BBA 45584

THE ATP POOL IN *ESCHERICHIA COLI*

I. MEASUREMENT OF THE POOL USING A MODIFIED LUCIFERASE ASSAY

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(Received April 6th, 1967)

SUMMARY

A sensitive modification of the luciferase assay for ATP is described. Light output is measured using a conventional liquid-scintillation counter. ATP over the range 2 to 20 $\mu\mu$moles can be assayed. The sensitivity of the method allows samples to be taken rapidly from growing cultures of bacteria without concentrating them from the medium. Chilling and anaerobiosis of growing cells before extracting with $HClO_4$ have been shown to cause a reduction in the ATP pool. ATP measurements in growing cultures of *Escherichia coli* show four different phenomena depending on culture conditions: (a) ATP production is in balance with growth. (b) ATP is consistently over-produced. (c) ATP is consistently under-produced. (d) Cyclic oscillations in ATP production are observed.

Doubling times for growth and ATP production are compared, and the ATP pool in cells grown under carbon-limiting conditions in a chemostat has been measured. Starvation experiments have shown that the ATP pool drops more rapidly anaerobically than aerobically.

INTRODUCTION

The amount of ATP produced by growing microorganisms appears to be well controlled, since the growth yield is proportional to the calculated amount of ATP produced from the energy source[1]. It has therefore been suggested that the intracellular ATP concentration is related to the energetic requirements of the organism[2]. In *Streptococcus faecalis*, the amount of ATP per known bacterial mass, *i.e.* the ATP pool, falls at a constant rate during exponential growth[3]. In yeast, the ATP pool also falls during growth[4]. The pool of ATP in harvested, resuspended *Aerobacter aerogenes* drops more slowly aerobically than anaerobically[5].

Since *Escherichia coli* can grow on complex or minimal media both aerobically and anaerobically, with a variety of energy sources, measurement of the ATP pool level throughout growth under these varied conditions should show the extent of its control by the organism. It has been reported that, at a constant absorbance, the ATP pool is constant in *E. coli* growing aerobically on a variety of media and

hence at greatly varying rates[6]. The present work examines the ATP pool at various stages of growth and starvation under different conditions.

METHODS

Growth of the organism

E. coli, strain K_{12}, was maintained and grown on the media described in a previous paper[7], except that tryptone (Oxoid) was used instead of casamino acids. Sterile glucose, glycerol or sodium pyruvate were added to the media, when desired, to give a final concentration of 0.4 % (w/v).

Inocula were grown in tryptone soya broth or in the same medium as subsequent growth and then either shaken aerobically or gassed with 95 % N_2–5 % CO_2 during growth at 37°.

Growth of batch cultures was in 5- or 15-l fermenters containing 3 or 11 l of medium, respectively, and a 1 % inoculum. The fermenters were attached to a Microferm (New Brunswick Model MF-107). The temperature was controlled at 37°. The culture was agitated mechanically and gassed with air or with a 95 % N_2–5 % CO_2 mixture. The pH was maintained at 7.0 ± 0.1 by an Analytical recording pH meter controlling the input of 5 M KOH through a peristaltic pump. In some experiments cells grown up to an absorbance of 0.6 in one fermenter were used as a 1 % inoculum for another fermenter.

The same organism was grown in a chemostat as described by ELSWORTH *et al.*[8]. Growth conditions were otherwise identical to those for batch culture.

The progress of growth was followed by measuring the absorbance of samples at 550 mμ in a Unicam Model SP 600 spectrophotometer. Cells were harvested in the cold and washed with 0.01 M potassium phosphate buffer (pH 7.4). They were resuspended in buffered saline containing 0.05 M potassium phosphate buffer (pH 7.4), 0.45 % NaCl and 5 g/l of the salts solution used in the growth medium. The suspensions were maintained at 37° in a water-bath and water-saturated air or 95 % N_2–5 % CO_2 was passed through by means of spargers.

