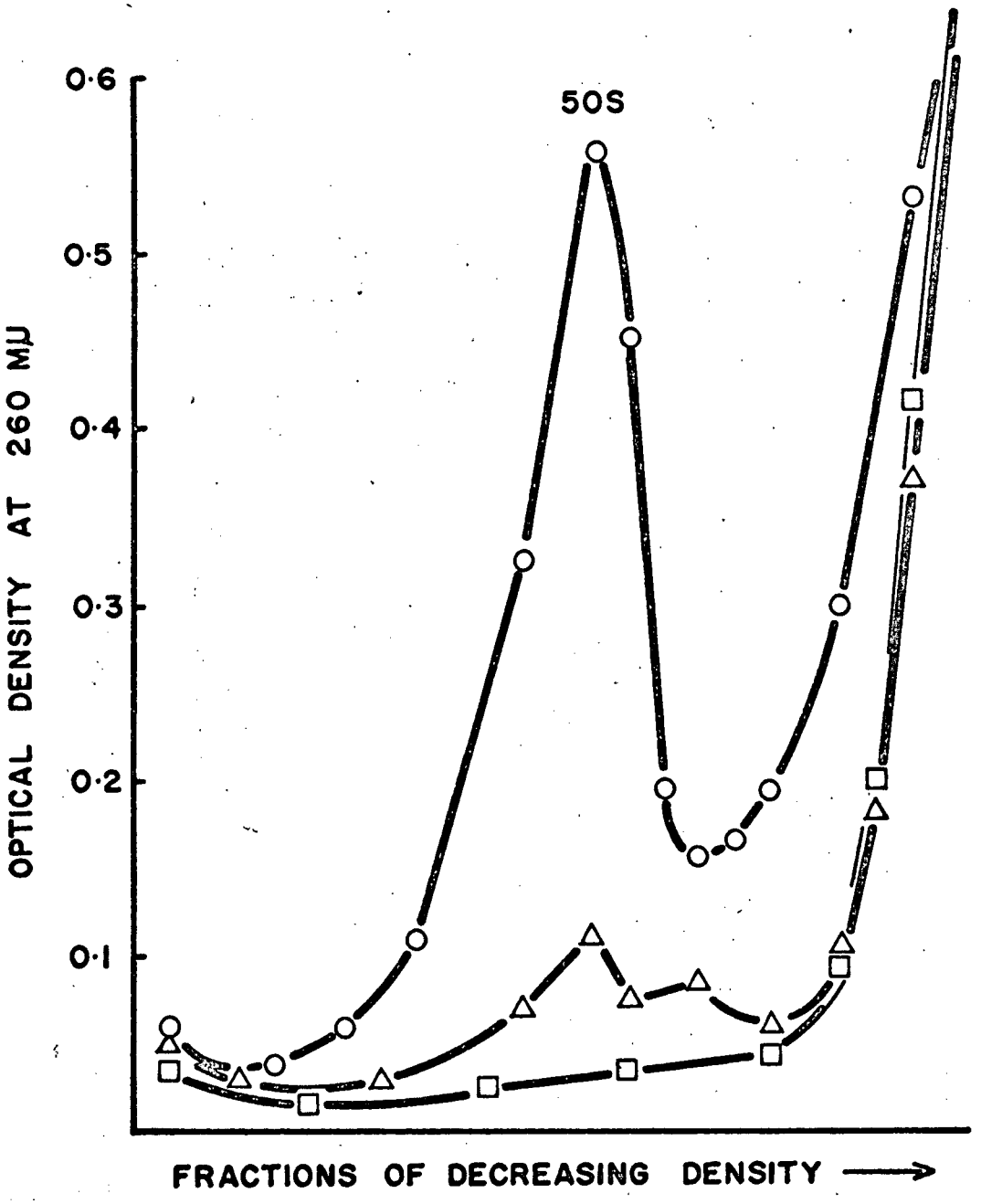

Figure 17. Ribosome sedimentation patterns of extracts of cells grown in the complete medium during 48 hr respiration. Gradients contained 10^{-4}M Mg^{++}.

Symbols: circles, 0 hr; triangles, 24 hr; squares, 48 hr.

Table 16. Optical densities of 50S ribosome peaks during the respiration period

Medium in which the cells were grown						
Nitrogen-limiting			Carbon-limiting		Complete	
Time	Optical density at 260 mμ of 50S ribosomes					
hr	OD at 260 mμ	% of 0 hr present	OD at 260 mμ	% of 0 hr present	OD at 260 mμ	% of 0 hr present
0	1.40	100	1.31	100	2.62	100
24	0.51	36	0.53	40	0.64	24
48	0.20	14	0.30	23	0.25	10

chemically-defined media, which is in agreement with the previous findings with regard to cell size. However, it is seen that the 50S ribosomes were very rapidly degraded during the first 24 hr of the respiration period. The appearance of material sedimenting in the region of the 30S can possibly be attributed to a break-down of the 50S ribosomes into nonfunctional fragments, since a 50S particle is not known to be capable of giving rise to a functional 30S particle. No significance can be attached to the small peak of heavier material which appeared in the 0 hr pattern of the carbon-limited cells without a further confirmation of its actuality.

The dispensability of the ribosomes in non-growing cells is obvious when it is recalled that the loss in viability of the cells grown in the chemically-defined

media is approximately 25% during the 48 hr starvation period (Table 13), whereas at the end of this same period the loss in ribosome content is approximately three times as great. Through the phenomenon of turnover, a pool of ribosomal precursors is available and there can be little doubt that these substances form a major proportion of the substrates for endogenous metabolism.

iv. Enzyme complement

Stier and Stannard (1936) reported that a gradual fall in the initial rate of oxygen consumption when glucose was added to Saccharomyces cerevisiae was a function of the physiological age of the culture, and Norris, Campbell and Ney (1949) could detect no appreciable decrease in the ability of P. aeruginosa to oxidise glucose after aeration for eight and one-half hours. The enzymatic competence of 48 hr cells of P. aeruginosa at intervals during subsequent starvation was observed by the addition of either 5 μm of glucose, or 7.5 μm of succinate or α-ketoglutarate to Warburg cups containing cells grown in the nitrogen-limiting, carbon-limiting defined media or the complete medium (5.3 mg, 6.1 mg and 7.1 mg dry weight of cells respectively). The values for endogenous uptake of oxygen have been subtracted (Norris, Campbell and Ney, 1949) and the results are shown in Tables 17, 18 and 19. The time indicated as "lag" was not necessarily a static period in oxygen uptake, but represents the time elapsed before the onset of the maximum Q_{O_2}.

