The Survival of *Peptococcus prevotii* in Relation to the Adenylate Energy Charge

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SUMMARY

Starvation of *Peptococcus prevotii* caused extensive loss of RNA which produced a large flux of adenine nucleotides. The adenylate energy charge remained essentially constant over the period of rapid decline of RNA but then fell when the residual RNA was being degraded more slowly. Loss of viability became more rapid when the adenylate energy charge fell below 0.4 to 0.5.

INTRODUCTION

The adenylate energy charge is a useful concept introduced by Atkinson (1968) to enable the energetic state of a biological system to be expressed quantitatively. It is defined as

$$\frac{[\text{ATP}] + 0.5 [\text{ADP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]}$$

and thus depends on the individual concentrations of ATP, ADP and AMP. Chapman, Fall & Atkinson (1971) have demonstrated that in *Escherichia coli* an adenylate energy charge of 0.5 or less is incompatible with high viability. We have investigated the changes in adenylate energy charge during starvation of the anaerobe *Peptococcus prevotii*, an organism that ferments purines and a limited number of amino acids while having an extremely limited ability to attack carbohydrates (Whiteley, 1957). Work in this laboratory (unpublished) has shown that the only amino acids fermented appreciably by *P. prevotii* are serine and threonine which are thus major energy-yielding substrates. Serine is metabolized by deamination to pyruvate, thioclastic fission of pyruvate to acetyl-CoA, CO₂ and H₂, and conversion of acetyl-CoA to acetate, via acetyl phosphate, with the generation of one mole of ATP.

METHODS

Growth of organism. *Peptococcus prevotii*, ATCC 14952, was grown at 37 °C in still culture in medium containing (g/l): KH₂PO₄, 25; NaCl, 5; Difco 'Bactopeptone', 20; Difco yeast extract, 10; and sodium thioglycollate, 1. The pH was adjusted to 7.2 with NaOH. Bacteria were grown in either 1 l flasks (small scale) or 10 l aspirators (large scale). Fully grown cultures yielded approx. 50 µg dry organisms/ml but the yield and characteristics of growth varied slightly, apparently according to the batch of yeast extract used for preparing the medium. The initial exponential period was followed by a long period of slow, arithmetic growth. It was the extent of this latter phase of growth which varied according to the batch of yeast extract.

The organism was maintained by subculturing 250 ml liquid cultures every 2 to 3 days.

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The organism was preserved as stab cultures in solid medium composed of growth medium supplemented with 15 g Oxoid no. 1 Agar/l.

Starvation of organisms. Bacteria were harvested late in the period of arithmetic growth and just before the onset of stationary phase. They were washed with 67 mM-KNa phosphate buffer, pH 6.8, and then resuspended in the same buffer at approx. 0.8 mg dry wt/ml. The suspension was placed in a starvation vessel comprising a round, glass flask (1 l) having facilities for gassing the suspension and for introducing and withdrawing solutions aseptically (Dawes & Holms, 1958). For small-scale starvation experiments a three-necked round flask (150 ml) was used, one neck carrying a tube for gassing the suspension. All operations were carried out under anaerobic conditions and with aseptic precautions. All glassware was treated with hot 10 % nitric acid and washed with distilled water. The buffer solutions were made up with double glass-distilled water.

In large-scale starvation experiments, 10 l of culture were harvested at 24 000 g on a Sorvall RC2-B centrifuge fitted with a Szent-Györgyi & Blum type KSB-R continuous flow system (Ivan Sorvall Inc., Norwalk, Connecticut, U.S.A.). About 3 h elapsed from the start of harvesting until it became possible to remove the first sample of starving organisms from the starvation vessel. In small-scale experiments, up to 1 l of culture was harvested at 5000 g in an M.S.E. Mistral 6 L centrifuge (M.S.E. Ltd, Crawley, Sussex). The harvesting and washing procedure occupied 1 h and was performed as near as possible to 37 °C, but it proved more difficult to achieve this in the large-scale experiments.