Extraction of ATP

This was extracted by $HClO_4$. A few millilitres of the medium in which the organisms were growing were taken every few minutes from the growth vessel. The liquid was stirred rapidly if the culture was aerobic or left still if it was anaerobic. A 2-ml sample was shot into 0.5 ml of ice-cold 30 % (w/v) $HClO_4$. After 10 min on ice the extract was shaken well and then neutralised with 1.5 ml of 1 M KOH; the precipitate was then allowed to settle. If the ATP in the supernatant could not be assayed immediately, the samples were kept at −15°.

Assay of ATP

ATP was assayed by a modification of the method of MCELROY[9]. About 0.02 ml of the extract was added to a mixture of 0.2 ml of 0.2 M glycylglycine buffer (pH 7.4) and 0.7 ml of water, at room temperature. Then 0.02 or 0.05 ml of filtered firefly lantern extract in arsenate–magnesium buffer (Sigma FLE-250), which had been kept at 2° for 24 h after reconstitution and then stored at −15°, was added. The shaken mixture was assayed immediately in an IDL Tritomat scintillation counter (Model

6020). One channel was set at 1385 V and the coincidence control was switched out.

The first 10-sec count was proportional to the ATP in the sample. An ATP standard of 2–20 $\mu\mu$moles, freshly diluted from a 1 mM solution, was assayed at the same time.

MATERIALS

ATP was purchased from Boehringer.

RESULTS

Assessment of the method

There was a standard delay time of 45 sec between the addition of firefly lantern extract to the sample and the first 10-sec count by the scintillation counter. Since the luminescent flash decays rapidly, it was necessary to check the linearity of the standard curve. We found that, under the conditions used, the luminescent decay was exponential for at least the first six 10-sec counts of repeat counting and so it would be easy to extrapolate back to zero time. Later this was found not to be necessary; Fig. 1 shows that there was a linear relationship between the first 10-sec count and the amount of ATP used, up to 15 000 counts/sec, the maximum rate of linear counting under these conditions. A similar linear relationship was found using arsenate buffer, although the counts after this time interval were about a third of those given using glycylglycine buffer. This linear relationship was not found by ADDANKI, SOTOS AND REARICK[10], using a different scintillation counter of rather lower sensitivity. Under the conditions of assay that they used, only the luminescence rate at 7 sec after the addition of ATP was proportional to the quantity of ATP added.

The size of the first 10-sec count given by a constant amount of ATP was proportional to the amount of firefly lantern extract used. The small amount of bacterial extract used did not quench the flash at all.

To find the percentage recovery of ATP, known amounts were added to cold $HClO_4$ immediately after the addition of a bacterial suspension. By comparison with

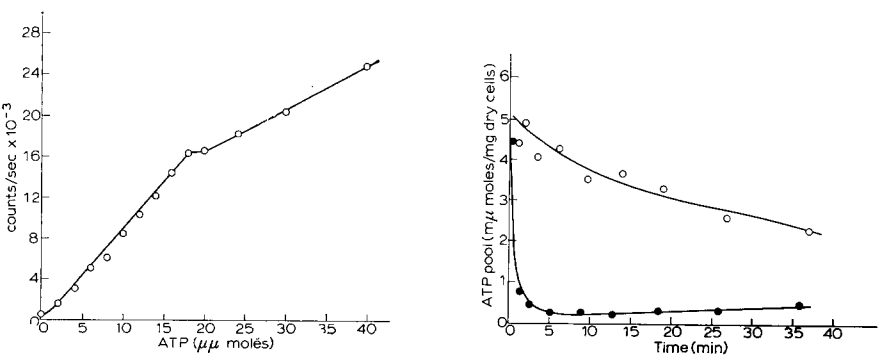


Fig. 1. Relationship of the first 10-sec count to the quantity of ATP, using glycylglycine buffer.

Fig. 2. The effect of cooling and anaerobiosis on the ATP pool in *E. coli*. Stationary phase cells grown aerobically in a synthetic medium at 37° were cooled rapidly in ice-water and were gassed either with air or with 95% N_2–5% CO_2. O—O, gassed with air; ●—●, gassed with N_2–CO_2.