Table 17. Oxygen uptake when 5 μm glucose were supplied at various times during the respiration period. Endogenous uptake has been subtracted

Medium in which cells were grown	Time substrate added after 0 hr	Max Q_{O_2}	% theoretical O_2 taken up
	hr		
Nitrogen-limiting	0	137	81
	3	131	79
	24	91	66*
	48	67	50*
Carbon-limiting	0	105	79
	3	102	67
	24	85	64*
	48	62	49*
Complete	0	99	70
	3	80	62*
	24	63	62*
	48	55	59*

* % of theoretical O_2 taken up after 4 hr, at which time the oxidation of the exogenous substrate was not complete.

Table 18. Oxygen uptake when 7.5 μm succinate were supplied at various times during the respiration period. Endogenous uptake has been subtracted

Medium in which cells were grown	Time substrate added after 0 hr	Length of Lag	Max. Q_{O_2}	% theoretical O_2 taken up
	hr	min		
Nitrogen-limiting	0	30	42	73
	3	30	42	85*
	24	65	32	70*
	48	90	21	54*
Carbon-limiting	0	35	80	79
	3	40	74	79*
	24	60	47	71*
	48	75	32	74*
Complete	0	30	82	76
	3	40	78	64*
	24	75	30	60*
	48	120	23	60*

*% of theoretical O_2 taken up after 4 hr, at which time the oxidation of the exogenous substrate was not complete.

Table 19. Oxygen uptake when 7.5 μm α-ketoglutarate were supplied at various times during the respiration period. Endogenous uptake has been subtracted.

Medium in which cells were grown	Time substrate added after 0 hr	Length of Lag	Max. Q_{O_2}	% theoretical* O_2 taken up
	hr	min		
Nitrogen-limiting	0	75	29	65
	3	40	35	72
	24	75	29	66
	48	+	14	36
Carbon-limiting	0	40	30	65
	3	120	30	65
	24	+	10	20
	48	+	5	15
Complete	0	90	32	52
	3	90	28	50
	24	+	13	26
	48	+	11	30

*% of theoretical O_2 taken up after 4 hr, at which time the oxidation of the exogenous substrate was not complete.

+gradual increase in Q_{O_2} during 4 hr; no evidence that maximum Q_{O_2} had been attained.

Glucose was oxidised without a lag by all the test cells; however, the maximum Q_{O_2} was seen to diminish with increased starvation. From data obtained on the concentration of extracellular protein, about 6% lysis had occurred during the experimental period (Table 21); thus with an approximate 50% decrease in maximum Q_{O_2} it would appear that the enzymes or cofactors, involved in the dissimilation of glucose were involved in turnover. The maximum Q_{O_2} for the oxidation of succinate also declined and the length of the lag period increased with the duration of starvation. Although the succinate permease mechanism has not been well studied in this organism, it is known to be chloramphenicol-sensitive (Duncan, unpublished data); and the induction to α-ketoglutarate by glucose-grown cells likewise involves protein synthesis (Duncan, 1962). Mandelstam (1958) was the first to suggest that one of the functions of protein turnover in starved bacteria was to maintain an adequate free amino acid pool for the synthesis of inducible enzymes should this be advantageous in changed nutritional circumstances. That the cells used in these experiments were able to adapt to α-ketoglutarate in the apparent absence of a 30S ribosome is remarkable since Tissières *et al.* (1960) have implicated the 70S ribosome as the functional protein synthesizing unit in *E. coli*. It must be concluded either that the 70S ribosome is not the functional ribosome in *P. aeruginosa*, or, more probably, that the low UV-absorbance of the 30S particles relative to that of the 50S

particle was responsible for the 30S ribosomes not being clearly defined. However, the increasing lag before the attainment of maximum Q_{O_2} noted with progressive starvation is a reflection of the diminishing ribosomal content as well as any loss that may have occurred in the enzymes, or cofactors, concerned in the oxidation of ∝-ketoglutarate. The only sharp induction to ∝-ketoglutarate was noted at 0 hr for the cells grown on the carbon-limiting medium. This is reasonable in view of the fact that, unlike the nitrogen-limited cells, which were in the presence of ∝-ketoglutarate until the time of harvesting, the carbon-limited cells were not exposed to extracellular ∝-ketoglutarate during growth. The decreased lag and the apparently higher Q_{O_2} exhibited by the nitrogen-limited cells at 3 hr, in contrast to 0 hr, may be attributed to ammonia limitation; for extracellular ∝-ketoglutarate is considered to enter the metabolic pathways of P. aeruginosa through the initial action of glutamic dehydrogenase (Duncan and Campbell, 1962).

v. Chemical fractions

At intervals during the 48 hr starvation period the variously grown cells were removed from the suspending fluid by centrifugation at 5000xg for 10 minutes at room temperature. Protein, DNA and RNA determinations were made on the Warburg cup supernatant fluids and cell fractions, which were obtained through the use of a modification of the method of Roberts et al. (1955). All values have been

corrected to a 5 mg dry weight of cells basis.

a. Protein

The results of protein determinations made on the Warburg cup supernatant fluids and on the hot TCA-insoluble fractions are given in Table 20, and it was calculated that, regardless of the medium in which the organism was grown, protein accounted for 60-65% of the dry weight of the cells. The net loss during the 48 hr of starvation was 15% for the nitrogen-limited cells, and 16% for both the carbon-limited cells and those grown in the complete medium. Table 21 indicates the percentages of the total protein which appeared in the Warburg cup supernatant fluids at 0 hr and 48 hr. The values at 0 hr appear to represent an excessive amount of lysis particularly with respect to the carbon-limited cells and those grown in the complete medium when compared with the O_2:NH_3 ratios for the first 3 hr of starvation given in Table 12. It is possible that an incomplete precipitation of the cells during centrifugation, or an accidental removal of a portion of the cells with the supernatant fluids may account in part for these values. In this connection it should be noted that the cells grown in the carbon-limiting medium and those grown in the complete medium, which were both obviously slimy, would not pack into a firm pellet upon centrifugation and very easily become detached from the wall of the centrifuge tube. However, on the assumption that this technical difficulty was common to all

