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Adenine Nucleotide Levels in *Neurospora*, as Influenced by Conditions of Growth and by Metabolic Inhibitors

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By a combination of luciferase and fluorescence methods adenine nucleotide pools in *Neurospora crassa* have been examined under various conditions of growth and metabolic inhibition. During sustained exponential growth (25 C, shaking liquid cultures), the intracellular adenosine 5'-triphosphate (ATP) concentration, $[ATP]_i$, rises slowly from the conidial level near 1 mM (1 mmol/kg of cell water) to a maximum of 2.0 to 2.5 mM at 14 h, after which it slowly declines. The adenosine 5'-diphosphate and adenosine 5'-monophosphate (AMP) curves show two peaks, at 8 and 20 h, with a minimum at 16 h. The "energy charge" function varies around a mean of 0.72 throughout the period of exponential growth. Transferral of growing cells to buffer lacking a nitrogen source stabilizes the $[ATP]_i$ near 2.5 mM, apparently independent of the cell age, and most studies of metabolic inhibitors were carried out on cells grown 14 to 16 h and then shifted to N-free buffer. Under these conditions sudden respiratory blockade (cyanide) produces exponential decay of ATP with a time constant of about 5.7 s (half-time of 3.9 s), and at a rate which implies a minimal ATP turnover of 0.44 mM/min. This figure is about one-third the rate (1.17 mM/min) which would be calculated from steady-state respiration, a discrepancy which may partly be accounted for by transphosphorylation from appreciable amounts of non-adenine nucleoside di- and triphosphates present in *Neurospora*. For all three adenine nucleotides, the transients associated with sudden respiratory blockade include overshoots or undershoots of several minutes duration, which are consistent with feedback regulation of glycolysis by the AMP/ATP ratio.

In the past 15 years numerous studies have been made of the relationship between adenine nucleotides, particularly adenosine 5'-triphosphate (ATP), and various physiological processes in microorganisms: growth (14, 16), cell division (8, 22), development of storage compounds (19), nucleic acid turnover (12, 17), clock-type oscillations of glycolysis (4), and transport (20, 21). The object of all these experiments has been to discover the manner in which energy metabolism regulates, and is regulated by, other physiologic processes. In view of current theories on photophosphorylation and oxidative phosphorylation (33), one aspect of the relationship between energy metabolism and transport has become particularly important: the generation of membrane potentials during the active transport of ions.

One cannot study this process directly either in mitochondria or in most microorganisms; but the fungi, particularly the ascomycete *Neurospora*, offer hyphae of suitable size and mechanical properties for microelectrode studies. The resting membrane potential of *Neurospora* hyphae is rapidly and reversibly abolished by low concentrations of respiratory inhibitors (39, 40), and under restricted conditions there is a proportionality between the fraction of respiration maintained and the minimal membrane potential reached in the presence of an inhibitor. Further data suggest that the voltage generating process (ion pump), which is reflected in the potential difference across the plasma membrane, must be fueled by an energy reservoir, such as ATP, rather than by an intermediate of electron transfer. In order to investigate the question in more detail, a precise comparison between membrane voltages and adenine nucleotide levels is needed, and the central purpose of the present experiments

is to describe the relationships among ATP, adenosine 5'-diphosphate (ADP), and adenosine 5'-monophosphate (AMP) under various normal and inhibited conditions. Corresponding studies of membrane potential will be presented in a subsequent paper.

MATERIALS AND METHODS

General. The wild-type strain RL21a of *Neurospora crassa* was used throughout these experiments. Methods for growing and handling the fungus have been described previously (41), and will be reviewed briefly. Shaking liquid cultures were prepared by inoculating 5- to 8-day conidia at a density of 10^6/ml into 160 ml of a standard minimal medium (Vogel, [48]; with 2% sucrose) and incubating the suspension on a reciprocating shaker (120–140 strokes/min) at 25 C. This treatment results in exponential growth (Fig. 2A), during which the cultures consist of unbranched filaments 2 to 3 μm in diameter and up to several hundred micrometers long. At the end of a specified period, normally 15 to 16 h, cultures were harvested on filter paper or cheesecloth, rinsed several times with distilled water, suspended at a density of 1 to 2 mg/ml (dry wt) in buffer, and distributed in 20-ml portions into 125-ml Erlenmeyer flasks. The buffered suspensions were allowed to preincubate 20 to 90 min (shaking, 25 C) before use. Control experiments (Fig. 1B) showed that the ATP level in normal cells remains constant throughout this interval. Stationary cultures were obtained by inoculating the conidia into 20 ml of minimal medium and incubating the suspensions at 25 C for 24 to 72 h. The resulting mycelial mats were handled in the same manner as the shaking cultures.

Buffers. Unless otherwise noted, all experiments were performed at pH 5.8 (the pH of the growth medium), in a standard buffer solution containing 20 mM 3,3-dimethylglutaric acid (DMG) brought to pH 5.8 with KOH (final concentration, 25 mM), 1 mM $CaCl_2$, and 1% glucose. DMG has proved to be an inert buffer, which neither stimulates nor inhibits the growth of *Neurospora*.

Preparation of cell extracts. Metabolism was halted (at measured intervals after the injection of inhibitor into shaking suspensions of cells) by pouring the suspension through a sintered glass suction filter and dashing the resulting pellet of cells into petroleum ether/dry ice. Each frozen pellet was lyophilized (maintained at −20 C), weighed, powdered in a Ten Broek homogenizer at −70 C, and extracted for 1 to 2 h in 1.5 ml of 6% perchloric acid (PCA) at 0 C. The resulting suspension was transferred quantitatively to a graduated centrifuge tube, diluted to 5.0 ml, and centrifuged at 2000 × *g* for 15 min. A sample (4.0 ml) of the clear supernatant fluid was removed for neutralization. After the addition of 1.2 ml of 200 mM glycylglycine, the sample was titrated to pH 6.8 with an automatic titrator delivering KOH. The resulting dense precipitate of potassium perchlorate was removed on a membrane filter (Type RA, 1.2-μm pore size; Millipore Corp., Bedford, Mass.). To prevent precipitate from forming in the reference junction of the pH electrode, an NaCl junction was used. The filtered precipitate was subsequently rinsed with saturated $KClO_4$ solution, which was collected with the original filtrate. The volume of total filtrate was brought to 12.0 ml with saturated $KClO_4$. Finally, all samples were frozen and stored at −20 C. Two standard samples of ATP (1.0 ml of about 1.5×10^{-4} M Na_2H_2ATP; Sigma Chemical Co., St. Louis, Mo.) were carried along through all steps (after the grinding), for each batch of 10 to 15 cell samples being extracted.

Measurement of ATP. ATP was assayed with fire-fly luciferase according to the method of Strehler (45), using the photomultiplier of an Aminco-Bowman spectrophotofluorometer (American Instrument Co., Silver Spring, Md.). The cuvette chamber of the instrument was fitted with a syringe port so that the sample could be injected directly, and all light output measured. Desiccated fire-fly lanterns, obtained from Sigma Chemical Co., were extracted in distilled water as recommended by McElroy (45); the extract was brought to pH 7.5 and frozen for storage. Two buffers were freshly prepared for each experiment: (i) 20 mM glycylglycine, 5 mM phosphate, 25 mM $MgCl_2$, brought to pH 7.4 with NaOH; and (ii) 20 mM glycylglycine, pH 7.4. A 250-ml sample of the latter, at room temperature (about 23 C), was put into the reservoir of an LI Repipet (Labindustries, Inc., Berkeley, Calif.). At the start of each analytical run, a batch of cell extracts and standards, along with several tubes of enzyme, were thawed, mixed thoroughly, and then packed in an ice-bath. For each assay, 50 μliters of cold enzyme and 0.95 ml of buffer (i), at room temperature, were put into a cuvette which was set into the spectrophotofluorometer. Then 100 μliters of cell extract was taken into the syringe of the Repipet, and the syringe tip was connected to the port of the cuvette chamber. The chamber was closed, the shutters to the photomultiplier were opened, and the sample was rapidly injected ahead of 0.9 ml of buffer (ii). This technique accomplished rapid mixing in a total volume of 2.0 ml, and gave a flash peak (550 nm, 5 mm slits: see Fig. 1A) in 1 to 3 s which was reproducible with a 1% standard error of the mean (SEM) and was proportional to the amount of ATP present up to 2.5×10^{-9} mol (1). Each batch of samples was assayed twice: in order and in reverse order, as a means of compensating for time-dependent decay of the thawed luciferase.

