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Nucleotide Metabolism During Differentiation in *Dictyostelium discoideum*

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Energy metabolism in *Dictyostelium discoideum* was studied by following the incorporation of [^{3}H]adenine and [^{32}P]Pi into the intracellular adenine nucleotides at two stages of differentiation. That the levels of nucleotides measured represented levels present in vivo was shown by demonstrating that the concentrations determined from cells quickly frozen in liquid nitrogen were identical to those from cells harvested and subsequently killed in perchloric acid. In addition, significant compartmentalization among the nucleoside triphosphates was not observed after 30 min, since uridine triphosphate, guanosine triphosphate, and adenosine triphosphate (ATP) were at radioactive equilibrium after exposure to [^{32}P]Pi. At the sorocarp stage of differentiation, the dominant role of adenylate kinase activity is indicated by the observations that (i) adenylate kinase activity was found to be present, (ii) the specific radioactivities of the beta- and gamma-phosphates of ATP were equal, (iii) the increase in specific radioactivity of adenosine diphosphate was one-half that of ATP in the presence of [^{32}P]Pi, and (iv) the steady-state equilibrium constant calculated (0.69) from the nucleotide levels equaled that of the apparent equilibrium constant reported in the literature (0.70). By using the same criteria, adenylate kinase activity was not the predominant reaction establishing the adenine nucleotide levels in cells at the pseudoplasmodium stage of differentiation.

This report concerns the metabolism of nucleotides during differentiation in the cellular slime mold, *Dictyostelium discoideum*. The slime mold offers a unique system for the correlation of nucleotide metabolism with development, since data are available concerning nucleotide levels and their role in the synthesis of the carbohydrate end products of differentiation. For example, the level of uridine triphosphate (UTP) is known, as well as its minimum rate of turnover in vivo, based upon its role as a precursor in the synthesis of uridine diphosphate glucose (UDPG; references 12, 13). The latter product, in turn, is the precursor in a number of biosynthetic pathways essential to differentiation (19). The cellular slime mold starves as it differentiates, using endogenous protein as an energy source. Differentiation is completed within 24 hr at 23 C and is characterized by an orderly sequence of morphological stages: amoeba, aggregation, pseudoplasmodium, culmination, and the final fruiting body, or sorocarp. That energy metabolism is altered over the course of differentiation is indicated by changes in the rate of respiration (6, 10). A 10-fold decrease in oxygen consumption occurs between the amoeba and sorocarp stages. In addition, glucose stimulates oxygen consumption at the sorocarp but not at the earlier stages of differentiation.

This report describes some properties of nucleotide metabolism in *D. discoideum* at the pseudoplasmodium and sorocarp stages of development. These stages were selected since they showed the most striking differences in their regulatory properties. Energy regulation at the sorocarp stage was examined in particular detail because of its relative simplicity: (i) the synthetic processes of differentiation have ceased essentially, (ii) endogenous energy reserves are at a minimum, and (iii) there is a low rate of oxygen consumption, and (iv) the effects of stimulating respiration on nucleotide levels can be studied.

MATERIALS AND METHODS

$Na_2H[^{32}P]O_4$ and [^{3}H]adenine were obtained from New England Nuclear Corp. (Boston, Mass.). Norite was purchased from Fisher Scientific Co., (Medford, Mass.) and activated by the procedure of Till et al. (17).

Cells of *D. discoideum* strain NC-4 were grown on a rich agar medium in the presence of *Escherichia coli*. Cells at the amoeba stage were harvested from the agar surface, washed three times with cold sterile distilled water, and replated onto 2% agar containing 0.01 M potassium phosphate buffer (*p*H 6.5) and 1.0 mM diso-