Table 20. Distribution of intracellular and extracellular protein during the respiration period

Time	Medium in which the cells were grown								
	Nitrogen-limiting			Carbon-limiting			Complete		
	mg Protein per 5 mg dry weight of cells								
hr	supernate	cells	total	supernate	cells	total	supernate	cells	total
0	0.15	3.11	3.26	0.14	3.03	3.17	0.15	3.06	3.21
3	0.21	3.00	3.21	0.16	2.80	2.96	0.16	2.90	3.06
12	0.21	2.88	3.09	0.20	2.69	2.89	0.16	2.72	2.88
24	0.22	2.75	2.97	0.22	2.56	2.78	0.23	2.60	2.83
36	0.24	2.53	2.77	-	-	-	0.23	2.48	2.71
48	0.26	2.50	2.76	0.25	2.35	2.60	0.27	2.36	2.63

Table 21. Percentages of total protein in the Warburg cup supernatant fluids at the beginning and the end of the respiration period

% Total protein in supernates			
Time	Medium in which the cells were grown		
hr	Nitrogen-limiting	Carbon-limiting	Complete
0	4.8	4.5	5.0
48	10.5	10.5	11.8

test samples it appears from Table 21 that about 6% of the cells lysed during the experimental period. At the termination of the starvation period, the Warburg cup supernatant fluids of the nitrogen-limited and carbon-limited cells were qualitatively examined for the presence of proteolytic activity, however none could be detected.

b. Deoxyribonucleic acid

The concentration of DNA has been reported to increase during the starvation of E. coli (Horuichi, 1959), P. aeruginosa (Gronlund and Campbell, 1963) and A. aerogenes (Strange, Wade and Ness, 1963). An increase was demonstrated during the starvation of the cells grown for 48 hr in the three media used in these experiments, and from Table 22 it was calculated that DNA accounted for between 3.3-4.9% of the total dry weight of the cells regardless of the medium in which they were grown. The DNA values given for the cells

in the table represent acid-insoluble DNA only, since no diphenylamine-positive material was found in the cold TCA fraction of the cells. However, no distinction was made between acid-soluble "DNA" and acid-insoluble DNA in the supernatant fluids, and it is assumed that any acid-soluble material which may have been present and which was capable of reacting with diphenylamine derived initially from DNA. The percentage increase during the 48 hr starvation period was 17%, 14% and 22% for the nitrogen-limited cells, carbon-limited cells and those grown in the complete medium respectively. The sudden increase of DNA in the Warburg cup supernatant fluid exhibited during the first 3 hr of starvation of the carbon-limited cells could have been the result of lysis. The viability figures shown in Table 13 are insufficiently reliable to offer denial or support to this suggestion, and the value for extracellular protein at 3 hr (Table 20) would not suggest that more lysis had occurred during the respiration of these cells than of those grown in the other media.

The possibility that extracellular slime may have contributed to an excessive concentration of UV-absorbing material in the supernatant fluid of respiring carbon-limited cells has already been discussed. In the event that there were any validity in this suggestion, it would seem to gain support from the noted rapid increase in diphenylamine-positive material found in the supernatant fluid during the first 3 hr of starvation of the carbon-limited cells. The decrease recorded for the cells could possibly represent a sloughing of

Table 22. Distribution of DNA in the cells and supernatant fluids during the respiration period

Time	Medium in which the cells were grown								
	Nitrogen-limiting			Carbon-limiting			Complete		
	μg DNA per 5 mg dry weight of cells								
hr	supernate	cells	total	supernate	cells	total	supernate	cells	total
0	5	200	205	9	244	253	8	160	168
3	5	211	216	66	187	253	8	161	169
12	6	215	221	59	202	261	13	169	182
24	8	218	226	64	202	266	19	179	198
36	14	218	232	79	190	269	29	175	204
48	20	221	241	99	190	289	30	175	205

bound extracellular DNA due to the agitation of the Warburg cup during respirometry, and this would make it appear that cellular DNA had decreased.

The increase in intracellular DNA seen in the nitrogen-limited cells and those grown on the complete medium could possibly indicate a progression towards polynucleism preparatory to septum formation should the environmental conditions become more favourable.

c. Ribonucleic acid

Gronlund and Campbell (1963) reported the presence of RNA, ribonucleotides, ribonucleosides and possibly free bases in the supernatant fluid of endogenously respiring P. aeruginosa previously grown for 20 hr in a carbon-limiting medium. Data on the status of the orcinol-positive material in the cells and supernatant fluids during the 48 hr starvation period currently being discussed are shown in Table 23. The cold TCA fraction of the cells accounted for less than 2% of the total intracellular quantity of substances which reacted with orcinol and for the purposes of this discussion acid-soluble "RNA" will be considered as though it were RNA.

It was calculated from these data that RNA accounted for 11%, 12% and 13% of the total dry weight of the nitrogen-limited cells, carbon-limited cells and those grown in the complete medium respectively. In similar order the percentage loss of intracellular RNA was found to be 61, 60 and 59; the values for extracellular RNA reveal an

Table 23. Distribution of intracellular and extracellular RNA during the respiration period

Medium in which the cells were grown									
	Nitrogen-limiting			Carbon-limiting			Complete		
Time	μg RNA per 5 mg dry weight of cells								
hr	supernate	cells	total	supernate	cells	total	supernate	cells	total
0	28	538	566	96	510	606	110	530	640
3	42	430	472	198	398	596	116	495	611
12	45	328	373	198	371	569	131	437	568
24	51	291	342	206	300	506	150	329	479
36	79	257	336	206	251	457	181	253	434
48	91	208	299	230	206	436	203	220	423

accumulation of orcinol-positive material in the supernatant fluids, particularly in that of the carbon-limited cells.

vi. Extracellular UV-absorbing material

The release of UV-absorbing material to the suspending fluid of respiring microorganisms has been reported by many workers (Strange, Dark and Ness, 1961; Postgate and Hunter, 1962; Strange, Wade and Ness, 1963). In P. aeruginosa, Gronlund and Campbell (1963) showed that this material reacted positively with orcinol and negatively with diphenylamine and identified the constituents as RNA, nucleotides, nucleosides and possibly free bases. Because of the known presence of DNA and/or its degradation products in the supernatant fluids during the respiration of the cells grown on the three media used in these experiments, the measurement of material which absorbed UV at 260 mμ was not a true reflection of ribosomal degradation. The values for acid-soluble UV-absorbing material which are presented in Table 24 show that whereas there was a relatively steady increase in this material in the supernant fluid during the respiration of the nitrogen-limited cells, there was a sharp increase during the first 3 hr of the respiration of the carbon-limited cells.