Determination of other nucleotides. ADP was assayed as ATP, following conversion with creatine kinase and phosphocreatine. Conversion was carried out in a total volume of 3.0 ml, containing 1.5 ml of cell extract, 8 mM additional glycylglycine (pH 7.4), 10 mM $MgCl_2$, 10 mM phosphocreatine, and 0.15 mg of creatine kinase (Nutritional Biochemicals Corp., Cleveland, Ohio). The reaction vessel was incubated at 25 C for 30 min, transferred to boiling water for 5 min, and then frozen for storage. Control experiments showed that 95 to 99% of ADP in standard samples was converted and recovered as ATP.

AMP was assayed fluorometrically, using the lactic dehydrogenase-pyruvate kinase system (13). Fluores-

cence was measured by the Aminco-Bowman spectrophotofluorometer, with excitation at 360 nm and emission at 465 nm (3-mm slits). Standard samples of AMP were prepared in a solution of 20 mM glycylglycine (pH 6.8) saturated with $KClO_4$.

Electrophoretic separation of nucleotides in *Neurospora* was carried out by the 2-dimensional method of Dahlberg (10). Cells were grown in the usual manner, but in a medium containing only 2 mM phosphate, labeled with $^{32}PO_4$ at a specific activity of 2.3 Ci/mol. The medium was chosen so the cells would essentially remove all phosphate from the medium, but not deplete their internal stores during the 15 to 16 h of growth. Cell samples were then processed as already described for ATP and ADP analyses.

For the first dimension electrophoresis, 50 μliters of each perchlorate extract was spotted onto Whatman 540 filter paper (W. & R. Balston, Ltd., Maidstone, England) and run in pyridinium acetate buffer (pH 3.5: 0.5% pyridine, 5% acetic acid, 2 mM ethylenediaminetetraacetic acid [EDTA]) at 4,000 V (Savant LT48A Electrophoresis Unit; Savant Instruments, Inc., Hicksville, N.Y.) for 45 min. The sample strip was then cut out, sewed onto Whatman DEAE paper, and run at right angles for 10 h at 1,400 V. The resulting electrophoretogram was autoradiographed for 72 h. Spots were identified by comparison with standard sample mixtures electrophoresed under the same conditions. All major spots were cut from the electrophoretogram, transferred to scintillation fluid, and counted on a Mark I Scintillation Counter (Nuclear Chicago Corporation, Des Plaines, Ill.).

Oxygen measurements. Oxygen consumption by *Neurospora* was measured on light suspensions of cells (0.5 mg/ml dry wt) in a closed vessel (18.6 ml) similar to that described by Pressman (32). The suspension was maintained at 25.0 ± 0.1 C by water circulating in an outer jacket of the vessel and it was stirred with a magnetic flea. A Clarke-type oxygen electrode (YSI No. 5331; Yellowsprings Instrument Co., Yellowsprings, Ohio) with a Teflon membrane was employed; it was calibrated against dense cell suspension for zero oxygen and against air-saturated (CO_2-free) buffer for 0.21 atm oxygen. The solubility coefficient of oxygen in the standard buffer was estimated by the method of Carpenter (5) and was found to be 27.2 μliters of O_2/ml at 1 atm, about 4% smaller than for distilled water. The time-constant for response of the oxygen electrode to a step change in O_2 concentration was 5.9 ± 0.1 s.

Computations. All data were obtained in units of μmol of nucleotide per unit dry weight of cells. This unit was converted to mmol per kg of intracellular water (mM) by using the ratio intracellular water/dry weight = 2.54 (41). In most cases the results are expressed as the mean ± 1 SEM. As is discussed in connection with Table 1, within some groups of experiments (Fig. 3 and 5) the variability of control measurements was quite large, so that, in order to make meaningful comparisons, it was necessary to convert the results from units of mmol/kg of cell water to units of percentage of control. Curves in Fig. 1 and 4 were fitted using a computerized method for nonlinear least squares estimation (28).

RESULTS

Evaluation of ATP assay. Because of the high rate of respiration in *Neurospora* and the high cell densities reached in filter pellets (100 mg dry wt/ml or greater), harvested cells would be expected to become anoxic in 5 s or less, so that conditions in which cell samples are taken become critical to the ATP analysis. In the present experiments, the total time required for harvesting and freezing was 3 to 4 s, of which about one-half was required for filtering, and one-half for transferral and freezing. In control experiments, shorter intervals did not result in elevated ATP levels, but intervals exceeding 6 s (for harvesting and freezing together) did produce depression of ATP levels.

The vulnerability of the fire-fly assay to reaction conditions such as salt concentration

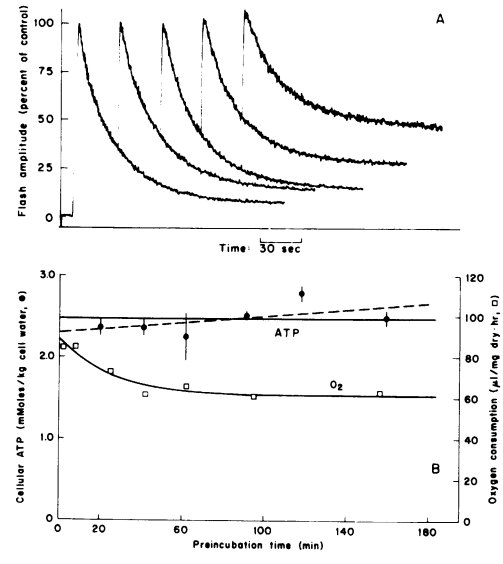

FIG. 1. *Control experiments to show (A) the nature of the data records for the luciferase assay, along with the effect of ADP on the flash response to ATP; and (B) the effect of preincubation time on the rate of oxygen consumption and the ATP level, after cells have been transferred from growth medium to buffer. The curves in A are flash records for samples containing 1.25 nmol of ATP plus (left to right) 0, 0.62, 1.25, 2.5, and 5.0 nmol of ADP. In B each plotted rate of respiration (□, right ordinate scale) represents the average value for duplicate determinations. The smooth curve is drawn according to the equation* $Y = 61.0 + 28.2\ e^{-0.040t}$, *in which the numbers are least-squares estimates. Each plotted value of ATP is the average for 4 determinations (●, left ordinate scale). The dashed line has been drawn by least squares and has a slope of 0.002 ± 0.001 mmol/kg of cell water·min, which is not significantly different from zero* ($p > 0.05$).

and the presence of nucleotides other than ATP has been pointed out by numerous authors (see esp. Strehler & Totter [46] and Aledort et al. [1]). In the present experiments the reaction buffer was designed to contain sufficient salt (Na + K = 15 mM, Cl = 24 mM, orthophosphate = 2.4 mM) so that the contribution of these substances from the cells would be negligible (K < 0.1 mM, orthophosphate < 0.01 mM). No attempt was made to assess the actual effects of various anions or cations, except for the perchlorate ion. That species sharply reduces the flash peak, but in these experiments the effect was held constant (at about 50% reduction) by preparing the extracts uniformly in saturated $KClO_4$.