Starvation was carried out at 37 °C. The starvation vessel was shaken gently on a flask shaker (Griffin & George Ltd, P.O. Box 11, Ledson Road, Manchester) and a slow stream of wet, sterile, oxygen-free nitrogen was passed through the suspension. Small samples (up to 10 ml) were removed through a port closed with a Suba Seal (Freeman & Co. Ltd, Barnsley, Yorkshire) using sterile needles and syringes. Larger samples were removed via a tap at the bottom of the vessel.

Determination of viability. The slide-culture method of Postgate, Crumpton & Hunter (1961), modified for use with anaerobic bacteria (Dawes & Large, 1970), was used except that the culture was not sealed with a coverslip and all manipulations were performed under a stream of sterile, oxygen-free nitrogen.

Measurement of ATP, ADP, and AMP. ATP in bacterial extracts was measured by the firefly luminescence method using a high-gain photomultiplier tube as described by Dawes & Large (1970) except that the output of the photomultiplier tube was measured by a Smith 'Servoscribe' linear chart recorder (Smith Industries Ltd, Wembley, Middlesex).

ADP and AMP in extracts were determined as ATP after enzymic conversion of each to ATP (Johnson, Hardman, Broadus & Sutherland, 1970).

Bacterial extracts were prepared by pipetting samples of bacterial suspension into sulphuric acid (to give a final concentration of 0.3 M). After 10 min a predetermined volume of M-NaOH was added to pH 7.3. Denatured material was removed by centrifuging and the extracts were stored frozen. The extracts were assayed for adenine nucleotides within 48 h of preparation. Correction was made for adenine nucleotides in the supernatants of samples taken simultaneously, and immediately centrifuged; it was negligible up to 12 h starvation and quite small thereafter. Because of the high ionic strength of the neutralized extracts, which decreases the light output in the luminescent reaction, the solutions were diluted tenfold with water before determination of the adenine nucleotides. The method of internal standards was used to determine the ATP in the extracts.

Protein and RNA estimation. Bacteria were harvested from starving suspensions by centrifuging, and RNA and protein determined by the methods of Herbert, Phipps & Strange (1971).

Fig. 1. Changes in protein ($\square$), RNA ($\circ$), total adenine nucleotides ($\triangle$) and adenylate energy charge ($\bullet$) during starvation of *Peptococcus prévotii* (large-scale experiment). Bacteria from a 10 l culture were harvested, washed and starved at 0.84 mg dry wt/ml (total volume, 450 ml). Samples (9 ml) of the suspension were taken at intervals for determination of adenine nucleotides. Protein and RNA estimations were made on 15 ml and 20 ml samples respectively of the starving suspension.

Serine and ammonia determinations. Serine was determined by the ninhydrin method of Yemm & Cocking (1955). With serine-supplemented suspensions of the organism, the ammonia produced by metabolism of the amino acid interfered and a correction was applied. Ammonia was determined by nesslerization.

RESULTS AND DISCUSSION

Adenine nucleotides and adenylate energy charge during starvation of Peptococcus prévotii

Changes in the levels of ATP, ADP, AMP, bacterial protein and RNA were measured in a large-scale experiment and the adenylate energy charge was derived (Fig. 1). The initial increase in total adenylate at 5 h was reflected by increases in the three individual nucleotide concentrations; thereafter all decreased as starvation continued. The typical microbial storage materials, namely glycogen, polyphosphate and poly- β -hydroxybutyrate (Dawes & Senior, 1973), were not detected. It is clear that RNA is the only endogenous substrate used substantially during starvation and that the small decline in protein is not significant as an energy source. These results were highly reproducible and all similar experiments demonstrated the large flux in adenine nucleotides corresponding to a period of rapid breakdown of RNA. The adenine nucleotides are presumably formed by the successive action of polynucleotide phosphorylase and adenylate kinase, both of which have been detected readily in normal and in starving cultures of *P. prévotii* (J. G. Morton, unpublished).

Table 1. *Intracellular levels of adenine nucleotides in starving Peptococcus prévotii (small-scale experiment)*

Bacteria from 800 ml of culture were harvested, washed and starved at 1.06 mg dry wt/ml (volume, 40 ml). Samples (3 ml) of the suspension were taken at intervals for determination of adenine nucleotides.