The reason for the decrease in extracellular UV-absorbing material during the first 12 hr of starvation of cells grown on the complete medium is not known. The presence of pyocyanine was suspected in the supernatant fluids of these cells, and because this green pigment has

Table 24. Optical density at 260 mμ of the supernatant fluids during the respiration period

OD at 260 mμ of acid-soluble material in supernates			
Time	Medium in which the cells were grown		
hr	Nitrogen-limiting	Carbon-limiting	Complete
0	0.295	0.610	0.750
3	0.500	1.27	0.530
12	0.570	1.68	0.450
24	0.700	2.08	0.490
36	0.905	2.48	0.830
48	1.13	2.80	0.880

a strong UV-absorption, the fluids were extracted three times with chloroform prior to the measurements of UV-absorption. The pigment was found to be extractable from the organic solvent with dilute acid, in which a red color became evident, which confirmed its identity as pyocyanine.

vii. Glucosamine

Cell-wall polymers have not been implicated as substrates for endogenous metabolism (Dawes and Ribbons, 1964). In order to ascertain the status of cell-wall material during the 48 hr starvation period of cells grown on the nitrogen-limiting medium, carbon-limiting medium and the complete medium, determinations of glucosamine, as a constituent of the cell-wall, were made on the whole cell suspensions at intervals during the experimental period. The results show that there was no change in the total concentration of glucosamine in the suspensions of

nitrogen-limited or carbon-limited cells (Table 25). A slight decrease was apparent for the cells grown in the complete medium and, in order to clarify this finding, a separate experiment was performed in which the cells were allowed to respire for 6 days. A decrease, although a less extensive one, was again demonstrated (Table 26).

Table 25. Glucosamine in whole cell suspensions during the respiration period. Values are corrected to 5 mg dry weight of cells

μg Glucosamine			
Time	Medium in which the cells were grown		
hr	Nitrogen-limiting	Carbon-limiting	Complete
0	54	46	50
3	52	47	51
12	56	45	48
24	54	45	45
36	55	47	45
48	54	45	45

Table 26. Glucosamine in whole suspensions of cells grown in the complete medium during six days of starvation. Values are corrected to 5 mg dry weight of cells

μg Glucosamine					
Time					
0 hr	3 hr	1 day	2 day	4 day	6 day
51	51	50	49	45	45

On the basis of two trials, a decrease in glucosamine concentration during the starvation of these cells appears probable, and an extension of the respiration period of the cells grown in the chemically-defined media possibly would have shown that glucosamine was being utilized to a similarly slight extent. However, the possible contribution of glucosamine, as a substrate for endogenous metabolism, was negligible when compared with that of RNA or protein.

viii. Oxidative assimilation

The incomplete oxidation of relatively small amounts of substrate by washed suspensions of microorganisms was first reported by Cook and Stephenson (1928) during manometric experiments with E. coli. Since this initial observation, much work has been devoted to the study of oxidative assimilation beginning with Barker (1936) who concluded that the assimilation product in Prototheca zopfii had the empirical composition (CH_2O). Holme and Palmstierna (1956) investigated the accumulation of a glycogen-like material in resting E. coli when held in the presence of glucose; and a good correlation was shown to exist between the accumulation of a polysaccharide and the assimilation of radioactive carbon from glucose-U-C^{14} supplied to resting S. lutea (Binnie, Dawes and Holms, 1959). Clifton (1962) found that the hot TCA-soluble and insoluble fractions contained most of the radioactivity when C^{14}-labeled glucose was added to resting cells of Bacillus cereus.

Endogenously-produced ammonia was reincorporated by P. aeruginosa when resting cells were supplied with exogenous glucose (Warren, Ells and Campbell, 1960), and this reincorporation was later concluded by Duncan and Campbell (1962) to occur through the initial mediation of glutamic dehydrogenase. Studies with glucose-U-C^{14} suggested that the availability of ammonia determined the rate at which carbon was assimilated into the cells and it was found that the major percentage of the incorporated radioactivity was located in the hot TCA-insoluble fraction.

The ability of the cells under study to oxidise glucose and to adapt to, and oxidise α-ketoglutarate at the end of 48 hr starvation has already been demonstrated. Changes in the cells and the Warburg cup supernatant fluids due to the addition of these substrates were examined seven hours following their addition to the carbon-limited cells at the end of the 48 hr respiration period. The uptake of endogenously-produced ammonia by the cells, as shown by a reduction in the ammonia concentration of the supernatant fluid, is indicated in Figure 18; and Figure 19 shows that there was a decrease in the concentration of UV-absorbing material in the supernatant fluid upon the addition of the substrates. Presumably the latter represents a reabsorption of free bases and nucleosides in view of the fact that phosphorylated compounds are not known to be able to penetrate viable cells at physiological temperatures.