Three nucleotides were examined for possible interference with the luciferase assay: AMP, ADP, and deoxyATP (d-ATP). AMP had no measurable effect on the luminescence curves. ADP, though conspicuously retarding the decay of the ATP flash, only slightly enhanced the initial flash peak. As is shown in Fig. 1A, for the largest ratio of ADP/ATP tested (= 4.0), peak enhancement due to ADP was 6%. d-ATP differed from ADP in eliciting a slow flash without ATP; it also slightly enhanced the flash due to ATP. But again, the effect on the initial flash peak was less than 6% with dATP/ATP ratios in a reasonable physiological range (less than 1.0). It is clear that the presence of either ADP or dATP could lead to serious errors in estimation of ATP if the flash intensity at any time other than the peak were used for the calculation.

Nucleotide levels during exponential growth. In order to establish a reference point for further experiments, adenine nucleotides were determined on shaking liquid cultures at various times along the growth curve. Contrary to the usual procedure, determinations in this experiment were made on cells kept in the growth medium, instead of the standard buffer. The results are shown in Fig. 2 (B and C), together with a standard growth curve (A) and a curve of respiration (B; right ordinate scale). The period of exponential growth lasts from 4 to 18 h, but neither the energy turnover—as measured by the rate of respiration—nor any of the adenine nucleotide levels remained constant (per unit cell mass) during that period. Oxygen consumption and all three nucleotides were low, as might be expected, in freshly harvested conidia. By the onset of exponential growth, oxygen consumption had risen fivefold (the same factor found by Zalokar [50] for succinic dehydrogenase), after which it slowly declined. AMP and ADP began to rise soon after inoculation, and reached peaks at 6 to 8 h after which they fell steadily until nearly the end of exponential growth. ATP remained at the conidial level until well into the period of exponential growth, rose more than twofold to reach a peak at 13 to 15 h, and then declined. Evidently, net synthesis of adenine nucleotides takes place during the first 8 h of growth; this is followed by a 4-h interval in which the sum of ATP, ADP, and AMP is approximately constant (at 3.0 mmol/kg of cell water, in this experiment) and then by an extended period in which the sum declines.

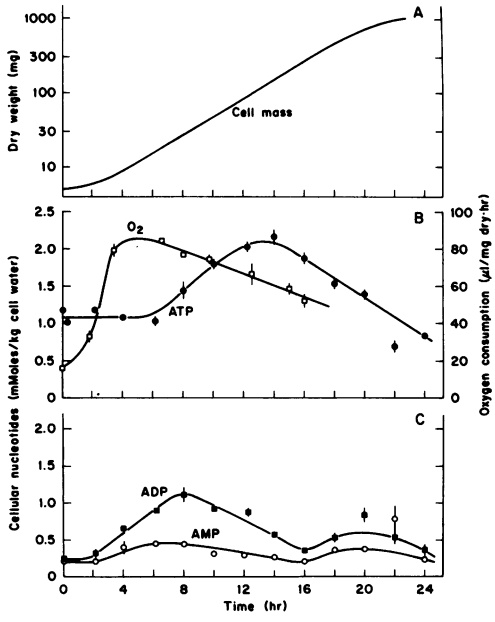

FIG. 2. *Adenine nucleotide levels during exponential growth of Neurospora. (A) Growth curve (41). (B) Oyxgen consumption (right ordinate scale) and ATP concentration in cell samples removed from exponentially growing cultures. (C) ADP and AMP concentrations. Each plotted value of nucleotide is the average for 3 determinations; each value of respiration represents the average for 2 to 6 determinations. Vertical bars. ±1 SEM. The respiration data were kindly provided by Alan Lambowitz.*

Effects of removing the cells from growth medium. From the manner in which ATP concentrations vary with cell age, it is obvious that cells in exponentially growing shaking cultures are not optimal material for study of the energy requirements of a specific physiological process, such as transport. Because a large variety of experiments had previously been carried out on cells grown in shaking cultures and then transferred to a standard buffer (lacking a nitrogen source; see Materials and Methods), a similar treatment was tested for the ATP

experiments. It results in at least two readily measured changes in energy metabolism: (i) respiration declines by about 30%; and (ii) ATP rises (usually) and stabilizes in the neighborhood of 2.5 mmol/kg of cell water. The effect of time on the respiration of cells incubating in buffer is shown in Fig. 1B (right ordinate scale). In the experiment shown, the initial rate was approximately 90 μliters per mg (dry wt) per h, and the steady-state level (near 60 μliters per mg (dry wt) per h was approached with a time constant of 25 min (rate constant = 0.04 min^{-1}).

Fortunately, the intracellular ATP concentration stabilizes more quickly, reaching the steady-state value after incubation for not more than 20 min in the buffer. In the experiment shown in Fig. 1B (left ordinate scale), ATP averaged 2.5 mM for all cell samples taken in the interval 20 to 160 min. Normally, cells were used for ATP measurements after 20 to 90 min in buffer.

Removal of cells from growth medium to the standard buffer has the further advantage of eliminating the age-dependence of the intracellular ATP concentration, at least for cells 10 to 18 h old. This point is demonstrated in Table 1, which shows the average ATP concentrations in cells either resuspended and preincubated for 20 min in standard buffer (lines 5–7, column 6), or sampled directly from the growth medium (lines 2–4, column 6). The mean ATP concentration in the three groups of preincubated cells was 2.8 mM, and the difference between the extremes (0.24 mM) was not significant ($P > 0.1$). The three groups of cells measured in growth medium gave a mean ATP concentration of 1.8 mM, and the difference between the extremes (0.63 mM) was significant at the level $P < 0.01$.

Incubation of the cells in buffer did not eliminate scatter in the ATP measurements, however, and, in order to make different experiments comparable, one standard condition of measurement was chosen for controls in all experiments: cells from 15- to 16-h shaking cultures, preincubated in buffer for 20 min. One hundred thirty such control samples gave a mean ATP concentration of 2.56 ± 0.06 mM; 30 of these samples gave mean ADP and AMP concentrations of 0.61 ± 0.05 mM and 0.34 ± 0.03 mM, respectively (Table 1, line 1). The control ATP values in lines 2 to 4 and 5 to 7 of

TABLE 1. *Effects of various conditions of growth and incubation on nucleotide concentrations in Neurospora*[a]

Type of expt	Conditions of growth	Conditions of preincubation	Control ATP concn (mmol/kg cell water)	Experimental ATP concn (% of control)	Measured nucleotide concn (mmol/kg cell water)		
					ATP	ADP	AMP
Control expt	15–16 h shaking (standard)	20–90 min DMG buffer + 1% glucose	2.56	100	2.56 ± 0.06	0.61 ± 0.05	0.34 ± 0.03
Effect of preincubation at different cell ages	10 h shaking	20 min in fresh growth medium + 1% glucose	2.11	85	1.80 ± 0.08	0.92 ± 0.02	0.32 ± 0.01
	14 h shaking	as above	2.11	102	2.16 ± 0.11	0.57 ± 0.02	0.26 ± 0.02
	18 h shaking	as above	2.11	72	1.53 ± 0.07	0.53 ± 0.07	0.36 ± 0.03
	10 h shaking	20 min in DMG buffer + 1% glucose	2.95	99	2.92 ± 0.02	—	—
	14 h shaking	as above	2.95	91	2.68 ± 0.10	—	—
	18 h shaking	as above	2.95	95	2.79 ± 0.15	—	—
Stationary cultures	71–72 h in stationary liquid	harvest without preincubation	2.24	91	2.05 ± 0.05	0.60 ± 0.11	0.22 ± 0.06
	71–72 h in stationary liquid	30 min in DMG buffer + 1% glucose	2.24	82	1.82 ± 0.05	0.49 ± 0.04	0.21 ± 0.02

[a] All data except those in the first line (Control experiment) represent average analyses for 3 to 5 cell samples. Averages in the first line were obtained from 130 samples (ATP) or 30 samples (ADP and AMP). Control ATP concentrations (column 4) are the mean values obtained from simultaneous measurements on 15- to 16-h shaking cultures, preincubated in DMG buffer + 1% glucose. Lines 2 to 4, 5 to 7, and 8 to 9 represent three separate groups of experiments.