dium ethylenediaminetetraacetate. The cells were allowed to differentiate to a desired stage of development and then were rinsed into a polypropylene centrifuge tube with Bonner's "standard solution" (4) buffered at *p*H 6.5 with 1.0 mM 2-(*N*-morpholino)-ethanesulfonic acid. The cells were dispersed by a few strokes of a loose-fitting Teflon homogenizer to obtain a homogeneous suspension. The packed-cell volume was determined on a 2-ml sample of this cell suspension by centrifuging at 1,000 × *g* for 15 min in a graduated test tube. Three milliliters of the original suspension was added to 50-ml Erlenmeyer flasks and gently swirled for 10 min at 23 C on a no. 249 rotary shaker (Fisher Scientific Co.) at a setting of 70. Labeled compounds were then added. After incubation for various time periods, 3 ml of cold 10% perchloric acid was added to the suspension. The extract was centrifuged, the supernatant fluid was neutralized with 5 N KOH and centrifuged again, and the supernatant fluid was removed. Nucleotides were separated from the extract by adding 15 mg of acid-washed Norite for each milliliter of extract. The Norite and bound nucleotides were then washed three times with water over a membrane filter (Millipore Corp., Bedford, Mass.). The adsorbed nucleotides were eluted from the Norite with 9 ml of cold ammoniacal ethanol (2:50:50, NH_4OH:ETOH:H_2O), frozen in an acetone-dry ice bath and lyophilized, taken up in approximately 200 μliters of water, and applied to PEI-cellulose thin-layer chromatograms. The nucleotides were separated by two-dimensional chromatography in LiCl and formate buffer and eluted, by the method of Randerath (15). Specific radioactivity of the eluted nucleotides was determined from the absorbance, measured at 260 nm, and the radioactivity determined by using an LS-200 liquid scintillation counter (Beckman Instruments, Inc.).

The specific radioactivity of the beta- and gamma-phosphates of adenosine-5'-triphosphate (ATP) was determined after exposing the cells to label for 30 min and isolating the nucleotides as described previously. The ATP was eluted from the chromatogram in a solution containing 10 mM glucose, 2 mM ATP, 0.7 M $MgCl_2$, and 0.02% bovine serum albumin in 1 ml of 0.02 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, *p*H 7.4. Hexokinase at 0.5 μg was added, and the reaction mixture was incubated at 25 C for 30 min, boiled for 3 min, cooled, and Norite was added. The nucleotides were then isolated from the mixture. An identical sample without added hexokinase was carried through all treatments. That 30 min was sufficient for total conversion of ATP to adenosine diphosphate (ADP) was shown by a parallel experiment in which the amount of glucose-6-phosphate formed was determined. Unlabeled ATP at a concentration equal to that in the test situation was added to the reaction mixture. After 10, 20, and 30 min, the tubes were boiled for 30 min and cooled. Nicotinamide adenine dinucleotide phosphate and glucose-6-phosphate dehydrogenase were added to give a final concentration of 0.5 mM and 0.5 μg/ml, respectively, and the reaction was followed at 340 nm. The per cent conversion of ATP to ADP was then calculated and shown to be complete after 20 min.

Adenylate kinase activity in broken-cell preparations was determined by the method of Colowick (5).

RESULTS

Effect of harvesting and extraction procedures on adenine nucleotide ratios. Since adenine nucleotide pools are among the most rapidly turning over in the cell, we tested whether the concentrations of the individual adenine nucleotides were affected by any stress that might occur during the usual harvesting procedure of the slime mold (Table 1). In treatment 1, cells were harvested in the usual manner by scraping them from an agar surface with a bent glass rod into cold 10% perchloric acid. In treatment 2, instead of scraping the live cells from the surface of the agar, liquid nitrogen was gently poured over the entire surface of the agar, thus freezing the plate in less than 1 sec. The frozen cells were then scraped from the agar surface into cold 10% perchloric acid, and the nucleotides were isolated as described above. At no time during this procedure were cells allowed to thaw before being added to the 10% perchloric acid, thus eliminating any stress that might occur during the usual harvesting procedure. That no significant differences in nucleotide levels were observed between treatments 1 and 2 indicates that, although these metabolite pools are rapidly turning over, the nucleotide levels are not disturbed by the harvesting procedures. Furthermore, treatment of cells with 10% perchloric acid extracted the total adenine nucleotide pool even at the sorocarp stage of differentiation, since further treatment of the extract by the French press or by sonic oscillator did not release additional adenine nucleotides.

Steady-state levels of adenine nucleotides. The steady-state levels for the adenine nucleotides at two stages of differentiation, the pseudoplasmodium and sorocarp, are shown in Table 1. The relative levels for the sorocarp stage are quite typical. Although there was little variation in the nucleotide levels between replications of a single-stage study, the levels measured from one experiment to another at the pseudoplasmodium stage showed considerably more variation. For example, in most cases a relatively high adenosine monophosphate (AMP) level was found in the pseudoplasmodium stage. In some experiments, however, including the one summarized here, the AMP levels were similar to that of the sorocarp stage. These variations in the steady-state levels of AMP in the pseudoplasmodium stage may indicate that a rapid change in the regulation of AMP occurs at this time during differentiation.