Time from start of harvesting (h)	Adenine nucleotides (nmol/ml suspension)				Adenylate energy charge
	ATP	ADP	AMP	Total	
1	5.76	2.21	0.43	8.40	0.82
1.5	1.94	0.55	0.14	2.63	0.85
2.25	1.30	0.52	0.26	2.08	0.75
3	1.82	0.46	1.21	3.49	0.59
4	2.39	1.92	1.59	5.90	0.57
6	2.33	1.64	1.37	5.34	0.59
9.5	1.30	1.04	1.04	3.38	0.54

We have not investigated the levels of ribonucleases in this organism but it is possible that these enzymes, if present, would cause a contribution to the pool of AMP if they are of the appropriate specificity.

The events which occur in the 3 h period after harvesting starts and before sampling of starving bacteria commences are clearly of interest. The organisms do not all enter starvation simultaneously but do so continuously as they are removed from the culture medium over the 90 min required for harvesting. Hence there will be a wide range of physiological states represented when sampling begins.

In some experiments small samples (up to 200 ml) of culture were harvested and washed rapidly, independent of the bulk of the culture which was still being harvested. Protein and RNA estimations on these rapidly harvested bacteria showed that very little decline in either polymer would have occurred over the 3 h required for harvesting and washing the bulk of the bacteria, i.e. the degradation of RNA shown in Fig. 1 had just started when the first sample was taken.

This conclusion is supported by small-scale starvation experiments. The quantity of organisms involved in such experiments did not permit a continuous assay of RNA, but the changes in levels of adenine nucleotides could be measured readily. Also, since the time required for harvesting and washing is about 1 h, these changes could be measured during very early starvation. The results from such an experiment (Table 1) were, as with the large-scale experiments, quite reproducible. There was a rapid drop in total adenine nucleotides during early starvation. We assume this was due to the fermentation of the purine moiety after dephosphorylation of AMP and, as AMP was removed, the equilibrium potentiated by adenylate kinase provided a continual source of AMP. This drop was followed by a very steep increase in the total adenine nucleotides which, from the experience of the large-scale experiment, we attribute to the degradation of RNA. Finally, the level of total adenine nucleotides fell as purines were fermented and supplies were not replenished fast enough by the decay of RNA, which occurred at a much lower rate as starvation proceeded.

The flux in adenine nucleotides occurred at approximately the same time, relative to the start of harvesting, with both the large-scale and small-scale experiments. However, there is one discrepancy between the two types of experiment. The adenylate energy charge over the period of rapid decay of RNA was approximately constant at just below 0.5 in the large-scale experiments, whereas in the small-scale experiments the value, although almost con-

Fig. 2. Loss of viability in starving *Peptococcus prévotii* in large-scale (open symbols) and small-scale (closed symbols) experiments.

stant, was nearly 0.1 unit higher. We can offer no explanation for this difference but it may be related to the fact that it was simpler to harvest and wash bacteria at or near 37 °C in the small-scale experiment than it was in the large-scale experiment. In the latter case the temperatures at which the washed bacteria were added to the starvation vessel may sometimes have been as low as 27 °C.

Despite the higher energy charge in the small-scale experiment there was no obvious difference in the decline in viability between large-scale and small-scale experiments (Fig. 2). Apart from one large-scale experiment (symbol, □) both types of experiment show a decline in viability followed by a more rapid decline after about 10 h starvation. (In this single experiment the 'peak' of ATP production was not so marked and was more spread than in the typical experiments.) Chapman *et al.* (1971) have indicated the critical nature of the value of 0.5 for the adenylate energy charge in *Escherichia coli* and possibly in other bacteria. The change in rate of loss of viability found in our experiments coincides with the attainment of a very low value for the level of ATP (about one-hundredth of the value for growing bacteria) and consequently a strong tendency for the energy charge to fall below 0.5. Thus although our results cannot distinguish whether it is the fall in ATP levels or the lower adenylate energy charge which might be responsible for the increased loss in viability after 10 h starvation, the results are not incompatible with the hypothesis of Chapman *et al.* (1971).