Table 1 illustrate the variability between experiments (2.11 mM versus 2.95 mM) and make clear that comparison of individual ATP values between different experiments is not as meaningful as a "percentage of control" comparison (column 5).

A comparison is also given, in Table 1 (lines 8, 9), of the adenine nucleotide levels in cultures grown on stationary liquid. Such cultures have been used by other laboratories for a variety of flux measurements (31), and are also very similar to the agar-grown cultures which must be used for electrophysiological experiments (39). Average ATP levels in stationary cultures are depressed 10 to 20% below those in control cells from shaking cultures (see also Fig. 4). The slight depression of ATP in stationary cultures preincubated with buffer + glucose is of marginal significance ($p \cong 0.05$).

Response of ATP to various metabolic inhibitors. From the large body of literature on transients in biochemical systems, it is natural to expect that any sudden change in energy metabolism will result in overshoot-undershoot

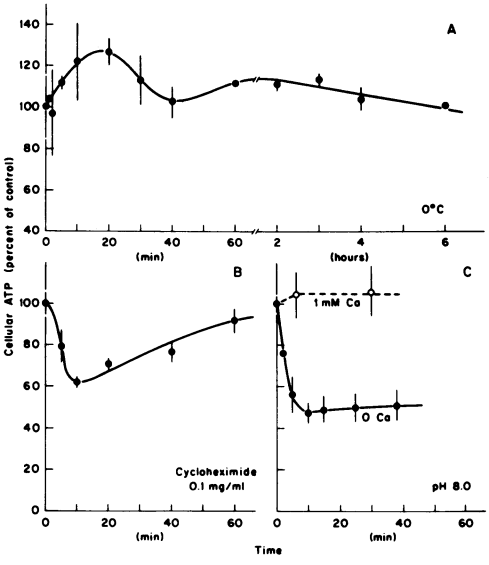

FIG. 3. *Effects of various treatments on ATP concentrations in Neurospora. (A) Lowered temperature. A step decrease in temperature from 25 to 0-2 C was obtained by diluting the suspension with iced buffer, followed by removal of the flask to an ice bath. (B) Cycloheximide. (C) High pH. The pH shift was accomplished by maintaining the cells with a pH stat, in 5 mM KCl + 1% glucose ± 1 mM $CaCl_2$, until (zero time) the following buffer solution was added: 30 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid) + 25 mM K(OH), pH 8.0. Control ATP concentrations: 3.06, 1.89, and 2.25 mmol/kg of cell water, respectively, in A, B, and C; vertical bars, ±1 SEM.*

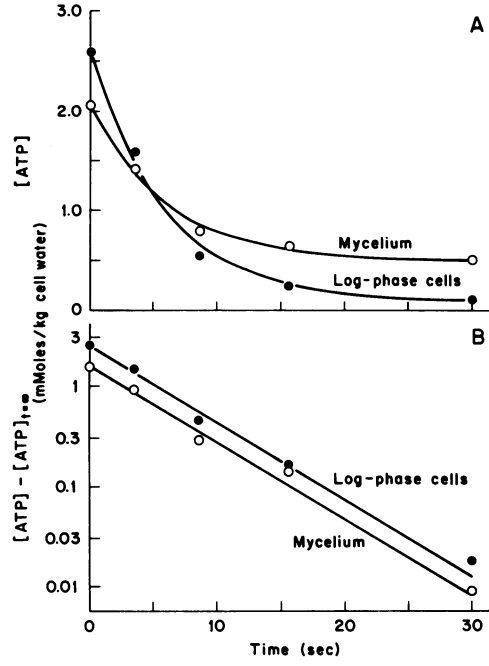

FIG. 4. *Initial decline of ATP with maximal respiratory inhibition. (A) Linear plot; (B) semilogarithmic plot. Open circles, mycelial mats from stationary liquid cultures; filled circles, cells from cultures growing logarithmically (exponentially) in shaking liquid. Each point plotted represents the average value for 3 determinations. Average SEM: exponential-phase cells, 0.023; mycelium, 0.054. The curves are drawn according to the equation* $Y = a_1 + a_2 e^{-kt}$, *in which the two coefficients and the rate constant have the following values (±1 standard error).*

	a_1	a_2	k
Exponential-phase	0.096 ± 0.068	2.518 ± 0.114	0.176 ± 0.020
Mycelium	0.503 ± 0.034	1.565 ± 0.058	0.172 ± 0.016

phenomena or even in oscillations of various metabolic intermediates. *Neurospora* certainly conforms to this expectation, at least in regard to the behavior of adenine nucleotides.

Some maneuvers, such as sudden lowering of the temperature or the introduction of a specific inhibitor of protein synthesis (cycloheximide), produce transient changes of ATP concentration without an appreciable effect on steady-state ATP levels. The data plotted in Fig. 3A show the response of ATP in *Neurospora* when the temperature was suddenly lowered from 25 to 0-2 C. Over the course of 20 min the intracellular ATP concentration rose about 30%. It subsequently declined, rose again, and then returned very slowly to the control value. Cycloheximide, on the other hand, gave a 40%

drop of ATP within 10 min, followed by a gradual return to the control level, over a period of 1 h (Fig. 3B).

While these experiments were being conducted, another factor governing ATP concentrations was discovered, quite unexpectedly. As is shown in Fig. 3C, upon transfer of the cells to buffer solutions at any pH above 7, intracellular ATP fell from normal values of about 2.3 mM to 1.1 mM in 10 min and stabilized at the latter value, with essentially no tendency to recover. The decline was prevented by putting 1 mM Ca^{2+} into the high pH buffers.

The effect of conventional respiratory inhibitors, such as cyanide or azide, on the ATP level in *Neurospora* has turned out to be unexpectedly complicated. The initial response, for example, to 10 mM KCN, is a rapid exponential decay of ATP. In the experiment shown in Fig. 4, the rate constant for decay was 0.176/s for shaking-culture cells and 0.172/s for stationary cultures; these two values are not significantly different, so that the average 0.174/s, should be taken as the best estimate. It corresponds to a time constant of 5.73 s, or a half-time of 3.95 s, for the decay process. The absolute initial rate of ATP breakdown in this preparation of shaking-culture cells was 0.44 mmol/kg of cell water·min.

At longer times—from 30-s inhibition to 1 h or more—partial recovery of the ATP concentration is observed. But, depending on the type of inhibitor and the concentration used, two elements in the recovery process can be discerned: one resulting from the functional disappearance of inhibitor (cyanide or azide), and the other representing a genuine physiologic adjustment. Plots of the intracellular ATP concentrations as functions of the cyanide concentration added to the medium are shown in Fig. 5. Maximal recovery, or maximal separation of the 30-s and 30-min curves, was found in the concentration range 3×10^{-5} M to 10^{-4} M KCN. The detailed time-course for recovery in 3×10^{-5} M KCN is shown in the inset to Fig. 5 for one experiment, in which ATP fell to 40% of the

