Studies on ATP, ADP, and AMP Concentrations in Yeast and Beer[1]

D. W. HYSERT and N. M. MORRISON, *Molson Breweries of Canada Limited, Montreal, Quebec H2L 2R5*

ABSTRACT

The bioluminescence adenosine triphosphate (ATP) assay has been used to study the yeast and medium ATP content and changes thereof during growth of 36 different ale, lager, and miscellaneous yeasts at 12°, 18°, and 23°C on brewery wort. Significant species-to-species differences were noted for these variables and for the influence of temperature on them. The contrasting patterns of changes in medium ATP levels for ale and lager yeasts, particularly at the lower temperatures, have been suggested as a means of yeast classification and differentiation. Adenosine diphosphate (ADP) and adenosine monophosphate (AMP) assays, based on the stepwise enzymatic conversion of these compounds to ATP and subsequent assay of the ATP by the aforementioned method have been developed. These assays were used to survey the adenine nucleotide concentrations in Canadian beers. Ale ATP, ADP, and AMP levels were generally much higher and covered a wider range than those for lagers. AMP levels in all types of beer examined were more than ten times higher than the ADP or ATP levels. These assays were also used to monitor yeast and medium adenine nucleotide concentrations during several brewery and laboratory fermentations. Significant increases in medium adenine nucleotide levels were observed in the early stages of ale fermentations, followed by gradual decreases in the latter period. The above data were used to derive yeast energy charge (EC) in terms of the ratio of adenine nucleotides. The EC values at various stages of yeast metabolism and fermentation are used to propose several potential practical applications of EC determinations in brewing research and development.

Key words: *Adenine nucleotides, Beer, Bioluminescence assay, Fermentation, Yeast.*

The importance of ATP analysis in biochemical, microbiological, and related fields of research and development is thoroughly appreciated and has been amply demonstrated (8). The availability of sensitive, rapid ATP assays such as the firefly bioluminescence method developed by Hysert *et al.* (8) has facilitated the study of ATP content of many organisms and changes thereof during metabolism and growth. The sensitivity and rapidity of the bioluminescence ATP assay coupled with the occurrence of ATP in all living matter and the general direct proportionality between cellular ATP and biomass have led a number of workers to develop this technique into procedures for the detection and enumeration of microorganisms. Such procedures which are applicable to brewery microbiological investigations and control have been described in the previously mentioned paper (8). The experiments to be described here are a continuation and extension of that work. In particular, the yeast and medium ATP content and changes thereof during fermentation of brewery wort at 12°, 18°, and 23°C by a selection of ale, lager, and wild yeasts have been studied. The purpose of these studies was three-fold: 1) to establish the variations in yeast ATP content during the growth cycle; 2) to study the phenomenon of ATP excretion by yeast during fermentation; and 3) to evaluate the possibility of using yeast ATP content, ATP excretion by yeast, and/or changes thereof during fermentation at various temperatures as methods for characterizing and/or differentiating between species and/or strains of yeast.

Rapid, sensitive adenosine diphosphate (ADP) and adenosine monophosphate (AMP) assays, based on the stepwise enzymatic conversion of these compounds to ATP and subsequent assay of the ATP by the aforementioned bioluminescence assay, have been developed as useful additions to the ATP assay (cf. 12,15). Specifically, the ADP to ATP and AMP to ATP conversions are effected by the reactions summarized in eqs. 1 and 2, respectively:

$$\text{ADP} + \text{PEP} \underset{Mg^{2+},\ K^{+}}{\overset{PK}{\rightleftarrows}} \text{ATP} + \text{Pyruvate} \qquad [1]$$

$$\text{AMP} + \text{ATP} \underset{Mg^{2+}}{\overset{MK}{\rightleftarrows}} 2\ \text{ADP} \qquad [2]$$

where PEP is the energy-rich compound phosphoenolpyruvate, PK is the enzyme pyruvate kinase, and MK is the enzyme myokinase. The equilibrium for eq. 1 lies far to the right ($K_2 \sim 1 \times 10^3$) and this reaction thus goes to completion. Although the equilibrium for eq. 2 lies approximately 60% to the left, quantitative conversion of AMP to ATP is achieved by coupling the two reactions: the ADP formed from AMP by reaction 2 is converted quantitatively to ATP by reaction 1. All three adenine nucleotides can thus be quantitatively determined as shown schematically in Fig. 1. Sample ATP content (A) is first determined. Appropriate amounts of PEP, PK, Mg^{2+}, and K^{+} are then added to the sample, the ADP to ATP conversion (eq. 1) is allowed to go to completion, and the converted reaction mixture is assayed for ATP. This ATP concentration, which is equal to [ATP] + [ADP] (hereafter abbreviated as AA) of

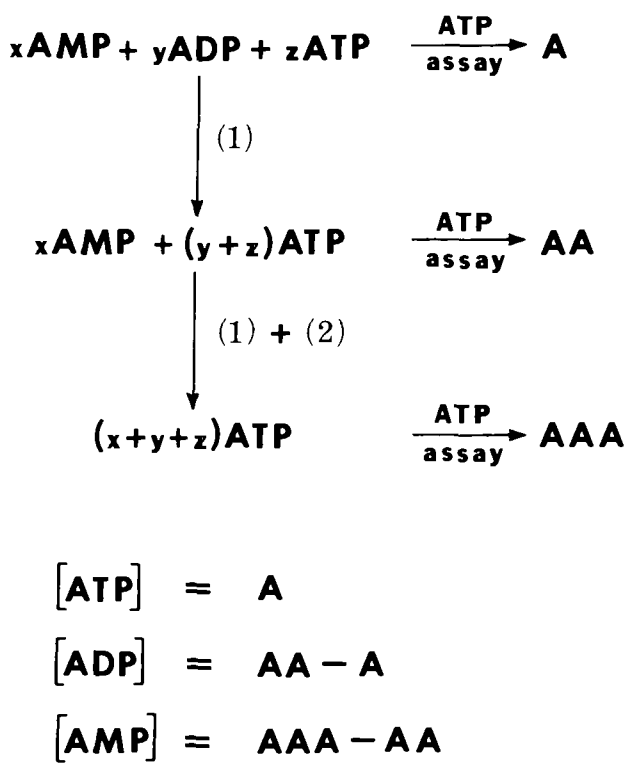

Fig. 1. Scheme for assay of ATP, ADP, and AMP concentrations on a single sample; 1 and 2 refer to the reactions shown in the respective text equations.