TABLE I

THE EFFECT OF HARVESTING PROCEDURES ON THE ATP CONCENTRATION IN *E. coli*

Cells were grown aerobically up to stationary phase in the medium stated, harvested in a refrigerated centrifuge and washed with cold 0.01 M potassium phosphate buffer (pH 7.4). The ATP pool is expressed as mμmoles/mg dry wt.

	Synthetic glucose	*Complex no glucose*
Before harvest	5.8	7.8
After harvest	3.1	3.1
After washing	0.8	0.34

an extract of the bacterial suspension alone and with the same, diluted solution of ATP, it was found that the percentage recovery was about 100 %.

The amount of ATP extracted from a bacterial suspension in $HClO_4$ at 20° was measured at periods of up to 1 h; the concentration fell 20 % in this time.

A standard solution of ATP in bovine serum albumin was extracted and assayed as described in METHODS. A total of 26 measurements were made from 10 identical samples. The mean count was 3600 counts/sec and the standard deviation from this figure was 340 counts/sec.

Previous workers, using less sensitive assays for ATP, have found it necessary to chill and harvest bacteria before acid extraction. This procedure has been examined. Table I shows the level of ATP measured before, during and after the harvesting of cells grown aerobically on both defined and complex media. This procedure clearly leads to a large reduction in the ATP pool.

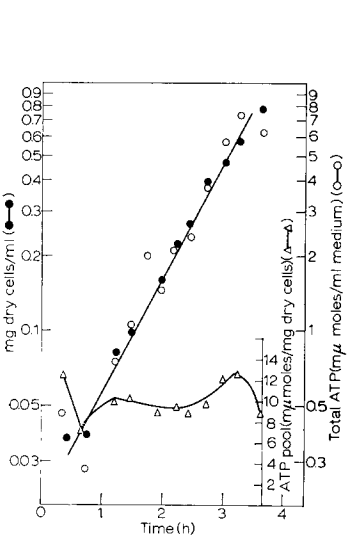
Suspensions of growing bacteria have been chilled aerobically or anaerobically and their ATP levels followed as a function of time (Fig. 2). It can be seen that the greatest reduction in level occurs during chilling anaerobically. These results throw doubt on methods for assaying ATP in which (a) a large lag exists between harvesting and extracting ATP for assay; (b) samples are harvested by centrifugation before extracting; (c) samples are chilled aerobically and especially anaerobically before extracting.

In all assays reported in this communication a delay of about 5 sec occurred between sampling and treating with $HClO_4$.

ATP in growing bacteria

ATP was measured during the logarithmic growth of *E. coli* under a variety of conditions. When the growth rate remained constant on a complex medium, the total ATP of the culture increased at the same rate as the growth rate (Fig. 3). Thus under these conditions the rate of ATP production was in balance with the rate of growth. This was confirmed by finding that when the growth rate changed, so did the rate of ATP production. The ATP pool (in terms of mμmoles ATP per mg dry wt. of cells) therefore remained fairly constant. This balance between ATP production and growth was observed in cells growing aerobically and anaerobically on a complex medium containing glucose as energy source and also in cells growing aerobically on a complex medium with no added energy source.

The rate of ATP production was usually more rapid than the growth rate in cells growing on a synthetic medium with glucose or glycerol aerobically or with glucose anaerobically (Fig. 4). In this case, the ATP pool increased during growth. On the other hand, cells growing anaerobically on a complex medium containing both glucose and yeast extract, had a rapid growth rate which was higher than their rate of ATP production; the ATP pool therefore decreased (Fig. 5).



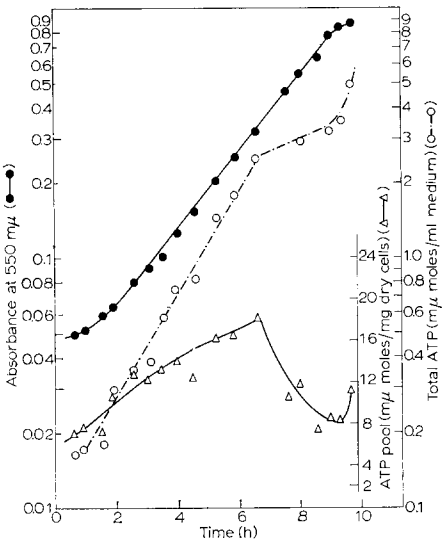


Fig. 3. ATP in *E. coli* during aerobic growth on a complete medium containing glucose. An inoculum of cells growing exponentially in the same medium was used. t_d cells and ATP = 42 min. ●—●, growth; ○—○, total ATP; △—△, ATP pool.