Test for compartmentalization of nucleotides. The extent to which compounds are compartmentalized during differentiation has often been a source of discussion. We therefore tested for

TABLE 1. *Effect of different harvesting procedures on the ratio of the adenine nucleotides*

Treatment	Nucleotide levels (μmole/ml)[a]					
	Pseudoplasmodium			Sorocarp		
	ATP	ADP	AMP	ATP	ADP	AMP
Perchloric acid	0.93 ± 0.09	0.30 ± 0.03	0.10 ± 0.03	1.37 ± 0.4	0.42 ± 0.03	0.08 ± 0.01
Liquid nitrogen	0.77 ± 0.12	0.30 ± 0.11	0.16 ± 0.07	1.45 ± 0.10	0.42 ± 0.05	0.09 ± 0.02

[a] Nucleotide levels were calculated from their absorbance at 260 nm by using a molar extinction coefficient of 1.54×10^4 cm^{-1} M^{-1}; concentration is expressed as micromoles per milliliter of packed cells. The mean ± standard deviations are the result of four determinations. Abbreviations: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate.

compartmentalization of nucleotides in the slime mold by incubating cells in the presence of [^{32}P] Pi and comparing the specific radioactivity of ATP, guanosine triphosphate (GTP), and UTP (Table 2). It is probable that the phosphorylation of guanosine diphosphate (GDP) and uridine diphosphate (UDP) to their respective nucleoside triphosphates is via the terminal phosphate of ATP (7). Therefore, if significant compartmentalization of the nucleotides does occur, the specific radioactivity of GTP and UTP could differ from the specific radioactivity of ATP, especially prior to saturation of these pools by radioactive Pi. Table 2 shows that, within the experimental error, the three nucleotides have the same specific radioactivity after a 30-min incubation with [^{32}P]Pi (saturation does not occur for at least 1 hr). Thus, the nucleoside triphosphates are at radioactive equilibrium, indicating the absence of significant compartmentalization of these compounds.

Incorporation of [^{32}P]Pi and [^{3}H]adenine into the adenine nucleotides. Incorporation of [^{32}P]Pi and [^{3}H]adenine into the adenine nucleotides was followed by incubating cells in the presence of label for various time intervals and isolating the nucleotides as described above. Table 3 shows that during a 20-min incubation, the adenine nucleotide levels do not change. This is also true for UTP (12). Thus, the experimental conditions used for the incorporation studies do not create a "stressed" condition in which altered cellular energy demands affect the level of adenine nucleotides. Furthermore, since no change in the nucleotide levels occurred over 20 min, increases in specific radioactivity over this time period are a valid measure of the rate of incorporation.

The labeling of the adenine nucleotides by [^{32}P]Pi may indicate the mechanism by which the levels of these compounds are regulated. Figure 1 shows that, at both the pseudoplasmodium and sorocarp stages, there was linear incorporation of [^{32}P]Pi into ADP and ATP, whereas AMP was only slightly labeled. At the sorocarp stage, the ratio of specific radioactivity of ATP to ADP is 2:1, suggesting that adenylate

TABLE 2. *Specific radioactivities of nucleoside triphosphates in sorocarps after 30-min exposure to [^{32}P] Pi*[a]

Expt no.	Nucleotide specific radioactivity (10^4 counts per min per μmole)		
	ATP	GTP	UTP
1	2.68	2.50	2.17
2	2.97	2.77	3.10
3	2.78	2.88	2.72

[a] Compounds were located by their fluorescence under ultraviolet light; by exposing the chromatogram to HCl vapors, the guanine nucleotide exhibited a characteristic fluorescence. Abbreviations: ATP, adenosine triphosphate; GTP, guanosine triphosphate; UTP, uridine triphosphate.