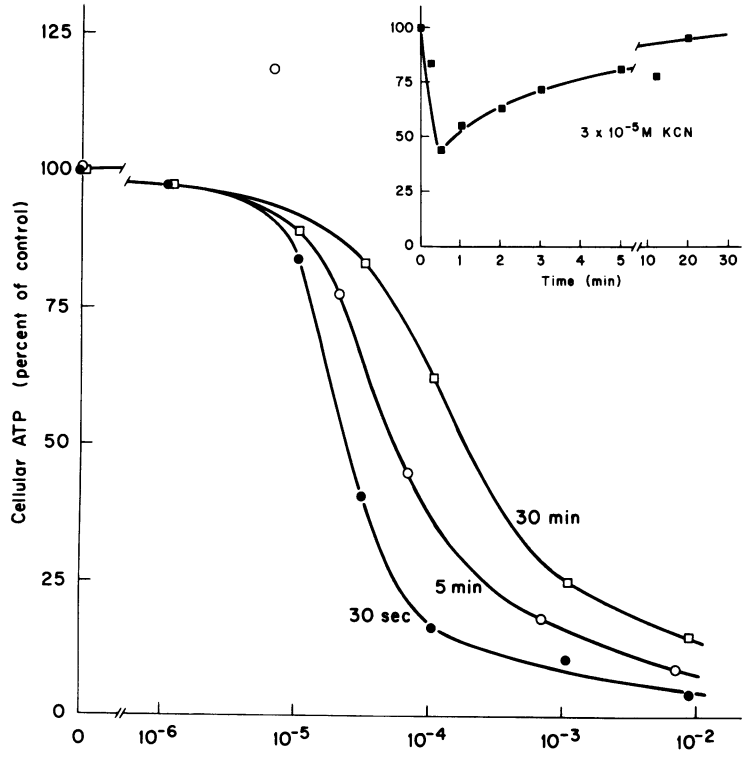

FIG. 5. *Influence of time of sampling on the dose-response (ATP) curves in Neurospora treated with potassium cyanide. Each point represents the average for 3 to 12 determinations. Control ATP concentrations: 2.56, 2.70, and 2.57 mmol/kg of cell water for the 30-s, 5-min, and 30-min curves, respectively. Average SEM: 0.16 mM at low cyanide concentrations ($\leq 10^{-4}$ M), and 0.06 mM at higher concentrations. Inset, actual time course of ATP at 3×10^{-5} M.*

control value in the first 30 s and then rose to 90% or more after 30 min. Table 2 (columns 6 and 7) reveals that, after 30 min in cyanide at several concentrations from 10^{-5} to 10^{-4} M, the percentage of recovery of ATP was essentially the same as the percentage of recovery of respiration, which suggested that the recovery at low inhibitor concentrations might result from inactivation of the inhibitor. Two other inhibitors were also tested: azide (5×10^{-5} M), which allowed a definite recovery of respiration, though less pronounced than that with cyanide; and antimycin (0.01 μg/ml) which did not permit recovery in the 30-min test period.

Confirmation of the idea that cyanide was being inactivated came from the observation that 3×10^{-5} M cyanide incubated with the standard buffer solution for 30 min before the addition of cells had no effect on respiration or the ATP levels. However, the nature of the inactivation process remains obscure; separate testing of each solution component (1% glucose, 1 mM $CaCl_2$, and 20 mM DMG) showed only partial effects of each. Furthermore, after cyanide had been inactivated in the buffer, its presence could still be measured (92–104%) with a cyanide electrode (Model 94-06, Orion Research, Inc., Cambridge, Mass.) under conditions in which most cyanide addition compounds would be expected to decompose to give ammonia (0.01 N NaOH added; [37]).

Physiologic adjustment leading to a partial recovery of ATP levels is seen at cyanide concentrations of 10^{-3} M and higher. The curves of Fig. 5 show a two- to threefold increase of ATP between the 30-s minimum and the value at 30 min; and the separation and slopes of the two curves would make sufficient cyanide loss or inactivation unlikely. This conclusion has been borne out by the facts that (i) preincubation of 10^{-3} or 10^{-2} M cyanide with buffer before the addition of cells does not elevate the ATP level at 30 s, and (ii) salicyl hydroxamic acid (SHAM), which inhibits an inducible alternate oxidase pathway in *Neurospora* (25) abolishes the partial recovery of ATP seen at 30 min with 10^{-2} M cyanide (Table 2; column 7, lines 16 and 18).

Transients of AMP, ADP, and ATP associated with the onset and release of maximal respiratory inhibition. In order to obtain a clearer picture of the energy depletion process which occurs during cyanide inhibition, several detailed experiments were undertaken to establish the time-courses for AMP and ADP. Figure 6 presents the data for the onset of cyanide (1 mM) inhibition. During the initial rapid decline of ATP, AMP and ADP both rose at rates which, added together, were approximately equal to the rate of ATP decline. ADP reached a peak in 10 to 15 s, fell to a minimum at about 50 s, and then very slowly rose, more or less parallel with the slow rise of ATP concentration. AMP reached a peak at 40 to 50 s, and thereafter declined steadily for the duration of the experiment. The sum of the three nucleotides declined exponentially from 3.9 to 2.9 mM with a time constant of 17.8 s.

In a general way, these results suggest that the relative levels of the different adenine nucleotides might be governed by the adenylate kinase reaction, coupled with a slow breakdown of AMP. Computer simulation studies were therefore undertaken applying the program of Garfinkel (18) to the following set of reactions:

$$\begin{aligned} ATP &\leftrightharpoons ADP + P_i \\ 2ADP &\leftrightharpoons ATP + AMP \\ AMP &\leftrightharpoons ADEN + P_i \end{aligned}$$

in which only the middle equation is intended to represent a defined chemical reaction (adenylate kinase). The other two equations represent the sum of all reactions leading to the synthesis and breakdown of ATP and AMP, represented as overall first order processes. Except for a short delay computed for the rise of AMP, the first 40 s of the curves in Fig. 6 can be simulated easily by the three equations above, but simulation of the later portions of the curves requires feedback control. The simplest way to produce the observed recovery of ADP and ATP on the proper time-scale is to make the rate constant for the reaction ADP + $P_i \rightarrow$ ATP proportional to AMP/ATP, with a single-compartment delay of 10 to 100 s. Control by the AMP/ATP ratio would be consistent with the known action of these adenine nucleotides on phosphofructokinase (2). The role played by the SHAM-sensitive alternate oxidase would be to provide a pathway for reoxidation of pyridine nucleotide required in glycolysis (25).

Inhibitors which primarily block glycolysis and which inhibit respiration only indirectly abolish the overshoot/undershoot features of the adenine nucleotide curves, as is demonstrated in Fig. 7 for 2-deoxyglucose (2-DOG). In this case ATP decayed slowly, with an apparent time constant of 105 s; the initial peak in ADP disappeared; and the partial recovery found with cyanide inhibition (Fig. 5 and 6) was missing, except perhaps for ADP. All of these results can reasonably be accounted for by the fact that 2-DOG blocks the initial reactions in glycolysis (49). Slow changes in the adenine

TABLE 2. *Adaptation of adenine nucleotides and respiration to low concentrations of cyanide*[a]

Inhibitor concn (M)	Time (30)	ATP concn (mmole/kg of cell water)	Control respiration[b]	Test respiration[b]	ATP[c]	Respiration[c]
Control	s	2.46 ± 0.11	63.9 ± 2.9	(63.9)	100	100
	min	(2.46)	57.0 ± 2.1	(57.0)	100	100
Cyanide 10^{-5}	s	2.43 ± 0.13	67.7 ± 4.0	51.0 ± 4.8	98.7	75.4
	min	2.48 ± 0.12	60.4	59.8 ± 3.0	100.8	99.1
1.7×10^{-5}	s	1.77 ± 0.08	63.3 ± 1.0	36.3 ± 4.8	71.9	57.4
	min	2.65 ± 0.08	56.5	61.4 ± 12.0	107.7	108.6
3×10^{-5}	s	0.81 ± 0.14	77 ± 20	12.0 ± 4.1	32.9	15.7
	min	2.66 ± 0.14	69	67.5 ± 4.0	108.0	98.7
5.5×10^{-5}	s	0.42 ± 0.08	63.1 ± 3.3	8.4 ± 0.8	17.1	13.3
	min	1.69 ± 0.15	56.4	51.7 ± 5.4	68.7	91.7
10^{-4}	s	0.30 ± 0.06	69.8 ± 5.5	4.3 ± 0.1	12.2	6.2
	min	1.32 ± 0.09	62.3	22.4 ± 3.8	53.6	36.0
10^{-3}	s	—	56.6 ± 3.5	0	—	0
	min	—	50.5	1.7 ± 0.5	—	3.4
10^{-2}	s	—	66.9 ± 4.0	0	—	0
	min	0.25 ± 0.08	59.7	1.9 ± 0.2	10.2	3.2
10^{-2} + 3mM SHAM	s	—	(66.9)	—	—	—
	min	0.11 ± 0.03	(59.7)	1.5	4.5	2.5
Azide 5×10^{-5}	s	—	66.9 ± 2.1	31.1 ± 0.7	—	46.5
	min	—	59.7	44.8	—	75.0
Antimycin 0.01 μg/ml	s	—	66.6 ± 2.2	55.4 ± 1.5	—	83.1
	min	—	59.5	51.0	—	85.7