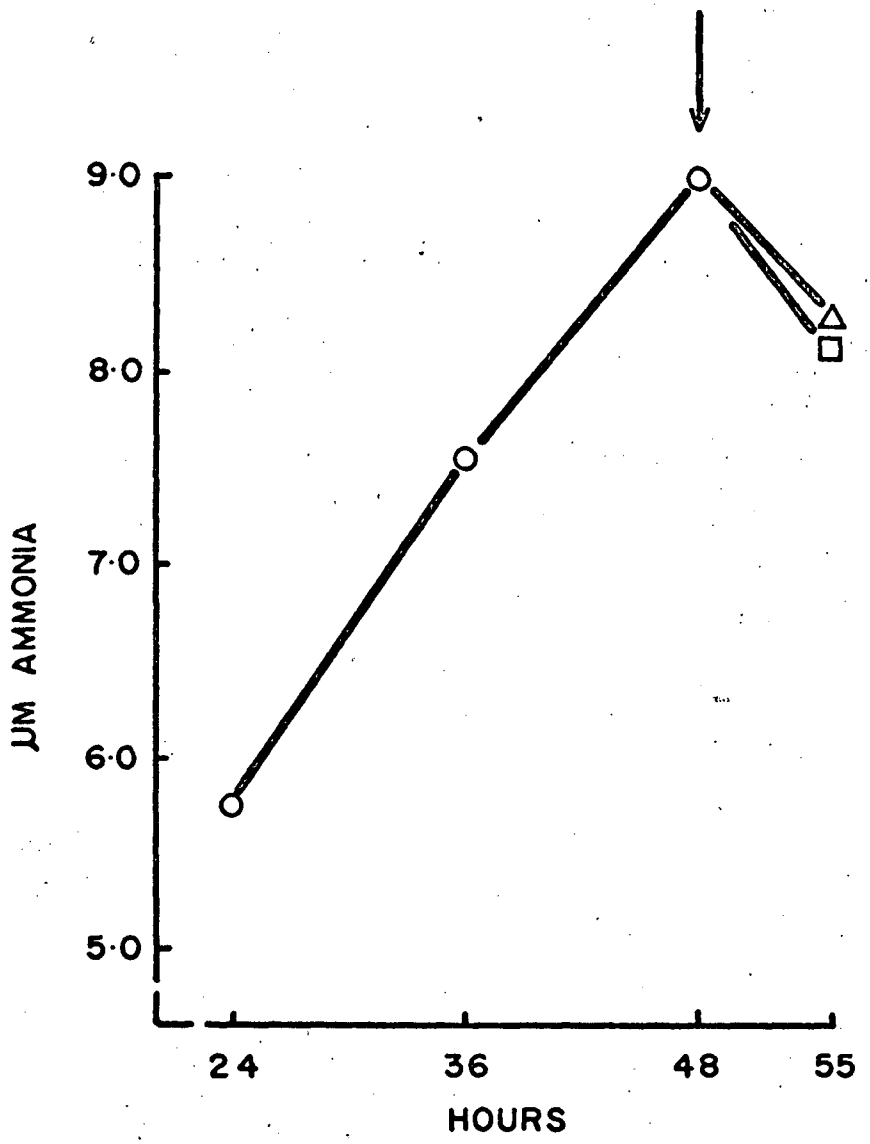

Figure 18. Uptake of endogenously produced ammonia by 6.1 mg dry weight of carbon-limited cells upon the addition of 5 µm of glucose or 7.5 µm of α-ketoglutarate after 48 hr respiration. Ammonia values per cup.

Symbols: circles, endogenous; triangle, glucose; square, α-ketoglutarate; arrow, time of substrate addition.