TABLE 3. *Levels of adenine nucleotides over the time period of incubation of cells at the sorocarp stage with [^{32}P] Pi*[a]

Time of shaking (min)	Nucleotide levels (μmoles/ml)		
	ATP	ADP	AMP
0	0.90	0.59	0.23
5	0.84	0.59	0.25
10	0.98	0.60	0.21
15	0.90		0.22
20	0.89	0.57	0.23

[a] Cells were harvested, placed in small flasks, and shaken on a rotary shaker for various time periods. The cells at zero time were treated the same, except they were not shaken. Abbreviations: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate.

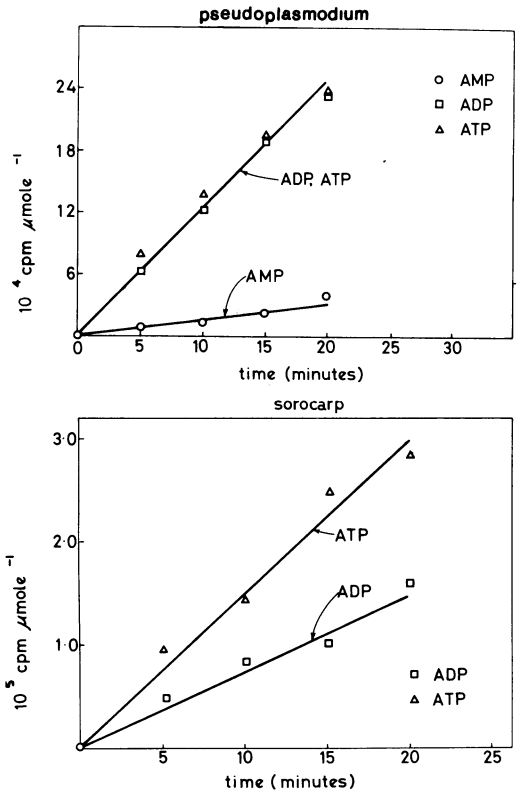

FIG. 1. *[^{32}P]Pi incorporation into the adenine nucleotides at the pseudoplasmodium and the sorocarp stages. A 20-μCi amount of [^{32}P]Pi was added to 3 ml of cell (0.3-ml packed-cell volume) and incubated at 25 C for 20 min. The nucleotides were then separated and counted as described in Materials and Methods.*

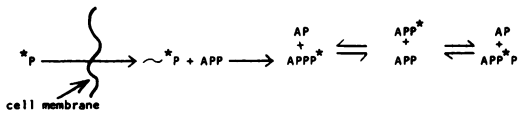

FIG. 2. *Entry of [^{32}P]Pi into the adenine nucleotides and equilibrium of the isotope via adenylate kinase.*

kinase is the primary enzyme active in distributing the label between the nucleotides. The incorporation of [^{32}P]Pi into the system would be represented by the reaction sequence shown in Fig. 2. Radioactive inorganic phosphate, which slowly permeates the cell membrane barrier, is incorporated via oxidative phosphorylation into the terminal phosphate of ATP. If the adenylate kinase system were regulating the nucleotide levels, two molecules of ADP would initially be formed from labeled ATP and the unlabeled AMP, as shown. Since the beta-position of ADP and the beta- and gamma-positions of ATP may be expected to come into isotopic equilibrium, the specific radioactivity of the ATP pool would be twice that of the ADP pool.

In contrast to the uptake of label at the sorocarp stage, the incorporation of [^{32}P]Pi into the adenine nucleotides at the pseudoplasmodium stage does not reflect regulation only by adenylate kinase (Fig. 1). In fact, the specific radioactivity of ADP is nearly the same as ATP. This relationship between ADP and ATP is difficult to understand in terms of known metabolic events. This result may be related to the fact that at this stage of differentiation cellular energy demands are probably near a maximum, and numerous synthetic reactions might well contribute to the adenylic nucleotide picture. However, no single specific enzyme system is implicated as was the case for the sorocarp stage of development.