[a] Each value of ATP listed represents the average for 3 determinations; each value of respiration, for 2 measurements. Although a control rate of respiration could be measured directly before a cyanide addition for the 30-s sample, the time-dependent change in respiration seen without inhibitors (Fig. 1B) made it necessary to *calculate* the control respiration for each inhibited sample at 30 min. The calculation was made by using the ratio 57.0/63.9, obtained from uninhibited cells (row 1, column 4).

[b] Values shown represent microliters per milligram of dry weight per hour.

[c] Values shown represent percentage of control.

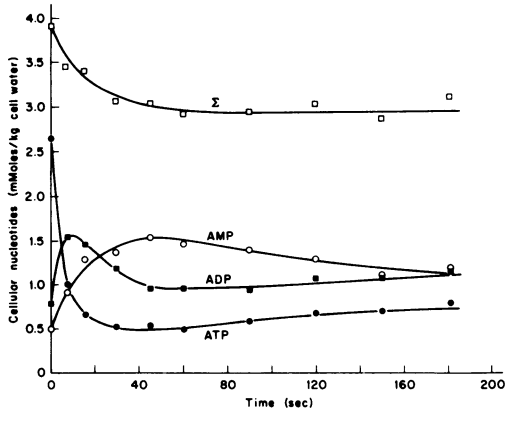

FIG. 6. *Time courses of all three adenine nucleotides in response to 1 mM cyanide. The points show the average values for 2 to 8 measurements. Average SEM: 0.10, 0.09, and 0.14 mmol/kg of cell water for ATP, ADP, and AMP, respectively.*

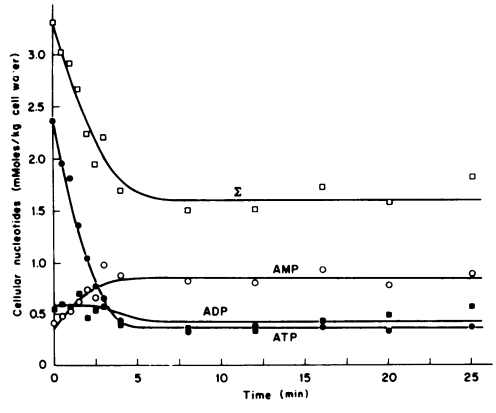

FIG. 7. *Adenine nucleotide response to 1% 2-deoxyglucose. Note that the abscissa scale is in minutes. Cells were preincubated in the normal buffer, but containing one-tenth of the normal glucose. At zero time 1% 2-deoxyglucose was added. Average SEM: 0.05, 0.06, and 0.11 mmol/kg of cell water for ATP, ADP, and AMP, in order.*

nucleotides would be the result of gradual depletion of 3-carbon glycolytic intermediates existing at the time of blockade; the disappearance of the ADP peak would result from a small effective rate constant for ATP hydrolysis, relative to that for transphosphorylation (adenylate kinase); and the absence of recovery would arise by blockade of glycolysis at or near the feedback control point.

Sudden release from maximal respiratory inhibition, as produced by the washout of sodium azide into high-pH medium (Fig. 8), resulted in a nearly linear rise of ATP and decline of ADP and AMP for about 15 s. Thereafter, the scatter of data was quite large, so that the time-course of approach to the new steady-state cannot be stated with certainty. However, for all three nucleotides the data suggest a low amplitude overshoot (ATP) or undershoot before the final steady-state is reached. Qualitatively at least, this result would also be accounted for by the feedback control operating through AMP/ATP.

Disposition of other nucleotides during inhibition. Preliminary data indicated that large quantities of nucleoside diphosphates were being generated under certain conditions of inhibition. This fact suggested that significant quantities of energy-storage compounds other than the adenine nucleotides might exist in *Neurospora*, and prompted a brief examination of general nucleotide levels by a technique of two-dimensional electrophoresis. Three conditions were chosen for analysis: (i) control cells, kept well aerated during 30-min incubation in standard buffer; (ii) cells incubated in buffer plus 10 mM KCN for 30 min; and (iii) cells incubated 30 min in the standard buffer minus glucose, to which 1% 2-DOG had been added. Autoradiograms of the electrophoresis patterns are reproduced in Fig. 9A-C.

It is clear from simple inspection of the autoradiograms that the levels of all nucleotides in *Neurospora* were strongly affected by respiratory or glycolytic inhibition. ^{32}P counts on all major spots are listed in Table 3. Among the nucleotides which were identified with reasonable certainty, ATP and the two cell-wall precursors, uridine diphosphate glucose (UDPG) and its *N*-acetyl derivative (UDPGNAc), were most prominent. These, along with ADP, remained prominent in cyanide-inhibited cells. In cells

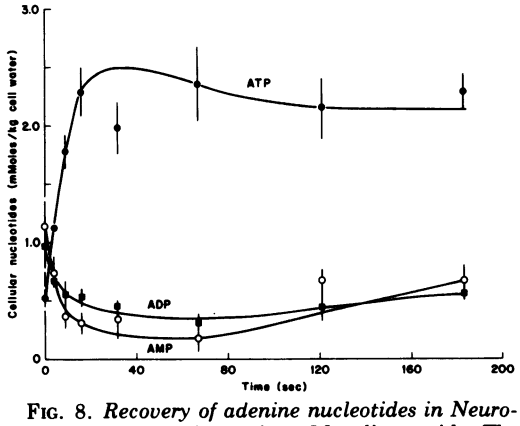

FIG. 8. *Recovery of adenine nucleotides in Neurospora following washout of 1 mM sodium azide. The inhibitor was removed by rapid harvesting of the cells to get rid of most of the suspension volume, followed by quick resuspension in HEPES buffer (see legend to Fig. 3) at pH 8.2. Each point represents the average for 6 measurements; vertical bars, ±1 SEM.*

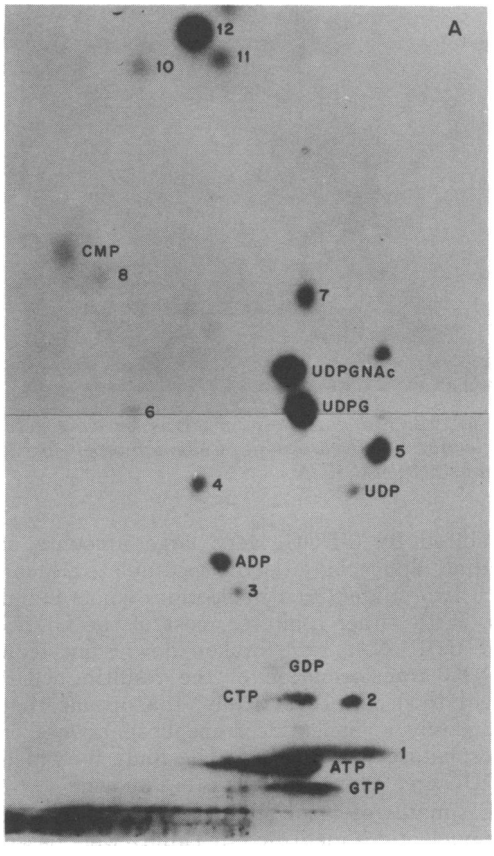

FIG. 9A. *Electrophoresis patterns showing the distribution of total nucleotides in Neurospora. Control cells. Cells uniformly labeled with $^{32}PO_4$. The electrophoresis sheets were autoradiographed for 72 h. Actual counts of ^{32}P are given in Table 3. UTP and TTP were accidentally lost at an early stage of the analysis.*