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the original sample, thus allows the calculation of sample [ADP]. MK is finally added to the sample mixture and, after quantitative AMP to ATP conversion (sum of eqs. 1 and 2), the reaction mixture is again assayed for ATP. This ATP concentration, the sum of all three adenine nucleotide concentrations, [ATP]+[ADP]+[AMP] (hereafter abbreviated as AAA), in the original sample, permits the calculation of sample [AMP].

The above methods have been used to survey the ATP, ADP, and AMP concentrations in Canadian beer. They have also been used to determine the levels of the three adenine nucleotides in yeast and medium during laboratory and brewery fermentations of wort. The latter data have been used to calculate values for energy charge (EC) of the adenylate pool of yeast which is defined as the ratio of energy-rich to total adenine nucleotides, as shown in eq. 3 (1).

$$EC = \frac{[ATP] + 1/2\,[ADP]}{[ATP] + [ADP] + [AMP]} \qquad [3]$$

Because all of the metabolic sequences of a living cell are coupled by ATP, ADP, and AMP, and because EC is a direct measure of the amount of metabolically available energy that is momentarily stored in the adenylate pool (5) or, alternatively, a measure of the charge on the adenylate "battery" (18), EC is a useful parameter in the study and understanding of cell growth, metabolism, and regulation (5). Although the capacity of the adenylate battery is very low, as indicated by the very fast ATP turnover rate for living cells (of the order of 1 sec for actively metabolizing yeast) its control is extremely well adjusted. This control is effected largely by allosteric activation by AMP or ADP, inhibition by ATP, or both, of key steps in the major energy-yielding metabolic pathways (18). Chapman *et al.* (5) have shown that EC in intact metabolizing cells is very strongly stabilized near 0.85, that cell growth can occur only above 0.8, that viability is maintained at values between 0.8 and 0.5, and that below 0.5 cells die. Comparison of the yeast EC values obtained in the previously mentioned experiments with these literature values suggests several potential practical applications of EC determinations in brewing research and development.

EXPERIMENTAL

ATP Assay

The materials and methods used for this assay have been described previously (8). In brief, beer, filtered culture media, and yeast culture extracts were assayed for ATP by adding 10 μl luciferase/luciferin premix to 10 μl of the sample in 1.0 ml *tris*-H_2SO_4-$MgSO_4$ buffer and measuring the resulting light output (LO).

Extraction Methods

Extraction by the previously described DMSO procedure (8) was used when only ATP assays were to be done. When the mono- and diphosphate assays were also to be done, extracts were prepared by the following procedure. The sample of interest—for example, yeast suspension—was injected into ten-fold its volume of boiling acetone contained in a vial immersed in a hot water bath; convenient volumes were 0.1 ml sample and 1.0 ml acetone. The acetone was removed to dryness by blowing nitrogen over the liquid surface while holding the vial in the hot water bath. The dry extracts were either assayed immediately or stored at −25°C (8).

AMP and ADP Assays

Reagents. The reagents and their preparation were as described by Lundin and Thore (12) with several modifications. Specifically, the sodium salts of ATP, ADP, and AMP, the tricyclohexylamine salt of phosphenolpyruvate (PEP), and the ammonium sulfate suspensions of crystalline rabbit-muscle PK and MK, were obtained from Sigma Chemical Company. Stock solutions of ATP, ADP, and AMP (10 ml m*M*) and of PEP (100 m*M*) were prepared in pH 8.0, 20 m*M* *tris*-H_2SO_4 buffer containing 2 m*M* EDTA and were stored at −25°C. Dilutions of the stock adenine nucleotide solutions and 2.5 m*M* PEP working solution containing 0.125 *M* $MgSO_4$ and 0.312 *M* K_2SO_4 were made in the same *tris*-H_2SO_4-EDTA buffer. These were stored at 0°C and were freshly prepared after several days. Working solutions of PK were prepared by ten-fold dilution of the PK suspension (10 mg protein/ml, ~ 400 units activity/mg protein) with pH 7.40, 10 m*M* *tris*-H_2SO_4 buffer containing 0.1% bovine serum albumin. Working solutions of MK were prepared by centrifuging the suspension (5 mg protein/ml, ~ 1700 units activity/mg protein) and dissolving the pellet in the original volume of the same buffer as used for PK. These enzyme solutions were stable for several weeks when stored at 0°C.

Assay Procedures

ATP, ADP, and AMP concentrations in beer, yeast extracts, and standard solutions were determined by the stepwise conversion of ADP and AMP to ATP and assaying ATP after each step. The enzymatic conversions of ADP and in turn AMP to ATP were done at pH 8.0 and room temperature. Beer samples were prepared for assay and conversion either by adjusting the pH to 8.0 with sodium hydroxide or by mixing beer with pH 8.1 *tris*-H_2SO_4-EDTA buffer in a 1:10 ratio. The latter procedure resulted in a solution pH (7.9–8.0) that was satisfactory both for the enzymatic conversions and the ATP assays. Yeast extracts were reconstituted with pH 8.1, *tris*-H_2SO_4-EDTA buffer; if the extracts had been prepared from 0.1 ml yeast suspension, 1.0 ml buffer was added. Standard adenine nucleotide solutions were prepared as described above. Interferences and/or inhibition by beer and/or yeast extract constituents were tested for by adding known amounts of the adenine nucleotides.

1. ATP Assay. PEP solution (40 μl) was added to 1.0 ml of sample solution prepared as described above. Duplicate ATP assays of the resulting well-mixed solution were done by the procedure described earlier (8), *viz.* 10 μl of solution was added to 1.0 ml pH 7.4, 10 m*M* *tris*-H_2SO_4, 3.5 m*M* $MgSO_4$ buffer, and the bioluminescence assay was initiated by adding 10 μl firefly luciferase/luciferin premix.