Figure 3 shows the incorporation of [^{3}H]adenine into the adenine nucleotides at the pseudo-

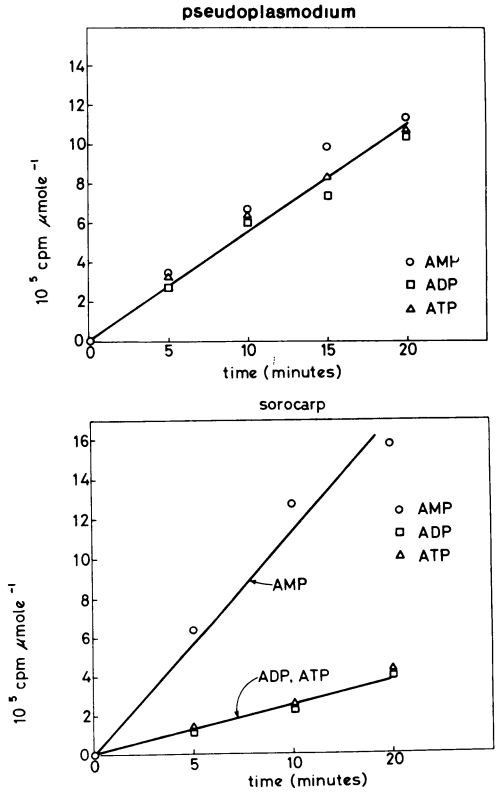

FIG. 3. *Incorporation of [^{3}H] adenine into the adenine nucleotides at the pseudoplasmodium and the sorocarp stages. Thirty microcuries of [^{3}H]-adenine was added to 3 ml (0.3-ml packed-cell volume) of cells and incubated at 25 C for 20 min. The nucleotides were separated and counted as described under Materials and Methods. The labeling patterns as shown occurred in 9 of 10 separate experiments.*

plasmodium and sorocarp stages. There were some similarities between the two stages; for example, the incorporation of adenine was linear over the 20-min incubation period. Furthermore, at both stages the specific radioactivity of ADP was always the same as ATP. However, significant differences occurred between the two stages in the relative rate of labeling of AMP and ADP. At the pseudoplasmodium stage, the rate of labeling was the same for all three adenine nucleotides. At the sorocarp stage, the rate of incorporation of label into AMP was four times the rate of incorporation into ADP and ATP. The phosphorylation of AMP to ADP is catalyzed by adenylate kinase and therefore may provide additional information concerning the role of this enzyme during differentiation.

The labeling of the adenine nucleotides by [^{3}H]adenine and [^{32}P]Pi was also carried out in the presence of added glucose. Liddel and Wright (10) have shown that glucose stimulated oxygen consumption at the sorocarp stage, but not at the earlier stages of differentiation. Therefore, the differences in energy metabolism observed between the two stages could be the result of the depletion of a readily available reserve material. However, the incorporation of isotope into the adenine nucleotides in the presence of glucose was the same as described earlier in the absence of added glucose. Therefore, although glucose stimulates oxygen consumption at the sorocarp stage, it does not appear to alter the mechanism by which isotope is incorporated into the nucleotides.

Enzyme activity of adenylate kinase at the pseudoplasmodium and sorocarp stages. The labeling patterns of the adenine nucleotides by [^{32}P]Pi indicated that at the sorocarp stage the nucleotide levels were controlled predominantly by adenylate kinase, whereas at the pseudoplasmodium stage they were not. We therefore measured the specific activity of the enzyme at both stages to determine if the dominance of adenylate kinase regulation at the sorocarp stage was due to a difference in enzyme activity at the two stages. The specific activity of adenylate kinase was assayed in both the forward and reverse directions and was found to be active at both stages (Table 4).

Position of radioactive phosphate in ATP. Another effect of adenylate kinase activity on the adenine nucleotides would be the randomization of label between the beta-phosphate and gamma-phosphate of ATP. This randomization of label occurs because of the reversibility of the reaction shown in Fig. 2, thus providing an equal chance for labeled or unlabeled ADP to enter the reaction. The specific radioactivities of the beta- and gamma-phosphates of ATP after exposure of cells at the sorocarp stage to [^{32}P]Pi for 30 min were 1.13×10^5 and 1.17×10^5 counts per min per μmole, respectively. That 30 min was sufficient for total conversion of ATP to ADP was shown by a parallel experiment in which unlabeled ATP at a concentration equal to that in the test situation was added to the reaction mixture. That both phosphates have equal specific radioactivities indicates, again, the participation of adenylate kinase in regulating these nucleotides.

TABLE 4. *Relative rates of adenylate kinase activity at two stages of differentiation*[a]

Stages	2ADP → ATP + AMP	AMP + ATP → 2ADP
Pseudoplasmodium	0.84 ± 0.3	2.59 ± 0.1
Sorocarp	1.37 ± 0.09	3.86 ± 0.5

[a] Reaction rates are expressed as micromoles per minute per milliliter of packed cells and represent the mean ± standard deviation of five determinations. Cell extracts were prepared by passage through a French pressure cell at 23,000 psi. The solution was then centrifuged at 35,000 × *g* for 15 min to remove whole cells, and the supernatant fraction was used for subsequent assays. Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; AMP, adenosine monophosphate.