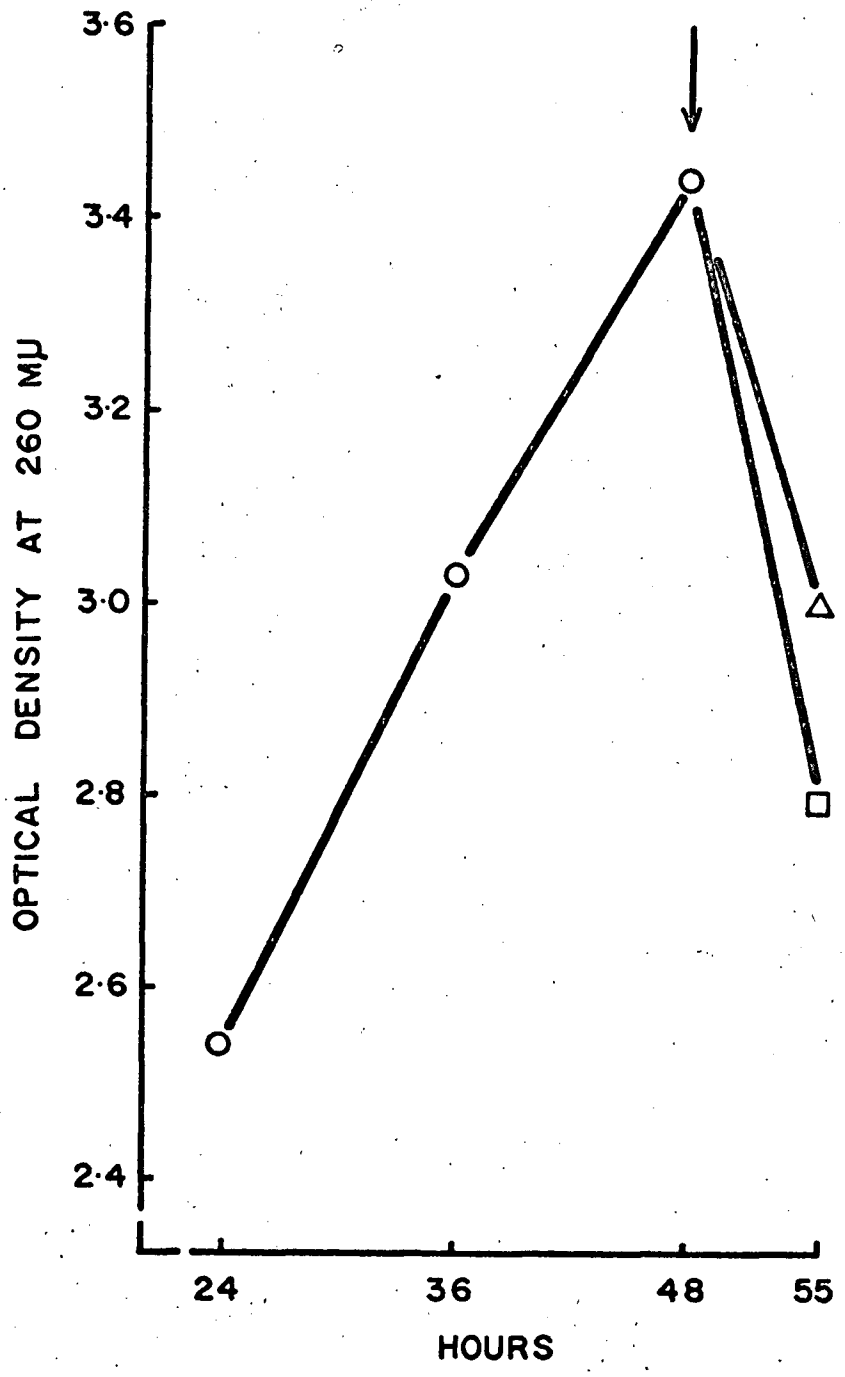

Figure 19. Uptake of endogenously produced acid-soluble UV-absorbing material by 6.1 mg dry weight of carbon-limited cells upon the addition of 5 μm of glucose or 7.5 μm of α-ketoglutarate after 48 hr respiration.

Symbols: circles, endogenous; triangle, glucose; square, α-ketoglutarate; arrow, time of substrate addition.

Ribosome sedimentation patterns were obtained from cell-free extracts in 10^{-4}M Mg^{++} at the time of the addition of glucose and seven hours later. From Figure 20 it is evident that there was an increase in the concentration of 50S ribosomes in conjunction with an apparent re-uniting of what are believed to be fragments sedimenting in the 30S region. This finding was confirmed in a separate experiment in which α-ketoglutarate was employed as the substrate, at approximately twice the concentration used in the adaptation studies. Cell-free extracts, containing 10^{-4}M Mg^{++}, were made at the times of adding the substrate to carbon-limited cells which had respired for 48 hr, and after 12 hr further respiration. The ribosome sedimentation patterns are shown in Figure 21, and plate counts made at these times showed a viability of 97×10^8 cells/ml and 95×10^8 cells/ml respectively. It has been suggested by Clifton (1962) and Duncan and Campbell (1962) that one of the functions of oxidative assimilation may be to replenish the endogenous reserves. This hypothesis is substantiated by the finding that, in the absence of significant cell division, there was a regeneration of the 50S ribosomes when starved P. aeruginosa was supplied an oxidisable substrate.

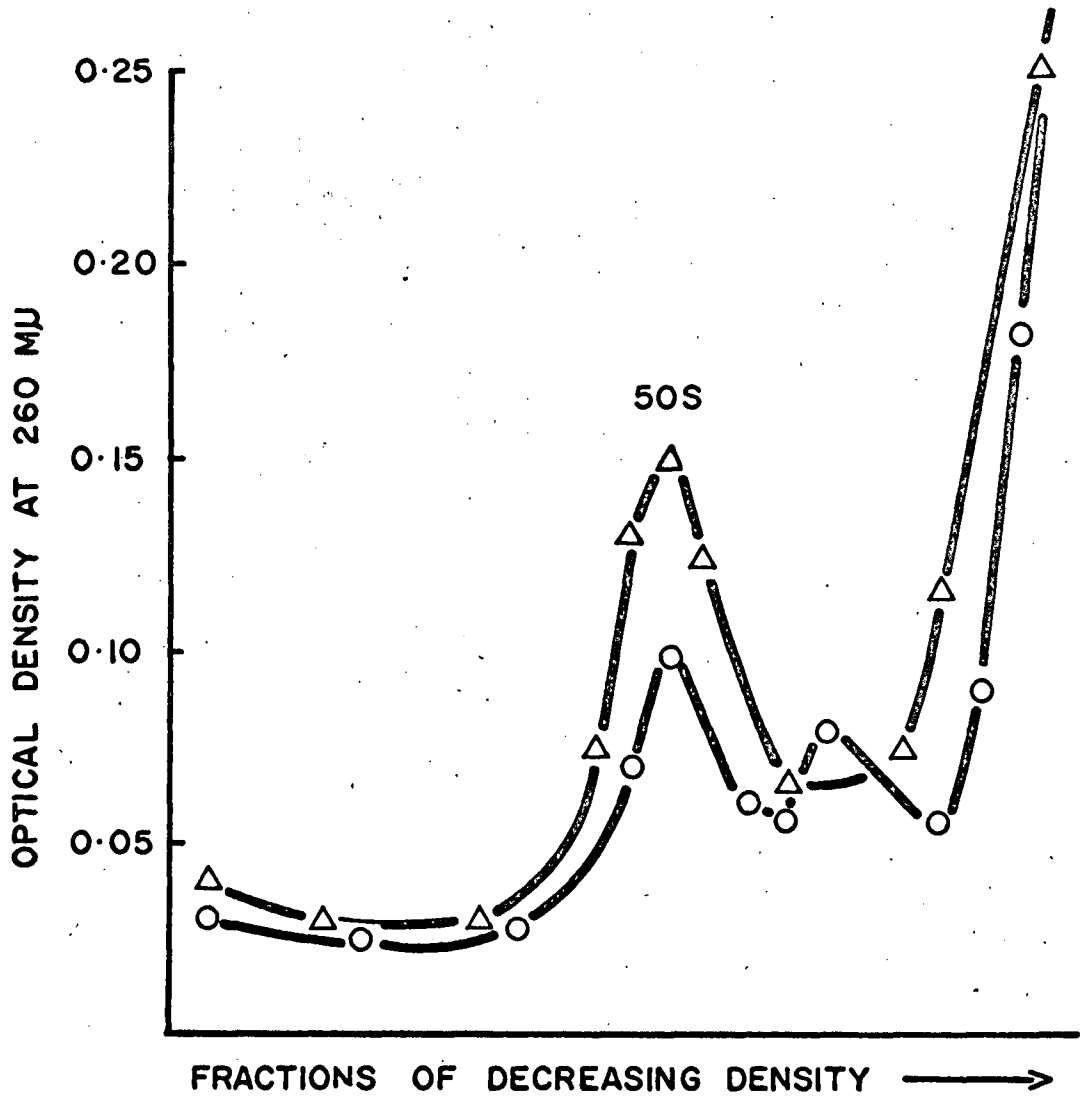

Figure 20. Ribosome sedimentation patterns of extracts of carbon-limited cells at the time of substrate addition and 7 hr later. Glucose added at rate of 0.83 μm per mg dry weight of cells. Gradients contained 10^{-4}M Mg^{++}.