2. ADP Assay. PK working solution (10 μl) was added to the above PEP-containing sample. After quantitative conversion of ADP to ATP had been effected by incubation of the resulting reaction mixture for a minimum of 5 min at room temperature, duplicate ATP assays were done as described above. Sample ADP content was calculated by subtracting the ATP content of the original sample from the ATP content of this converted sample:

$$[ADP] = AA - A$$

3. AMP Assay. MK working solution (10 μl) was added to the above PEP- and PK-containing sample. After quantitative conversion of AMP to ATP had been ensured by incubation of the reaction mixture at room temperature for a minimum of 10 min, duplicate ATP assays were done. Sample AMP content was calculated from the difference of these latter ATP assays (AAA) and the preceding ATP assays:

$$[AMP] = AAA - AA$$

All adenine nucleotide contents, determined by the above-described duplicate A, AA, and AAA assays of at least triplicate samples, were the means of at least six values. All ATP assays were corrected for background or endogenous LO, as described previously (8). Because of the very high sample dilutions afforded by the present ATP assay technique, interferences and/or inhibitions by sample components were generally found to be negligible compared to method variance.

Microbiological Procedures

Microbiological procedures were essentially as described previously (8).

RESULTS AND DISCUSSION

ATP Content of Yeasts and Their Culture Media

In a previous paper (8), the ATP content of a selection of yeasts in a resting or dormant metabolic state was determined. In an extension of these studies, the ATP content of a wider selection (36 strains or varieties) of ale, lager, and miscellaneous yeasts and of their respective culture media were determined during active growth and metabolism at 2, 4, and 7 days from pitching, using small-scale laboratory cultures of sterile plant wort at 12°, 18°, and 23°C. Summaries of the results for the ale, lager, and miscellaneous yeast experiments are presented in Tables I, II, and III, respectively. At 12°C, the ATP content of most yeasts studied had a narrow range and remained relatively constant during the 7-day growth period. As the temperature was successively increased to 18° and 23°C, the strain-to-strain range of this variable likewise increased and its pattern of change during growth became more divergent. The ATP content of the miscellaneous yeasts showed considerably more variability, both in terms of response to temperature and changes during culture growth, than did the other two groups, as would be expected since the former group comprised 12 different species, whereas the ale and lager yeasts were all strains of *Saccharomyces cerevisiae* and *S. uvarum*, respectively. The general homogeneity of the latter two groups is demonstrated by their narrow range of ATP content. The variations that were observed, particularly for the miscellaneous yeasts, are probably indicative of variations in cell size since various workers have shown that ATP as a percentage of cellular organic carbon is virtually constant for a

TABLE I
ATP Content of Ale Yeasts and Their Growth Media[a]

		Yeast ATP		Medium ATP[b]		Medium ATP/Culture ATP[c]	
		Mean fmol/cell	Range fmol/cell	Mean μg/l.	Range μg/l.	Mean %	Range %
12°C							
	2 days	0.15	0.074–0.45	8.6 (9.1)	1.9 –19	0.25 (0.21)[d]	0.14 –0.48
	4 days	0.14	0.089–0.27	9.9 (7.7)	4.6 –35	0.18 (0.096)	0.076 –0.61
	8 days	0.11	0.052–0.20	13 (5.7)	2.5 –69	0.18 (0.059)	0.039 –0.78
18°C							
	2 days	0.12	0.071–0.37	12 (13)	6.8 –26	0.63 (0.30)	0.14 –4.3
	4 days	0.17	0.13 –0.28	16 (15)	9.5 –25	0.19 (0.15)	0.071 –0.72
	7 days	0.20	0.12 –0.50	4.5 (4.2)	0.25–16	0.051 (0.017)	0.0013–0.35
23°C							
	2 days	0.22	0.12 –0.38	30 (25)	13 –77	0.24 (0.17)	0.087 –0.39
	4 days	0.22	0.068–0.35	88 (22)	8.5–502	0.63 (0.14)	0.035 –3.6
	7 days	0.20	0.12 –0.39	47 (4.9)	1.8 –267	0.42 (0.029)	0.0080–2.9

[a]Results of experiments done with 12 commercial and culture collection brewing strains of *Saccharomyces cerevisiae*. The cultures were grown in sterile brewery wort (20 ml), unstirred, and at the three temperatures shown using a thermostated water bath. Samples for analysis (1 ml) were withdrawn aseptically from the well-stirred cultures at the indicated intervals after inoculation. Three 100-μl portions of the whole culture were extracted with DMSO (8) and the remainder was passed through a 0.45-μm membrane filter. Duplicate ATP assays of the DMSO culture extracts and of the filtered medium were done as described in the **Experimental** section.
[b]ATP content of the cell-free growth medium.
[c]Ratio of medium ATP content to total culture ATP content, in per cent. The latter ATP data, obtained from ATP assays of DMSO extracts of the whole culture, are the sum of medium ATP plus the yeast cell ATP.
[d]Values in parentheses are the means excluding the data for three strains with unusually high values.

TABLE II
ATP Content of Lager Yeasts and Their Growth Media[a]

		Yeast ATP		Medium ATP		Medium ATP/Culture ATP	
		Mean fmol/cell	Range fmol/cell	Mean μg/l.	Range μg/l.	Mean %	Range %
12°C							
	2 days	0.15	0.080–0.30	0.88	0.28– 1.7	0.030	0.0069–0.075
	4 days	0.13	0.015–0.23	4.5	0.66– 7.7	0.096	0.015 –0.41
	7 days	0.22	0.11 –0.34	18	6.6 –31	0.18	0.062 –0.40
18°C							
	2 days	0.11	0.055–0.16	9.5	5.7 –14	0.28	0.12 –0.59
	4 days	0.19	0.077–0.35	13	6.0 –28	0.14	0.051 –0.33
	7 days	0.20	0.055–0.34	15	1.6 –33	0.14	0.017 –0.29
23°C							
	2 days	0.36	0.16 –0.68	23	12 –52	0.22	0.13 –0.38
	4 days	0.33	0.14 –0.51	48	18 –95	0.30	0.083 –0.56
	7 days	0.21	0.027–0.46	36	5.8 –87	0.35	0.062 –0.80

[a]These results are from experiments done with 12 commercial and culture collection brewing strains of *Saccharomyces uvarum*. The experimental procedures were as described in Table I, footnote a.

large variety of microbial cells (7). Because the requirements of viability maintenance and growth dictate that cell ATP content be closely controlled, the narrow range and relatively constant ATP pool size of the 36 yeasts examined here is not surprising. The prognosis for using such variables to characterize and/or differentiate yeasts thus seems unfavorable.