Fig. 4. ATP in *E. coli* during aerobic growth on a synthetic medium containing glycerol. An inoculum of cells growing exponentially in the same medium was used. t_d cells = 66 min; initial t_d ATP = 46 min. ●—●, growth; ○···○, total ATP; △—△, ATP pool.

Periods of marked over- or under-production of ATP were also observed during growth, induced by a number of different and apparently unrelated conditions. A period when the ATP production rate was faster than the growth rate was eventually followed by a decrease in the production rate, which was not necessarily correlated with a decrease in the growth rate (Fig. 4), although the variations in ATP concentration were more marked when the growth rate altered at the same time (Fig. 6).

When ATP production and growth occurred at the same time, a reduction in the growth rate was followed by over-production of ATP for about one generation time and then by under-production for about the same length of time (Fig. 7). This type of oscillation was observed under all the growth conditions used, except on a complex medium containing no added energy source. They were most commonly produced by a slowing of the initial rapid growth found when an inoculum growing in tryptone soya broth was used.

When an aerobically-growing culture was made anaerobic suddenly by changing the gas supply, a short cessation of growth was accompanied by a rapid drop in the ATP pool (Fig. 8). The slower growth that followed was again accompanied by an oscillation in the ATP pool.

The mean ATP doubling times (t_d) for growth and ATP formation and the mean ATP pool levels were calculated for all conditions of growth tested and are summarised in Table II. On average, ATP is formed more rapidly than cell mass under all the conditions used except aerobic growth on a complete medium. The mean ATP pool level is lowest during aerobic growth on a complete medium with no added energy source and highest during aerobic growth on a salts medium with glucose or glycerol as energy source, although growth is much slower on the glycerol medium.

Measurements were also made on the ATP pool in *E. coli* growing in continuous culture under conditions of glucose limitation. From two runs on a complete medium the mean ATP concentration is 5.4 mμmoles/mg dry wt. aerobically and 3.5 mμmoles per mg dry wt. anaerobically. In one run on a defined medium these figures are 7.5 and 3.0 mμmoles/mg dry wt., respectively.

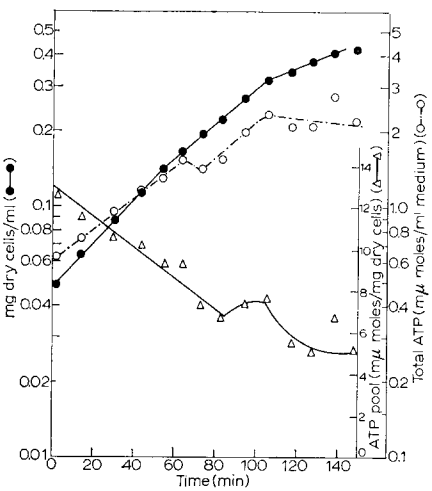


Fig. 5. ATP in *E. coli* during anaerobic growth on a complex medium containing both glucose and yeast extract. An inoculum of cells growing exponentially on the same medium was used. Mean t_d cells = 35 min; mean t_d ATP = 45 min. Symbols as in Fig. 4.