DISCUSSION

The adenine nucleotides are among the most rapidly turning over compounds in the cell; therefore, their true levels in vivo are often difficult to determine due to sudden changes that may occur during experimentation. In fact, it has been assumed that many metabolites can be estimated only roughly, if at all, due to the high metabolic turnover rate relative to the steady-state levels of metabolites (1). As we have shown in this study, however, the nucleotide levels in *D. discoideum* are not affected by the harvesting procedures used.

For an evaluation of the reactions regulating nucleotide levels, it is useful to formulate mass action ratios (8). Therefore, mass action ratios (K_{ss}) for adenylate kinase at the sorocarp stage of differentiation were calculated from the steady-state substrate levels and compared to apparent equilibrium constants taken from the literature (3, 14). If adenylate kinase regulates the adenine nucleotide levels, the values for K_{ss}, $[(ATP)(AMP)/(ADP)^2]$ obtained from the steady-state nucleotide levels should be similar to the apparent equilibrium constant. It is known that the apparent equilibrium constant of adenylate kinase in vitro varies as a function of *p*H or concentration of Mg^{2+} (2, 9, 11); a summary

from the literature, given by Bomsel and Pradet (3), shows such values to vary from 0.3 to 1.0 The values for K_{ss} at the sorocarp stage varied from 0.59 to 0.74. That the values for K steady-state at the sorocarp stage are similar to reported values for the K equilibrium of this reaction is evidence in favor of equilibration of the nucleotides by adenylate kinase at this stage. In contrast, however, the K_{ss} obtained from the nucleotide levels at the pseudoplasmodium stage of development range from 1.1 to 2.8, indicating a disequilibrium from the adenylate kinase system.

Assuming no compartmentalization, a minimum rate of ATP turnover was calculated by incubating cells in the presence of [^{3}H]adenine for very short time intervals. After 5 sec of incubation, the specific radioactivities of ADP and ATP were identical, indicating that the complete ATP pool had turned over. Since the ATP pool is approximately 1 μmole of packed cells per ml, a minimum rate of ATP turnover for both pseudoplasmodium and sorocarp was determined to be 12 μmoles per min per ml. This value is intermediate between the minimum rate of ATP turnover in rat liver of 25 μmoles per g per min (16) and that in *Rhodospirillum rubrum* of 2 μmoles per g per min (18). This rate of ATP turnover in *D. discoideum* is more than sufficient to account for the rate at which UTP is being used for the synthesis of UDPG and end product saccharides (12).

In pseudoplasmodia, various systems utilizing and regenerating ATP are active, e.g., oxidative phosphorylation, glycolysis, and numerous synthetic reactions. Therefore, the role of adenylate kinase in maintaining a balance between the adenine nucleotides may be masked, i.e., the relative contribution of this enzyme to the establishment of nucleotide levels may be hidden by the effects of other systems utilizing and producing the nucleotides. Conversely, at the sorocarp stage, the rate of oxygen consumption is lower, and glycolysis as well as many synthetic reactions occur to an insignificant extent; the consequences of adenylate kinase activity may, therefore, be more easily observed.

Atkinson (1) has suggested that regulation of the interactions between ATP regeneration and processes that consume ATP respond to the "energy charge" of the cell, rather than to the concentration of a single adenine nucleotide. The energy charge, formulated as (ATP)+(0.5 ADP)/(ATP)+(ADP)+(AMP), represents the average number of energy-rich bonds per molecule of adenine nucleotide. A completely discharged system would have an energy charge of zero since only AMP would be present. Conversely, in the completely charged system only ATP is present, and the energy charge is 1.0. A basic assumption of the energy charge concept is that the level of adenylate kinase is sufficient to maintain a virtual equilibrium of the adenine nucleotides. This criterion for adenylate kinase equilibrium is met in vivo at the sorocarp stage, as judged by the steady-state levels in Table 1 and the labeling patterns in Fig. 1. This stage therefore represents an example in which the energy charge principle may apply.

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