A more promising criterion for these purposes appears to be the ATP content of the culture growth medium. As shown in Tables I–III, much wider ranges of this variable and changes thereof during yeast growth at the three temperatures were observed than for cell ATP pool. Significant differences in the pattern by which ale and lager yeasts excrete ATP into the media are apparent. In particular, the mean medium ATP level for the ale yeasts approached a maximum within 2 days and generally decreased thereafter, whereas for the lager yeasts the mean medium ATP level increased throughout the 7-day growth period except at 23°C. The mean medium ATP levels for the miscellaneous yeasts showed diverse patterns at the three temperatures. Medium ATP levels significantly increased with increasing culture temperature for the majority of yeasts studied. The patterns of ATP excretion at the various temperatures generally varied as just described. Such significant strain-to-strain and particularly species-to-species differences in levels of medium ATP and in changes thereof during growth at various temperatures may, as mentioned above, be useful for classifying yeasts and/or their performance. Although the basis for such a method would appear to be in hand, more extensive testing, particularly evaluations of variation, reliability, and reproducibility, would be necessary before the method could be realized. These results were obtained with batch cultures using an incompletely characterized medium and uncontrolled aeration conditions. Such systems are obviously extremely complex and probably should be simplified, more closely controlled, defined, and adjusted to allow easier comparisons with plant fermentations and to enhance repeatability and reliability.

Also presented in Tables I, II, and III are the ratios of medium ATP to total-culture ATP, in per cent. This variable permits the direct comparison of medium ATP levels since it takes into account variations in culture growth. For ale yeasts grown at 12°C (excluding several atypical strains), this ratio decreased relatively uniformly from a maximum of 0.21% after 2 days to 0.059% after 8 days. The same general pattern was also observed for the 18° and 23°C cultures. The patterns of ratio change were much different for the lager yeasts: for 12°C cultures, the ratio increased from 0.030% after 2 days to 0.18% after 7 days; for 18°C cultures, a general decrease from 2 to 7 days was observed; and for 23°C cultures, diverse patterns were noted. The miscellaneous yeasts again exhibited their diverse nature. Their medium:total-culture ATP ratios varied widely with species, temperature, and growth time.

These data indicate some fundamental differences between ale and lager yeasts, suggesting again that such variables could be useful for taxonomic purposes. These differences are particularly distinct at the lower temperatures. The early maximum for the ale yeasts and subsequent decrease suggest that most excretion occurs during the early part of the ale growth cycle and that, subsequently, ATP reabsorption and/or degradation by extracellular enzymes and/or cell-wall enzymes outweigh any continuing ATP excretion. In contrast, the lager yeasts at 12°C appear to excrete ATP at an increasing rate throughout the growth period, suggesting fundamental differences in cell-wall structural features which allow ATP excretion and inhibit ATP reabsorption as well as possible differences in cellular makeup which lead to ATP degradation.

Development of AMP and ADP Assays

The basis for assay of all three adenine nucleotides studied here is the firefly bioluminescence ATP assay. As indicated previously(8), the features of this assay which contribute to its high sensitivity, selectivity, and freedom from interferences are the use of purified firefly luciferase and synthetic firefly luciferin at optimum concentrations, the use of a stabilized luciferase/luciferin premix, and the use of high sample dilution (100-fold for the standard assay). These same features are also advantageous for the ADP and AMP assays. As discussed in the introduction, these two assays are based on the stepwise conversion of ADP and AMP as shown in eqs. 1 and 2, ATP assay after each conversion step, and calculation of the individual nucleotides by difference (see Fig. 1). The present methods for the ADP and AMP conversions are essentially as described by Lundin and Thore (12) with the exception of a conversion reaction mixture pH of 8.0 instead of 7.75. At this higher pH, the ADP and AMP conversions for standard nucleotide solutions, beer, and yeast extracts were quantitatively complete in less than 5 and 10 min, respectively. Furthermore, quantitative conversions could be achieved in the same time periods with pHs from about 7.8 to 8.2. Although pH 8.0 was used when practicable, this was a useful feature when exact pH adjustment was difficult, for example, when only small samples of medium, beer, and/or yeast extracts were available. The acetone extraction method described in the **Experimental** section rather than the DMSO method was used to prepare yeast extracts. The acetone could easily

TABLE III
ATP Content of Miscellaneous Yeasts and Their Growth Media[a]

		Yeast ATP		Medium ATP		Medium ATP/Culture ATP	
		Mean fmol/cell	Range fmol/cell	Mean μg/l.	Range μg/l.	Mean %	Range %
12°C							
	2 days	0.10	0.010–0.24	6.4	0.42 –26	0.51	0.016 –1.9
	4 days	0.14	0.059–0.37	9.4	2.3 –27	0.26	0.030 –0.55
	8 days	0.11	0.038–0.33	12	1.2 –42	0.12	0.022 –0.38
18°C							
	2 days	0.14	0.043–0.43	20	4.2 –56	0.76	0.082 –1.74
	4 days	0.16	0.069–0.37	16	1.8 –41	0.43	0.035 –2.3
	7 days	0.12	0.046–0.24	7.9	0.088–20	0.14	0.0020–0.69
23°C							
	2 days	0.21	0.071–0.55	25	0.23 –77	0.34	0.0054–0.97
	4 days	0.15	0.070–0.28	25	1.9 –96	0.25	0.035 –0.73
	7 days	0.10	0.023–0.32	25	9.1 –94	0.48	0.078 –1.2

[a]Results of experiments done with 12 miscellaneous species of yeasts including 7 species of *Saccharomyces* other than *cerevisiae* or *uvarum*. The experimental procedures were as described in Table I, footnote a.

be totally removed after extraction, thus avoiding sample dilution and inhibition. Because the AMP to ATP conversion rate has been found to be the limiting factor in AMP assays at low concentrations, sample concentrations in the conversion mixture were maintained as high as practicable.