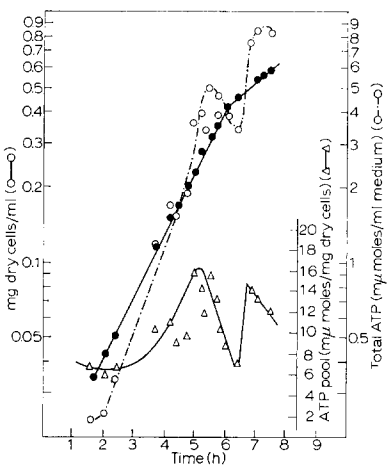


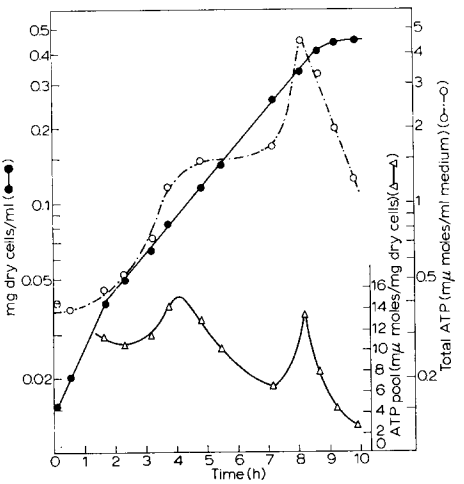
Fig. 6. ATP in *E. coli* during aerobic growth on a synthetic medium containing glucose. An inoculum of stationary phase cells in the same medium was used. t_d cells = 54 min; t_d ATP = 37 min. Symbols as in Fig. 4.

TABLE II

ATP FORMATION IN *E. coli* UNDER DIFFERENT GROWTH CONDITIONS

Medium	*Growth condition*		*Number of experiments*	*Mean cell t_d (min)*	*Mean ATP t_d (min)*	*Mean ATP pool (mμmoles/mg dry wt.)*
	Carbon source	*Aerobic or anaerobic*				
Complete	—	Aerobic	2	40	38	6.6
Complete	Glucose	Aerobic	4	36	37	9.1
Complete	Glucose	Anaerobic	3	42	37	7.4
Salts	Glucose	Aerobic	3	59	43	12.3
Salts	Glycerol	Aerobic	3	81	77	13.5
Salts	Glucose	Anaerobic	2	100	71	9.4

The ATP pool in rapidly harvested, fresh, stationary phase cells fell markedly but was partially restored by resuspension in phosphate–saline at 37°. The subsequent fall-off in the pool was followed in cells grown aerobically on either a minimal medium, or a complete medium with no added energy source. The overall behaviour under starvations of both types of organism was very similar. Aerobically, the pool rose slowly and remained high for 12–20 h before dropping; anaerobically, the concentration both rose and fell much more rapidly (Fig. 9). A similar, transient rise was found in anaerobically-grown cells. Aeration maintained a higher pool level in these cells throughout their starvation.



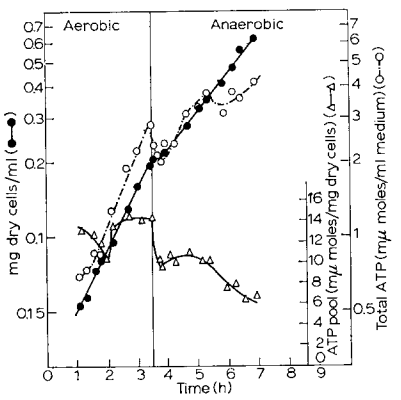


Fig. 7. ATP in *E. coli* during anaerobic growth on a synthetic medium containing glucose. The inoculum was grown in tryptone soya broth. t_d cells = 124 min; t_d ATP = 120–140 min. Symbols as in Fig. 4.

Fig. 8. ATP in *E. coli* changed rapidly from aerobic to anaerobic growth on a synthetic medium containing glucose by changing the gas supply. An inoculum of cells growing exponentially in the same medium was used. Aerobically, t_d cells = 76 min; t_d ATP = 64 min; anaerobically, t_d cells = 118 min; t_d ATP = approx. 200 min. Symbols as in Fig. 4.

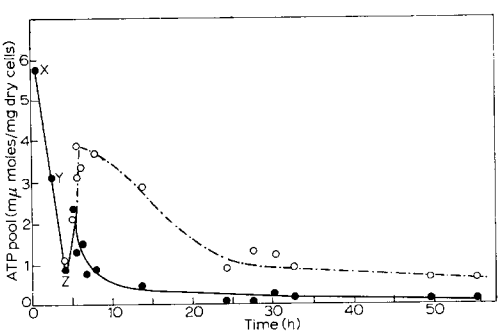


Fig. 9. The ATP pool in *E. coli* starved aerobically and anaerobically. Cells grown aerobically to stationary phase on a synthetic medium containing glucose (X) were harvested, washed in phosphate buffer (Y), resuspended in buffered saline at room temperature (Z) and gassed at 37° with air or N_2–CO_2. O·-·-·O, aerobic starvation; ●—●, anaerobic starvation.