Symbols: circles, time of substrate addition; triangles, 7 hr after substrate addition.

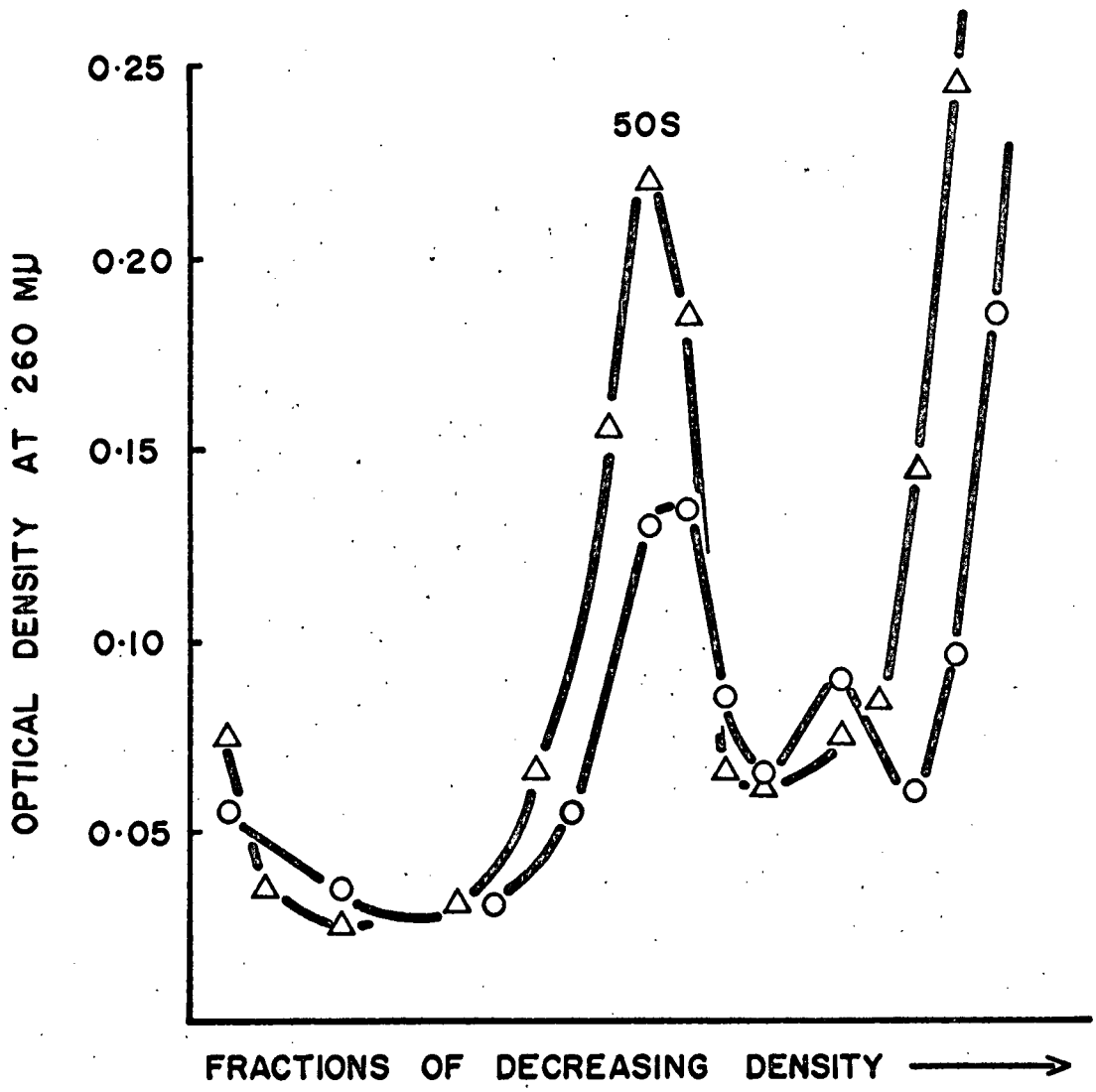

Figure 21. Ribosome sedimentation patterns of extracts of carbon-limited cells at the time of addition of substrate and 12 hr later. α-ketoglutarate added at rate of 2.5 μm per mg dry weight of cells. Gradients contained 10^{-4}M Mg++.

Symbols: circles, time of substrate addition; triangles, 12 hr after substrate addition.

GENERAL DISCUSSION

I. Cold-shock

The susceptibility of log phase cells and the immunity of stationary phase cells to the effects of exposure to the cold have been stressed by most of the workers engaged upon an investigation into the phenomenon of cold-shock (Hegarty and Weeks, 1940; Gorril and McNeil, 1960; Strange and Dark, 1962). The effect of cold-shock was not specifically studied on cells well advanced in the stationary phase, although Strange and Dark noted the sensitivity of A. aerogenes, after 20 hr of starvation, to exposure to the cold.

From the results of the present studies it is apparent that P. aeruginosa, following prolonged starvation, is highly susceptible to cold-shock as evidenced by a loss in viability, and the release of intracellular enzymes and an excessive amount of UV-absorbing material to the suspending fluid. The latter could possibly be attributed to an accelerated degradation of RNA, similar to that which is reported to occur in A. aerogenes as a result of exposure to the cold (Strange and Postgate, 1964). The traumatic effect of cold-shock most widely implicated has been damage to the permeability mechanisms of the cell membrane; however, the presence of 50-60% of the total catalase and

glucose-6-phosphate dehydrogenase in the suspending fluid after 3 hr respiration of 48 hr P. aeruginosa shows that a major breach of this barrier had occurred, and a general debility in these cells is indicated.

A changed pattern of endogenous metabolism due to cold-shock was seen in the elevation of the O_2:NH_3 ratios, and this is attributable to the presence of an oxidisable substrate, or substrates, found to be present in the filtrate of a shocked suspension of P. aeruginosa. The ability of this material to incorporate ammonia suggests that it is comprised of carbonaceous substances or substances with a high carbon to nitrogen ratio.