ATP, ADP, and AMP assays have been successfully developed for use with standard adenine nucleotide solutions, yeast extracts, fermentation media, and beer. Table IV presents typical per cent relative standard deviations (RSDs) for these assays. The RSDs for the A, AA, and AAA assays were generally in the 2–4% range. These RSD values, as indicated previously (8), increase with decreasing ATP concentration. Because the ADP and AMP concentrations were derived by difference, their RSDs are correspondingly larger than those of the sums from which they were derived. Detection limit, defined here as that amount of nucleotide required to give a signal equal to twice the standard deviation of the background, may be easily calculated for the A, AA, and AAA assays. The RSD for background or endogenous LO is in the 12–20% range. The upper background RSD, a typical background LO of 500 counts per (cp) 0.1 min and a typical premix activity of 100,000 cp 0.1 min/pmol ATP thus define a standard assay detection limit of 2 fmol/10 μl sample which is equivalent to 0.2 nM or 0.1 μg/l. ATP. Lowest quantitatively determinable (LQD) concentration, defined as that amount of material necessary to produce a signal that is five times the detection limit, has been suggested as a more realistic measure of assay sensitivity (4). A typical LQD for the ATP assay technique used here is thus 10 fmol/10 μl sample (= 1 nM or 0.5 μg/l. ATP). Although ATP detection limit and thus LQD, which depend on background LO and premix activity, can be reduced 10- to 100-fold by additional effort and cost (8), the indicated assay sensitivities were adequate for the present purposes. The above comments on detection limit and LQD obviously also apply to the AA and AAA assays. However, because individual ADP and AMP determinations involve calculation by difference, the detection limits and LQDs in these cases are much higher than the values mentioned above and depend directly on the concentrations and ratios of the three adenine nucleotides in the sample. For calculation of illustrative ADP and AMP detection limits and LQDs, the LO or ATP concentration from the previous assay, A and AA, respectively, is considered the background signal. The RSD of A and AA assays is typically 2.5%. The ADP assay detection limit and LQD are thus 0.05 and 0.25 times the sample ATP concentration—for example, 5 and 25 nM, respectively, for a sample containing 100 nM ATP. Similarly, the AMP detection limit and LQD are 0.05 and 0.25 times sample ATP + ADP concentrations.

TABLE IV
Per Cent Relative Standard Deviations (RSDs) of Adenine Nucleotide Assays[a]

	A	AA	AAA	ADP	AMP
Standard solution[b]	2.5	2.6	3.2	5.5	9.3
Yeast extracts[c]	3.7	3.9	3.1	8.8	12.5
Beer					
Ale[d]	3.6	2.7	6.9	5.1	7.9
Lager[e]	2.7	1.5	18	4.1	20

[a]Each RSD was calculated from the data for five to ten replicate assays. A, AA, and AAA are the ATP, ATP + ADP, and ATP + ADP + AMP stages of the adenine nucleotide assay scheme (see Fig. 1).
[b]This solution contained 100 nM each of ATP, ADP, and AMP.
[c]Acetone extracts of a yeast suspension were prepared. The ATP, ADP, and AMP concentrations of the reconstituted extract conversion mixtures were 220, 200, and 210 nM, respectively.
[d]Degassed ale was adjusted to pH 8.0 with NaOH. The mean ATP, ADP, and AMP concentrations were 69, 74, and 960 nM, respectively.
[e]Degassed lager beer was adjusted to pH 8.0 with NaOH. The mean ATP, ADP, and AMP concentrations were 4, 2, and 31 nM, respectively.

Additions of standard amounts of ATP, ADP, AMP, and combinations thereof were used to demonstrate that, for beer, fermentation media, and acetone yeast extracts, the methods described herein provided quantitative conversions and recoveries of the three nucleotides added singly and in various combinations (cf. 11,16). Furthermore, these experiments showed that analytical interferences and/or inhibition of the bioluminescence assay by sample components were negligible, presumably due to the 100-fold dilution of sample in the ATP assay mixture.

Lundin and Thore (12) have reported the presence of heat-stable nucleotide-converting enzymes in variously prepared extracts from several bacterial strains. Such enzymes could rapidly decompose extract nucleotides, particularly ATP. Dried acetone extracts of several yeast strains in a variety of metabolic states were examined for such activity. In all cases studied, both the dried and reconstituted extracts appeared extremely stable, even at room temperature, with respect to all three adenine nucleotides. However, to avoid any possible loss of the adenine nucleotides, the extracts were either assayed immediately or stored in the dried state at −25°C and reconstituted with buffer immediately before assay.

Chapman *et al.* (5) have observed that, during the latter part of the stationary phase of *Escherichia coli* cultures, the medium contained enzyme activity which could rapidly destroy the adenine nucleotides present. Cell-free media from actively metabolizing and stationary yeast cultures were examined for such extracellular enzyme activity. No changes in the ATP, ADP, or AMP concentrations were observed during incubation of the media at room temperature for 1 hr. Such stability of the adenine nucleotides in these media apparently demonstrates the absence of significant amounts of the aforementioned enzymes. In studying the adenine nucleotides of yeast cultures, the media were not extracted but, as a precautionary measure, they were either assayed immediately after sampling or stored at −25°C until assayed.

As mentioned above and as discussed in more detail previously (8), possible analytical interferences from the myriad of components present in biological growth media and extracts of biological materials have been minimized in the present assay by using purified luciferase preparations and by at least 100-fold sample dilution. AMP, ADP, and GTP (guanosine triphosphate), the most probable interfering substances, were tested for interferences (cf. 11) both individually and in various combinations, in the absence and presence of ATP. With the present reagents and assay technique, such interferences were found to be negligible.