Energy of maintenance and starving Peptococcus prévotii

The concept of energy of maintenance (Dawes & Ribbons, 1964) suggests that there is a specific rate of consumption of ATP during starvation to provide energy for essential processes and reactions. If the rate of regeneration of ATP is high then a pool of ATP will be established. When the endogenous substrates available for ATP regeneration become

Fig. 3. Stimulation of ATP levels in starving *Peptococcus prevotii* by serine. Bacteria from a 10 l culture were harvested, washed and suspended in buffer (450 ml) at a concentration of 0.70 mg/ml. The suspension was distributed equally between three starvation flasks. Soon afterwards, samples (9 ml) were removed alternately from two flasks for estimation of ATP. A solution of serine in phosphate buffer (to give a final concentration of 10 mM) was added to one of the two flasks and sampling of both flasks continued. The following day, serine (final concentration, 10 mM) was added to the third flask and samples (9 ml) for ATP estimation were taken before and after the addition. (A) Serine added 3 h 30 min after harvesting started (○), and no serine added (●). (B) Serine added 27 h after harvesting started.

exhausted, or nearly so, the rate of consumption of ATP is limited by the rate of ATP regeneration and the ATP pool will be very small. As a consequence the ADP and AMP pools become large relative to the ATP pool. This happened after 10 to 12 h starvation of *Peptococcus prevotii* under our conditions, when the ATP levels became too low to be measured accurately by our methods. The situation is a little complicated by the fact that purines are fermented by *P. prevotii*, so that the total quantity of adenine nucleotides was being depleted continuously although the fermentation did lead, of course, to regeneration of ATP.

We have therefore studied the ability of starving *Peptococcus prevotii* to regenerate ATP when supplied with a suitable energy source. Serine (10 mM) produced an immediate and large increase of ATP when added to organisms starved for about 3 h (Fig. 3). A much smaller response was given by organisms starved for 27 h and which had zero viability at that time. Nevertheless, ATP production occurred and although the actual amount detected after addition of serine at 27 h was low by comparison with the levels of ATP generated by serine during early starvation, there may be several reasons for this:

1. After lengthy starvation the permeability characteristics of the bacterial membrane may have altered so that the organisms were less able to take up serine. Assay of serine in the suspending medium supports this view, e.g. in one such experiment the rates of serine disappearance after starvation for 1 h and for 22 h 44 min were, respectively, 22.2 and 1.1 $\mu\text{mol/mg dry wt/h}$.
2. The levels of ADP and AMP are lower after prolonged starvation than during early starvation (this was true of the experiment of Fig. 1), and this may restrict the rate of regeneration of ATP.
3. The ATP-regenerating pathways may become impaired during starvation. We have checked this possibility by assaying the enzymes serine dehydratase, thioclastic enzyme,

Fig. 4. Changes in RNA, viability and adenylate energy charge in starving *Peptococcus prévotii* supplemented with serine. RNA estimations (○, ●) were made on 20 ml samples of the starving suspensions used in the experiments described in Table 2. Viabilities (□, ■) were also determined. Adenylate energy charges (Δ, ▲) are calculated from Table 2. Open symbols: control bacteria; closed symbols: serine-supplemented bacteria. The RNA contents at the time of first sampling were 93 and 86 $\mu\text{g/ml}$ suspension respectively for control and serine-supplemented bacteria (12.9 % of the dry bacterial weight in each case).

phosphotransacetylase and acetate kinase (C. M. Bentley, unpublished) and, of these, only serine dehydratase lost activity (about 40 %) over 24 h starvation.

We conclude that *Peptococcus prévotii* organisms starved for a long period are capable of regenerating some ATP and that the loss of viability observed when the bacteria starve is not necessarily due to failure of the ATP-regenerating system. If the energy of maintenance concept is valid, then death is due to failure of essential processes caused by an inability to regenerate ATP at a fast enough rate when the endogenous fuel (purines) is depleted.