DISCUSSION

Many workers have measured the ATP pool in microorganisms using the luciferase assay. The method used, though simple and relatively sensitive, was not generally capable of measuring the ATP in growing cultures of bacteria without first separating the cells from the medium. In the present paper, the sensitivity of the method was much improved by the use of a conventional liquid-scintillation counter: approx. $2 \cdot 10^{-12}$ mole ATP could be determined and so the ATP in 1 μg (dry wt.) of cells could easily be measured. Because no separation of the cells from the medium was necessary, the delay between sampling and extracting with $HClO_4$ was between 1 and 5 sec. STRANGE, WADE AND DARK[5] used thick suspensions of *Aerobacter aerogenes* to which $HClO_4$ was added directly, so the lag in this case was negligible. We examined the effect of the subsequent extraction procedure used by these workers and observed only a small drop in ATP concentration.

Other workers separated cells before extracting. FRANZEN AND BINKLEY[6] filtered a chilled suspension through Millipore membranes; about 10 min elapsed between sampling and extracting. POLAKIS AND BARTLEY[4] centrifuged yeast cells and froze them in liquid N_2, with a delay time of about 2 min. Our examination of the effect of sampling techniques has shown that low temperature, especially when coupled with anaerobiosis, causes a rapid reduction in the measured ATP pool of *E. coli*. It is possible that the reactions producing ATP in this organism are more sensitive than the utilisation reactions to low temperature and anaerobiosis.

In view of these results, some of the figures quoted by earlier workers for the ATP pool in different microorganisms may be too low, but no careful comparison of the methods has been made.

Measurements of the ATP pool in *E. coli* have shown various effects depending on the conditions of cultivation:

(i) The rate of formation of ATP can be in balance with the rate of growth. This may represent the ideal situation and is normally observed during rapid growth on complete media under aerobic or anaerobic conditions.

(ii) The rate of ATP formation can be greater than the rate of growth. This is observed during growth on a simple salts medium containing glucose or glycerol, aerobically or anaerobically. In this case energy production may not be limiting. Since growth is slower under these conditions, there may be a rate-limiting step in biosynthesis that is not energy dependent.

(iii) Under some conditions there may be an under-production of ATP, as is found during very rapid growth on an enriched complex medium containing glucose, yeast extract and tryptone. The ATP formation rate here is lower than the growth rate and so the ATP pool falls slowly. Similar results have been observed by FORREST[3] during the growth of *Streptococcus faecalis* on a similar medium. It is interesting that the doubling time for ATP production is less variable than the cell doubling time.

(iv) Over-production of ATP during growth can be followed by a period of under-production. This situation can be induced either by (a) using an inoculum dissimilar to the subsequent growth, in which case changes in levels of key catabolic enzymes may be responsible; or (b) changing from aerobiosis to anaerobiosis; or (c) changes in the growth rate. It is presumed that overshoot or hunting mechanisms are responsible for the periodicity of these effects.

Preliminary work with glucose-limited growth in a chemostat indicates that pool levels of ATP under these conditions are lower than in cells grown in similar media in batch culture. The pool level of anaerobic cells is also lower than in their aerobic counterparts. The difference between growth on defined and complete media is eliminated under these conditions, and provides further evidence for the suggestion made earlier that growth on defined media in batch cultures is not energy limited. In similar experiments on *E. coli* grown in phosphate-limited continuous culture, DAMOGLAU AND DAWES[11] found that the ATP pool was constant and very low under all conditions used.

Experiments with washed cells show that the ATP pool is maintained for a longer time aerobically than anaerobically. These results agree closely with those of STRANGE, WADE AND DARK[5] for *Aerobacter aerogenes*.

ACKNOWLEDGEMENT

One of the authors (H.A.C.) was in receipt of a Research Studentship from the Medical Research Council.

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