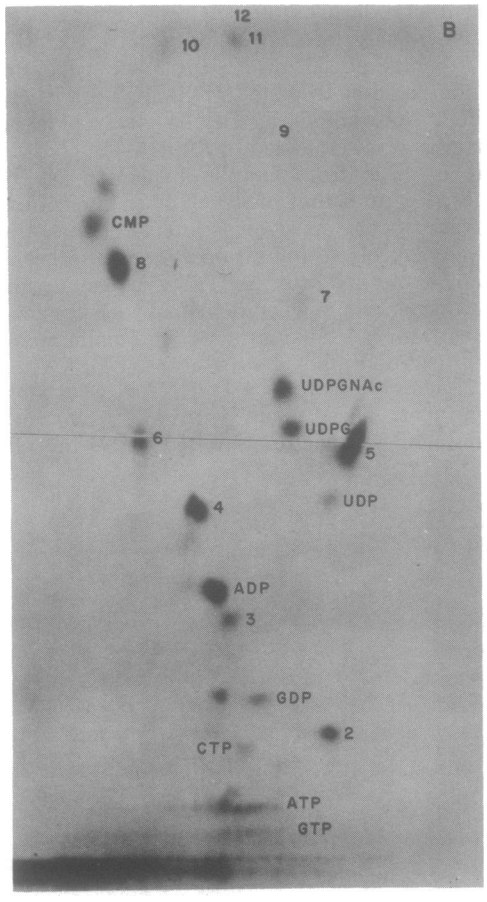

FIG. 9B. *Electrophoresis patterns showing total nucleotides in Neurospora. Cells inhibited for 30 min with 10 mM KCN.*

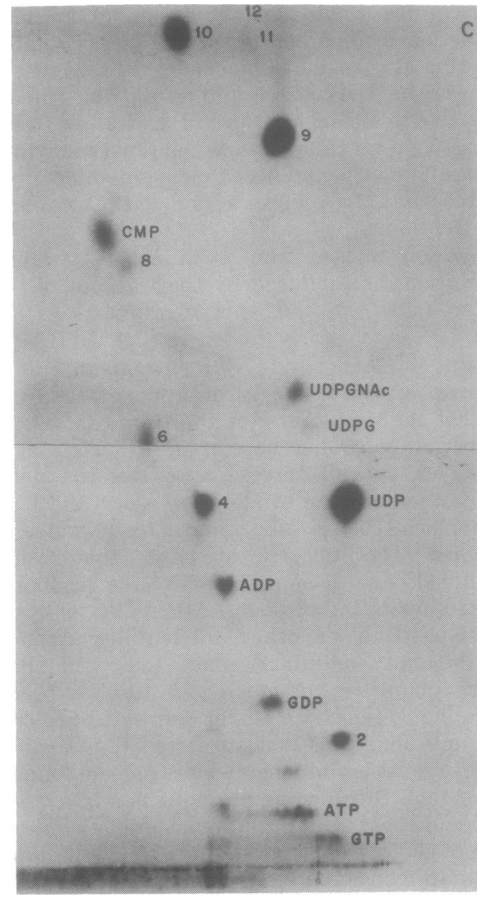

FIG. 9C. *Electrophoresis patterns .showing total nucleotides in Neurospora. Cells blocked for 30 min with 1% 2-deoxyglucose.*

inhibited by 2-DOG, very large amounts of uridine diphosphate (UDP) accumulated, along with two unidentified nucleotides (spots 9 and 10). With either inhibitor most of the UDPG and UDPGNAc were broken down; but with 2-DOG transformation of the resulting UDP failed to occur. Presumably, this means that ultimately a nucleotide transphosphorylase or phosphatase was blocked. The route by which 2-DOG creates such a block is not known.

Estimates of intracellular concentrations of the nucleotides identified in Table 3 were based on fire-fly assays of ATP in the control cells and are listed in Table 4. The total quantity of stored "high-energy" phosphate bonds dissipated by the breakdown of nucleoside di- and triphosphates can be calculated from Table 4 to be 8.3 and 6.7 mmol/kg of cell water, respectively, for cyanide and deoxyglucose inhibition. If the additional bonds presumably contained in the unidentified spots 1 to 6 (Table 3) are considered, the figures become 9.0 and 8.2 mmol/kg of cell water. In this same experiment breakdown of ATP and ADP together accounted for 3.5 to 3.8 mmol of phosphate bonds per kg of cell water. It is apparent that, given the appropriate transphosphorylases, the other nucleoside di- and triphosphates could act as significant buffering reserves for energy funnelled through ATP.

DISCUSSION

Nucleotide levels. Previously reported maximal sustained levels of ATP in several microorganisms are as follows (note new units: mmol per kg dry wt): *Streptococcus faecalis*, 20; *Escherichia coli*, 18; *Aerobacter aerogenes*, 6; *Saccharomyces cerevisiae*, 13; *Physarum poly-*

cephalum, 10; *Methanobacterium*, 9; *Chromatium* D, 7.5; and *Bacillus megaterium*, 5 (8, 9, 15, 20, 29, 34, 38, 43, 44). The mean level obtained in the present experiments for control preparations of *Neurospora*, 6.5 mmol/kg dry wt (2.56 mmol/kg of cell water; Table 1), thus would appear to lie at the lower end of the range for microorganisms generally. Nevertheless, 6.5 mmol/kg dry wt is higher than most previously reported values for *Neurospora*. In those cases where the documentation of methods is adequate (19, 42), it is clear that the lower values (0.34 and 3.97 mmol/kg dry wt) were obtained from anoxic or partially anoxic cells. A very recent report by Scarborough (36) quotes values of 4.2 to 4.6 mM in exponentially growing cultures, but without specifying how intracellular water was estimated. Owens et al. (30) found 2.3 mmol/kg dry wt for ATP in conidia, which is close to the value of 2.97 mmol/kg dry wt (1.17 mmol/kg of cell water) shown in Fig. 2B.

Smith and Wheat (42) and Kulaev and Mel'gunov (24) investigated the total nucleotide spectrum of *Neurospora* by ion-exchange chromatography and found the following to be present in detectable amounts: UDPGNAc, UDPG, ATP, AMP, uridine 5'-triphosphate (UTP), cytidine 5'-diphosphate (CDP)-choline, uridine 5'-monophosphate (UMP), and guanosine 5'-triphosphate (GTP). The most conspicuous among these nucleotides were UDPGNAc, UDPG, and ATP. The rather small number of nucleotides detected in these experiments can be attributed to insensitivity in the method. Similar but more sensitive experiments on the yeast *Candida utilis* have resolved almost all of the different ribonucleotides (35) in that organism.