Adenine Nucleotides in Beer

Numerous workers have studied yeast nucleotide excretion (9, and references therein) and beer nucleotide concentrations (2,3,6,14,17). Many of these studies have been accomplished only through great effort, often because of tedious, time-consuming, and

TABLE V
Adenine Nucleotide Concentrations[a] in Canadian Beers[b]

	ATP		ADP		AMP	
Type[c]	Mean	Range	Mean	Range	Mean	Range
Ale [23]	19	0.13–68	26	1.4 –92	380	11 –1500
Lager [30]	1.9	0.01–14	2.4	0.33– 5.9	42	0.49–390
Malt liquor [3]	17	2.2 –43	16	4.3 –38	70	16 –150
Low alcohol [5]	0.9	0.11– 2.6	4.2	2.0 – 8.5	16	3.5 –31
Porter [2]	6.5	4.8, 8.3	20	10, 30	380	230, 540

[a]All concentrations are in μg/l. Degassed beer with pH adjusted to 8.0 was assayed successively for ATP, ADP, and AMP by the methods described in the **Experimental** section. All assays were done in duplicate.
[b]The beers tested were a representative selection of products from the majority of Canadian breweries.
[c]The number of different products of each type examined are given in brackets.

demanding separation and determination techniques. Consequently, relatively limited numbers of assays have been done. The recent application of high-pressure liquid chromatography to determination of beer nucleotides appears to be a promising, relatively easy technique which may facilitate more extensive studies (6). The availability of the present fast, sensitive assays for ATP, ADP, and AMP led the authors to survey a cross section of Canadian beers for these three 5'-adenine nucleotides. Table V presents the mean values and ranges for ATP, ADP, and AMP concentrations determined in this survey. These data demonstrate that the ATP, ADP, and AMP levels in ales tend to be about ten-fold higher than those for lagers. On the basis of this observation and the data given in this table, malt liquors and porters would be classified as ales and the low-alcohol products as lagers. Figure 2, which presents the corresponding frequency-distribution graphs for the adenine nucleotides present in the ales and lagers examined in the survey, also demonstrates the points mentioned above. Furthermore, it shows that the lagers were a much more homogeneous group of products in terms of adenine nucleotide concentrations than the ales: they had considerably lower levels and narrower ranges of the nucleotides. The data in Table V and Fig. 2 also demonstrate that AMP is the major adenine nucleotide in beer, generally being present at more than ten times the concentration of the other two. This would probably be expected in view of the lower charge on AMP and consequently easier passage through the cytoplasmic membrane. Although ADP has been identified as one of the nucleotides excreted from yeast under certain conditions (10), the authors are otherwise unaware of any previous reports of ATP or ADP analysis on beer; the very low levels of these compounds may have precluded their determination or detection by less sensitive techniques. No comparisons of this laboratory's ATP and ADP data with literature values can thus be made. Most of the studies mentioned above have been concerned only with the monophosphate nucleotides. The following levels of AMP in beer, all in milligrams/liter have been variously reported: 1.2 – 1.9 and 0.5 – 1.2 for ales and lagers, respectively, by British workers (14); 0.1 – 0.5 for various domestic and imported beers by American workers (6); 3 for an ale by Canadian workers (3); 0.5 – 0.6 by Japanese workers (9); and 1.4 – 31 for beer at various stages of maturation by Belgium workers (13). The AMP data reported in this study are similar to many of the above values. Since even the highest AMP levels reported here are far below the flavor threshold level (17), these adenine nucleotides probably have little flavor impact. However, they may be significant in other ways. They indicate that other di- and triphosphate nucleotides probably occur in beer at levels similar to those for ADP and ATP. Confirmation of this will await development of more sensitive and selective assays. As indicated by the above results and discussions and as discussed in more detail by Lee and Lewis (10), nucleotides occur in beer largely as a result of yeast excretion. The factors involved in the extent and kinetics of this excretion include yeast strain, fermentation temperature, medium pH, medium Ca^{2+} and Mg^{2+} concentrations, and concentration of fermentable sugars. Adenine nucleotide assays could thus be useful in such diverse activities as monitoring fermentations and auditing beer. The former activity will be discussed in the next section. Adenine nucleotide assays in beer audits could be useful for characterizing beer, monitoring yeast and process changes, and assessing yeast performance and fermentation conditions. Because the three adenine nucleotide

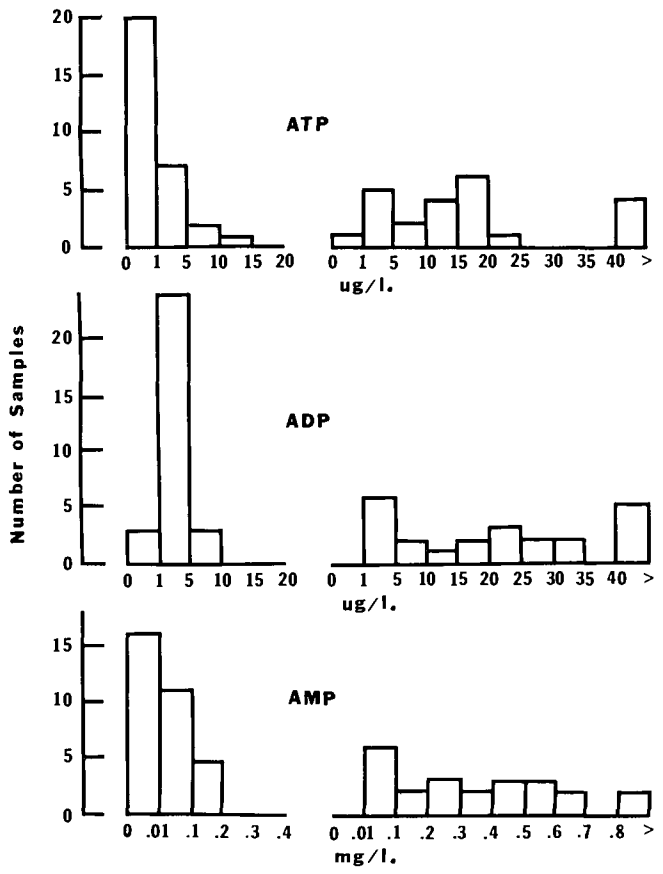

Fig. 2. Frequency distribution of adenine nucleotide concentrations in Canadian beers. These data were obtained as described in Table V footnotes. (Histograms to left of figure are for lager beers, and to the right are for ales.)