One feature of our results is puzzling, namely the quantity of ATP produced in response to serine during early starvation. At the time serine is added to the suspension the total of adenine nucleotides is 3 to 5 nmol/mg dry weight of bacteria (Fig. 1, Table 1). After addition of serine the ATP concentration alone is over 10 nmol/mg (Fig. 3). Since it is extremely unlikely that adenine nucleotides are synthesized *de novo* in the short time in which the ATP levels rise, the results suggest that there may be a store of polyadenylic acid or an adenylate-rich portion of RNA which is broken down in response to a stimulation of ATP

Table 2. *Effect of serine on levels of adenine nucleotides in starving Peptococcus prévotii*

Bacteria from a 10 l culture were harvested: half were washed with 67 mM-phosphate buffer, pH 6.8, and half with similar buffer containing serine (20 mM). The washed organisms were re-suspended in fresh quantities (225 ml) of the wash buffers and the suspensions placed in separate starvation flasks, serine-supplemented bacteria at 0.67 and control bacteria at 0.72 mg dry wt/ml. Samples (3 ml) were withdrawn at intervals for estimation of adenine nucleotides.

Time from start of harvesting	Serine concentration (mM)	Adenine nucleotides (nmol/mg dry wt)					
		Control organisms			Serine-supplemented organisms		
		ATP	ADP	AMP	ATP	ADP	AMP
2 h 50 min	15.75	0.33	0.73	0.29	6.60	0.73	0.73
4 h 30 min	0.50	1.78	1.78	2.20	13.20	3.63	1.14
6 h	0	2.53	2.42	2.65	5.26	3.01	1.70
8 h 25 min	0	2.47	0.85	2.30	4.29	1.15	2.02
11 h 20 min	0	1.04	0.34	1.13	3.35	1.26	1.41
16 h	0	0.06	0.47	0.36	1.80	2.01	1.73

production. Polyadenylic acid has not been isolated from natural sources although recent work (Ohasa & Tsugita, 1972; Ohasa, Tsugita & Mii, 1972) has demonstrated the presence of polyadenylate synthetase in *Escherichia coli* which is capable of polymerizing ATP in the absence of primer. Adenylate-rich sequences at the 3'-terminus of messenger RNAs in mammalian systems are well documented (Edmonds, Vaughan & Nakazato, 1971; Darnell, Wall & Tushinski, 1971; Lee, Mendecki & Brawerman, 1971) and are probably universal in eukaryotic messenger RNA.

A consequence of the conclusion that death of *Peptococcus prévotii* during starvation is due to depletion of energy reserves would be that loss of viability should be delayed if the bacteria are supplied with an exogenous energy supply during early starvation. Alternatively, the presence of an external energy supply may not affect the utilization of the endogenous energy source and loss of viability may not be delayed if excess ATP is 'run to waste'. We have investigated these possibilities by allowing the initial stages of starvation to occur in the presence of serine, which should maintain the adenylate energy charge at a high value. Table 2 and Fig. 4 show the results of such an experiment in which 10 l of culture were harvested and the organisms divided into two batches which were then washed and starved separately. With one batch the washing and starvation were performed in the presence of 20 mM-serine and, in this case, the adenylate energy charge was maintained initially but then fell as the serine was utilized completely. After an initial lag the RNA was degraded but at a slower rate than in the unsupplemented organisms. There is thus some evidence that RNA degradation is controlled by adenylate energy charge. However, kinetic factors are possibly of considerable importance in any control mechanism. Our normal procedure for starving bacteria favours RNA breakdown, since at the beginning of starvation the extra-cellular phosphate ion concentration is high and the ADP concentration low. With serine-supplemented organisms the ADP concentration was higher than with unsupplemented bacteria and RNA breakdown should have been less favoured with serine-supplemented organisms. Fig. 3 shows this to be so. We cannot apportion the contribution of adenylate energy charge and kinetic factors to the control of RNA breakdown as, to our knowledge, no work has been published on the controls governing the activity of polynucleotide phosphorylase and we do not know the effect of adenylate energy charge on this enzyme.

Thus loss of viability in starving *Peptococcus prévotii* can be delayed by supplying the

bacteria with an energy source, and this supports the conclusion that death of this organism is a result of the depletion of its energy reserves.

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