II. The Effect of the Growth Medium upon Endogenous Metabolism

A comprehensive search failed to reveal the presence of any of the recognised storage compounds when cells were grown in a medium considered favourable for their formation. Also, the immediate release of ammonia during the respiration of cells which had been grown for 12, 24 and 48 hr in the presence of excess carbon in a chemically-defined medium, indicates that no specific reserve product is laid down by P. aeruginosa. These findings confirm an earlier suggestion to this effect which was made when oxidative assimilation was studied in this organism (Duncan and Campbell, 1962).

Nevertheless an elevated O_2:NH_3 ratio was evident in the early stages of respiration of 48 hr nitrogen-limited cells in contrast to similarly aged carbon-limited cells,

and this was not found attributable to a carry over of carbohydrate from the medium in the intracellular pools. In view of the previous experience with cold-shocked cells, it is suggested that the 48 hr nitrogen-limited cells may suffer some degree of damage during the harvesting and washing procedures at room temperature, and to which similarly aged carbon-limited cells are, for some reason, immune. The carbon-limited cells were noticeably slimy before harvesting and it is possible that this extracellular slime may have afforded some measure of physical protection against the rigours of centrifugation and resuspension which was denied to the nitrogen-limited cells. The latter cells, however, gave rise to a froth in the washing and suspending buffer, presumably caused by leakage of intracellular material, and this may be indicative of more delicate surface structures. It is debatable whether carbon starvation is more, or less serious to the bacterial cell than nitrogen starvation; but it is conceivable that under conditions of nitrogen starvation, where endogenously produced ammonia is reincorporated, the cells may endeavour to divide with a consequent stretching of the available nitrogen to limits which endanger the integrity of the cell when subjected to harsh treatment.

III. Endogenous Metabolism during Protracted Periods of Respiration

1. Intercellular reaction

The contribution that cannibalism may have made to the survival of P. aeruginosa during prolonged starvation is difficult to assess. A net loss in viability of approximately 25% was demonstrated in the cells grown in the chemically-defined media during 48 hr of starvation, and possible regrowth is masked in this value. The percentage of the total protein found to be extracellular increased by only 6% during the starvation period, and this may be taken as a more reliable criterion of cell lysis in view of the fact that an inability of a cell to multiply on plate count agar does not necessarily indicate that it is devoid of all metabolic ability. However, leakage from moribund cells, in the absence of gross lysis, may have contributed to the presence of extracellular RNA, some of the degradation products of which have been shown to be oxidized without lag, although at a slow rate, when supplied exogenously to P. aeruginosa (Campbell, Gronlund and Duncan, 1963). With the disappearance of approximately 60% of the total RNA in 48 hr respiration it is unlikely that some degree of cannibalism can be excluded as a factor in the survival of those cells which were allowed to respire for a further 4 days.

2. Glucosamine as a substrate for endogenous metabolism

On the basis of the data which have been presented,

glucosamine cannot be considered as other than an incidental substrate for endogenous metabolism in P. aeruginosa. In contrast to Gram-positive bacteria, the cell walls of Gram-negative organisms, while being rich in lipid content and variety of amino acids, contain a relatively low proportion of amino sugars (Salton, 1960). The latter are found in the corseting lattice (mucocomplex substance) which confers rigidity upon the bacterial cell, and it is questionable whether much of this structure could be sacrificed, particularly by a Gram-negative organism, without detriment to the integrity of the cell. It is nevertheless possible that other constituents of the wall may be utilized during endogenous respiration, and thus determinations of glucosamine may be unreliable as an index of the utilization of cell wall material.

3. The ability of P. aeruginosa to survive

The relationship between the presence of specific reserve products and survival has been discussed by Dawes and Ribbons (1964). The tenacity to life demonstrated by starving suspensions of P. aeruginosa makes it questionable whether survival would have been enhanced had this organism the ability to lay down a storage product. In the absence of growth, the ribosomes have been shown to be dispensable, and thus prior to the commencement of the respiration period, which was approximately 30 hr after the exhaustion of the

growth-limiting element, the ribosome complement was already depleted. Although these bodies were largely solubilized during subsequent starvation, 40% of the 0 hr concentration of RNA still remained available for endogenous utilization after 48 hr, thus showing that the population was not in danger of immediate extinction.

That this organism was able to synthesize a permease for α-ketoglutarate at the end of the experimental starvation period despite an apparent solubilization of the ribosomes indicates that some functional ribosomes were present. These were probably relatively few in number, but the concentration was seen to increase during the oxidation of exogenously supplied α-ketoglutarate in the presence of the byproducts of endogenous metabolism, and thus a remarkable recuperative ability in this organism was demonstrated. A scavenger organism such as P. aeruginosa metabolises a broad variety of substrates and it is suggested that its adaptive capability, even after the cells have experienced extreme conditions of starvation, contributes to the survival of the species.

SUMMARY

P. aeruginosa, when well advanced in the stationary phase of growth, was found to be highly sensitive to exposure to the cold as seen by an extensive loss of intracellular enzymes during a subsequent period of respiration at 30 C, and the effects of cold-shock were shown to be mitigated by the presence of magnesium ions in the suspending buffer.

The presence of a specific reserve product for utilization during endogenous metabolism could not be demonstrated in cells incubated for an extended period of time in a chemically-defined medium which contained glucose in excess of that required for purposes of growth, and ammonia was found to be released immediately upon the respiration of the organism regardless of the medium in which it was cultured or the age at which it was harvested.

Prolonged starvation in the chemically-defined growth media showed that there was a reduction in the ribosome complement after the cessation of growth, and the ribosomes were found to disappear almost completely from 48 hr cells harvested from the defined or complete media when starved for a further 48 hr. The concentrations of protein and RNA were found to decrease, and the concentrations of DNA to increase during endogenous metabolism, and glucosamine was shown not to be a major metabolite.

Constituitive enzymes and/or the cofactors involved in the oxidation of glucose were found to diminish with progressive starvation, and an ability to adapt to, and oxidise α-ketoglutarate was demonstrated after a starvation period which had reduced the ribosome complement to negligible proportions. The addition of an exogenous substrate was shown to reincorporate endogenously liberated ammonia and material absorbing UV at 260 mμ , and a concurrent reformation of the 50S ribosomes was noted.

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