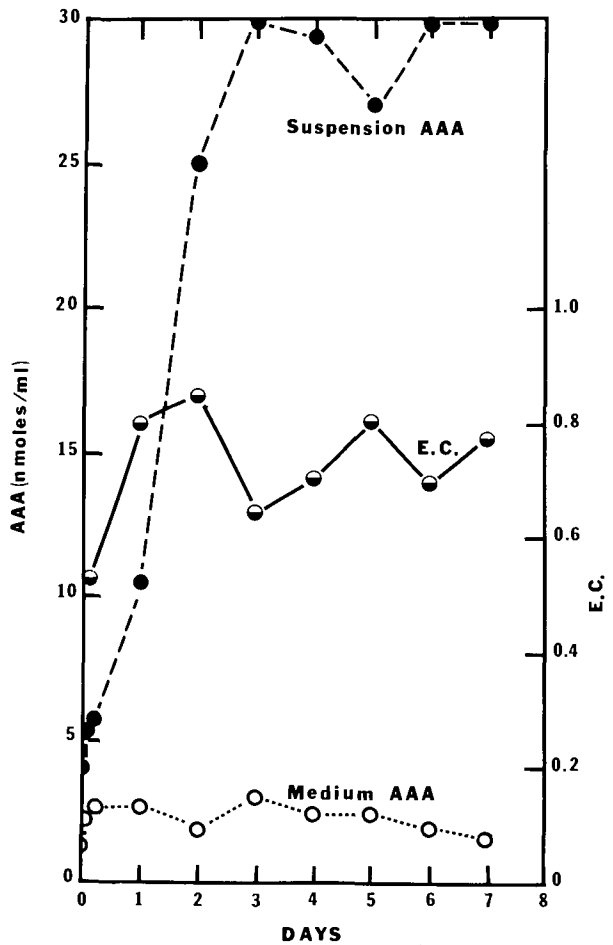

Fig. 3. Total adenine nucleotides (AAA) and Energy Charge (EC) during a laboratory fermentation of wort by ale yeast at 18°C (cf. 8).

levels are generally closely related, the rapid, facile ATP assay could be used as an indicator of overall adenine nucleotide excretion.

Yeast EC

The adenylate pool EC (see eq. 3) was determined for yeast suspensions in various physiological states. These EC were calculated from adenine nucleotide levels of yeast only, which were obtained by subtracting the sometimes considerable medium levels from the levels in acetone extracts of the total culture. The EC of ale-pitching yeast varied from 0.51 to 0.53, values which are consistent with previous observations that EC between about 0.5 and 0.8 permit maintenance of viability but not growth (5). Ale-yeast EC were determined at various stages of brewery fermentations. Values of 0.97, 0.83, and 0.90, observed 1, 3, and 4 days after pitching, respectively, indicate vigorous growth and fermentation and agree with the general observation that EC of microorganisms must be 0.8 or greater for growth to occur (5).

Figure 3 presents yeast EC and total adenine nucleotide concentrations of yeast and medium during a laboratory fermentation of brewery wort by ale yeast at 18°C. The average EC during the main part of this fermentation, 0.75, which is much lower than the 0.90 average observed for the above-mentioned plant fermentation, suggests slow yeast growth and a sluggish fermentation, which was indeed the case. The significance of the decrease in EC from the second to the third day is unclear but, since the difference is greater than the sample-to-sample method variance (± 0.05), an unusual two-phase fermentation is suggested.

Figure 3 also indicates that the medium makes a significant contribution to total-suspension adenine nucleotides particularly during the early stages of the fermentation. Rapid increases in adenine nucleotide content mainly due to AMP (see Fig. 4) were observed for both the medium and total suspension during the first several hours after pitching. Such behavior, presumably induced by the drastic change in medium that results from pitching, may be ascribed to the degradation of yeast ribonucleic acid and excretion of the nucleotides thus formed into the medium (10,11). Figure 4 presents the medium ATP, ADP, and AMP concentrations observed for this fermentation. All three nucleotide concentrations increased rapidly during the first day, again probably for the reasons described above. ATP and particularly ADP levels generally decreased during the remainder of the fermentation. Because the AMP assay has a larger uncertainty than the other two assays, the significance of the AMP fluctuations is uncertain. However, it appears that the general AMP level does not change much after the initial increase. It may be noted that the ATP, ADP, and AMP concentrations present at the end of fermentation, 0.060, 0.13, and 2.0 μM (equivalent to 33, 55, and 690 μg/l.), respectively, are in the same range as the mean values found for commercial ales (cf. Table V).

EC values and medium as well as suspension total adenine nucleotide concentrations for an ale fermentation carried out at 12°C, but otherwise identical to the 18°C one described for Fig. 3, are presented in Fig. 5. The general features of changes in the three variables during the fermentation are similar to those observed for the 18°C fermentation. The major differences undoubtedly resulted from the slower yeast growth and fermentation at the lower

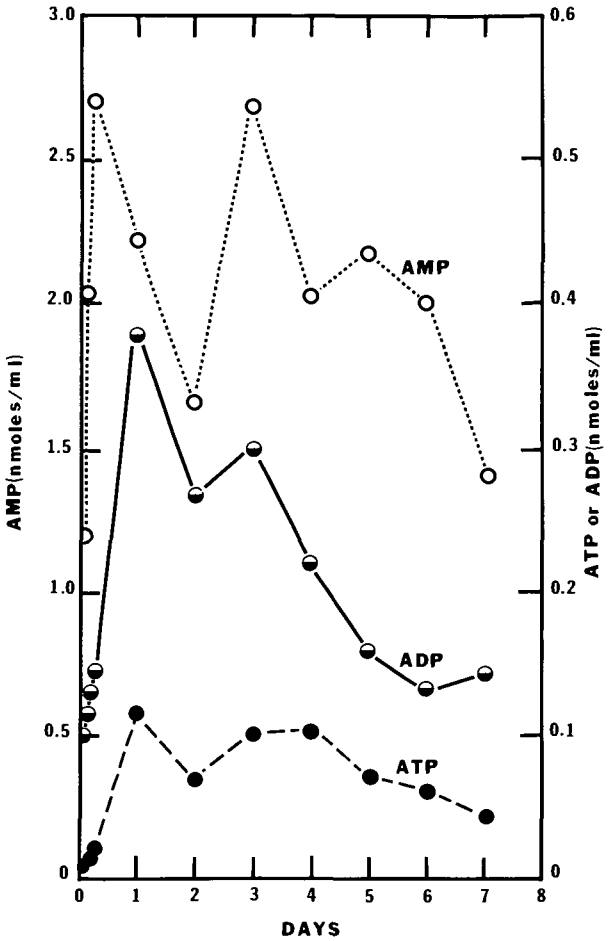

Fig. 4. Changes in medium adenine nucleotides during fermentation of brewery wort by ale yeast at 18°C. These data were obtained from the fermentation described for Fig. 3.

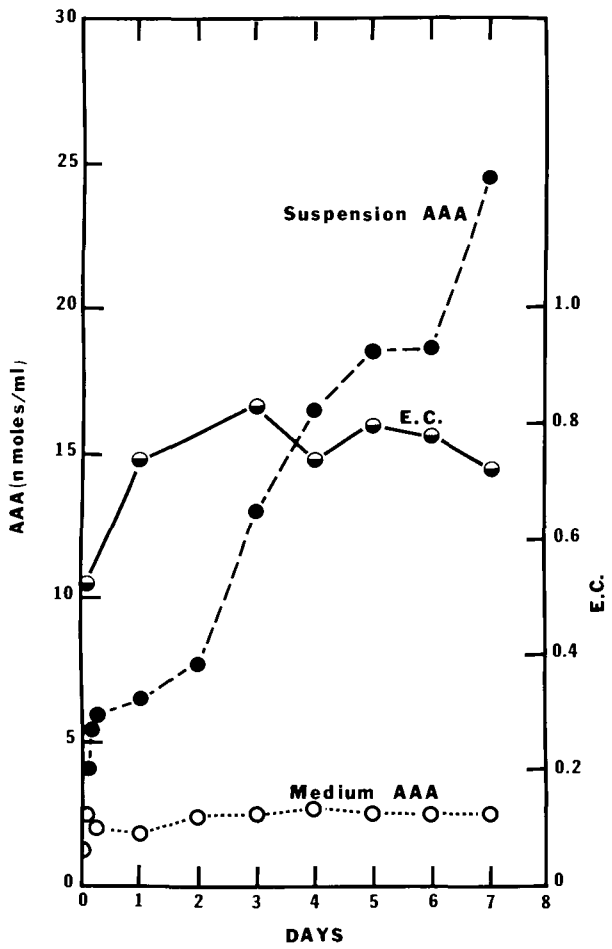

Fig. 5. Total adenine nucleotide concentrations (AAA) and Energy Charge (EC) during a laboratory fermentation of wort by ale yeast at 12°C.

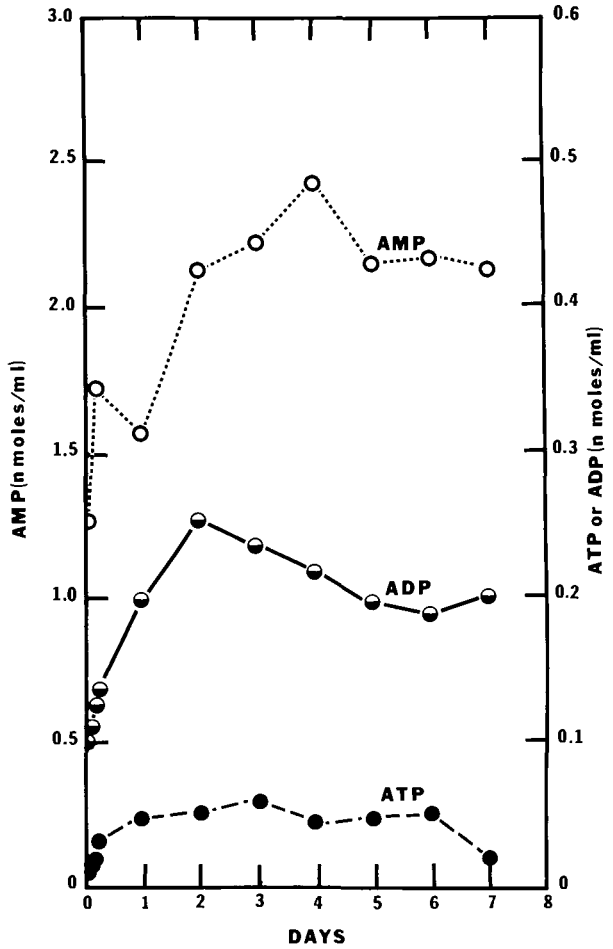

Fig. 6. Changes in medium adenine nucleotides during fermentation of brewery wort by ale yeast at 12°C. These data were obtained from the fermentation described for Fig. 5.

temperature. The medium ATP, ADP, and AMP concentrations during this fermentation are presented in Fig. 6. Again the general patterns of these curves are similar to those discussed for the 18°C fermentation.

The above observations and results suggest that EC has several potential practical applications in brewing. These include developing its use as a rapid method for determining yeast viability and predicting yeast fermentation performance and thus adopting it as a criterion for yeast management, including treatment and rejection. These suggestions may be illustrated by the following experiment. Brewery wort pitched with 11×10^6 cells/ml of apparently normal acid-washed lager yeast failed to ferment either at 12° or 18°C. The reason became apparent when the EC of the pitching yeast was found to be 0.36—well below 0.5, the minimum level required for maintenance of viability (5). Further confirmation of the suggested practical potential of EC determinations will hopefully be forthcoming from continuing experimentation in these laboratories.

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