Turnover. The most striking aspect of ATP metabolism in *Neurospora* is the fast turnover. A maximal rate of turnover can be calculated from the steady-state oxygen consumption, which averages about 60 μliters of O_2 per mg dry wt per h, under the conditions of these experiments. This converts to 17.5 mmol of O_2/kg of cell water·min (see notes on *Computations*, in Materials and Methods). Oxidative phosphorylation in *Neurospora* seems to occur mainly at sites II and III in the respiratory chain (data from isolated mitochondria; 27), so that the P/O ratio is 2. Therefore, the steady-state ATP turnover should be 70 mmol/kg of cell water·min, or 1.17 mmol/kg of cell water·s. A conservative estimate of the rate of ATP turnover was obtained from the initial rate of decay with maximal respiratory inhibition (Fig. 4), at 0.44 mmol/kg of cell water·s. The discrepancy between these two estimates must arise in part because the inhibitor permits continuing, albeit retarded, resynthesis of ATP, both from ADP + P_i and from the adenylate kinase reaction, and may also be affected by

TABLE 3. *Total counts per minute ($^{32}PO_4$) in major spots from 2-dimensional electrophoresis*[a]

Probable nucleotide	Control	30 min KCN	30 min 2-DOG
ATP	15,645	2,895	3,513
ADP	2,052	2,826	1,056
GTP	2,946	438	924
GDP	494	592	896
CTP	1,722		183
CMP	780	1,067	1,137
UDP	1,022	368	8,594
UDPG	12,116	1,140	208
UDPGNAc	6,322	1,210	684
Major unidentified spots (run with triphosphates)			
1	2,436		162
2	1,630	820	1,261
(run with diphosphates)			
3	578	547	146
4	1,212	1,400	1,627
5	3,952	3,946	124
6		697	452
(run with monophosphates)			
7	1,468	218	
8	282	2,276	444
9		193	7,987
10	496	349	3,188
11	931	411	213
12	7,762		293

[a] Spots 1, 5, 8, 11, and 12 lie close to the positions which would be expected for ITP, TDP, AMP, TMP, and UMP, respectively.

TABLE 4. *Apparent intracellular concentrations of nucleotides, calculated from ^{32}P label*[a]

Probable nucleotide	Control	30 min KCN	30 min 2-DOG
ATP	2.24 (2.24)	0.41 (0.30)	0.50 (0.27)
ADP	0.44 (0.29)	0.60 (0.60)	0.23 (0.24)
GTP	0.42	0.06	0.14
GDP	0.11	0.15	0.21
CTP	0.25		0.03
CMP	0.34	0.46	0.05
UDP	0.23	0.08	1.97
UDPG	2.60	0.24	0.04
UDPGNAc	1.45	0.28	0.16

[a] Computations are based on the counts listed in Table 3, assuming that 15,645 CPM = 2.24 mM ATP. Values in parentheses represent chemical assays for ATP and ADP.

resynthesis of ATP by transphosphorylation from the other nucleoside di- and triphosphates.

Fast turnover of ATP, coupled with the facts that *Neurospora* lacks conventional energy storage compounds (e.g., creatine phosphate, arginine phosphate) and cannot extract the energy from its storage polyphosphates (19, 47), would seem to leave the organism very vulnerable to metabolic accidents. While nonadenosine nucleoside di- and triphosphates might double or triple the effective ATP supply (Tables 3, 4), they could not prolong the time constant for ATP decay by more than 10 to 15 s in the event of complete respiratory blockade. At least two elements of a compensatory mechanism can be identified. The first, operating on a time scale of several minutes (Fig. 6), is probably the well-known adenine-nucleotide control of glycolysis. However, *Neurospora* is an obligate aerobe (11) which is unable to grow on the energy from glycolysis in the total absence of oxygen; this presumably means that pyridine nucleotide oxidation via alcohol production (23) is limiting. The second element of a compensatory mechanism then, is the induction, over a period of hours, of a cyanide- and antimycin-insensitive oxidase (inhibited by SHAM) which, though non-phosphorylating (27), rapidly reoxidizes pyridine nucleotides.

Regulation during growth. The finding that $[ATP]_i$ is not constant during exponential growth (Fig. 2) is contrary to results previously published on yeast and *E. coli*. Chapman and Bartley (7) found AMP, ADP, and ATP in yeast to be constant at about 1.2, 2.5, and 5 mmol/kg dry wt respectively, during 4 to 5 h of exponential growth under anaerobic conditions. Cole, Wimpenny, and Hughes (9), working with *E. coli*, found ATP to be roughly constant at 8 to 14 mmol/kg dry wt during exponential growth both aerobically and anaerobically. In *S. faecalis*, however, the situation is more complicated with $[ATP]_i$ generally falling during sustained exponential growth and stabilizing during linear growth (14). From Fig. 2B, the situation is evidently still more complicated in the case of *Neurospora;* the cellular ATP concentration is first stable, then rises, and eventually falls during the same exponential growth process. Very recently, Huzyk and Clark working with carefully synchronized cultures of *E. coli* have been able to demonstrate that nucleoside triphosphate levels (including ATP) oscillate in phase with cell growth and division (22). However, this phenomenon does not enter the interpretation of present results on *Neurospora* both because the time scale of fluctuations is too long and because no attempt was made to synchronize nuclear division.

In an effort to simplify the description of adenine nucleotide levels and to test for possible control mechanisms operating during growth of *Neurospora*, two commonly used functions were computed: Atkinson's "energy charge" function (EC = [ATP + 0.5 ADP]/[ATP + ADP + AMP]; reference 3), and the apparent equilibrium constant for adenylate kinase (K_{app} = ATP·AMP/ADP^2). Values of the first are listed in column 2 of Table 5. It can be seen that all values lie between 0.84 and 0.62. The average standard error of values is 0.085, so that differences are only barely significant even though the numbers tend to rise and fall cyclically. The overall average value of energy charge, 0.72, lies well within the range of values calculated by Atkinson and his collaborators for a wide variety of actively metabolizing cells and tissues (6).

By contrast, values of the second function, the apparent equilibrium constant for adenylate kinase, are very far from being constant. As shown in column 3 of Table 5, they vary between 3.39 and 0.52, compared with an average standard error of 0.11. It is worth considering, then, some possible causes of the inconstancy of K_{app}. (i) The most obvious would be that concentration changes during growth significantly displace the nucleotide ratio. The maximum rate of change of adenine nucleotides in growth, however, is very small (0.2 mmol/

TABLE 5. *Calculation of various parameters for adenine nucleotide reaction during exponential growth of Neurospora crassa*[a]

Time (h)	EC	K_{app}	K′
0±	0.79	3.39	0.34
2.2	0.78	2.42	0.18
4	0.66	1.00	0.52
6.2	0.62	0.58	0.33
8	0.67	0.52	0.29
10	0.74	0.69	0.27
12.2	0.77	0.77	0.26
14	0.82	1.73	0.45
16	0.84	3.13	0.24
18	0.74	1.96	0.92
20	0.70	0.76	0.37
22	0.48	1.90	
24.2	0.71	1.31	0.26
Average SEM	0.085	0.37	0.20

[a] EC, "energy charge" (see text; also Ref. 3). K_{app} = apparent equilibrium constant for adenylate kinase. K′ = K_{app} adjusted by assuming that 0.19 mM AMP is not accessible to the reaction. All parameters have been calculated from the analytic data, in Fig. 2.

kg of cell water·h) compared with the rate of turnover (0.4–1.2 mmol/s). Since the computer simulation studies indicate the rate constants for adenylate kinase and for ATP hydrolysis to be of the same magnitude, concentration changes during growth should have little effect on K_{app}. (ii) On the other hand, the steady-state condition (d[ATP]/dt = d[ADP]/dt = 0 can be expected to give an apparent equilibrium for adenylate kinase only if no other significant pathway for the synthesis of ATP or ADP exists (e.g., by phosphorylation or pyrophosphorylation of AMP). (iii) Finally, sequestering of one or more of the adenine nucleotides into a special intracellular compartment could produce variations in K_{app} without affecting the true equilibrium constant for the reaction. The simplest version of this idea would place 0.19 mM AMP in an isolated compartment. The resulting values of K_{app} are listed in column 4 of Table 5. Except for one value, the extremes are now 0.18 and 0.52, and the average standard error (0.20) would again indicate that most of the variations are not statistically significant; however, independent evidence of AMP compartmentation is lacking.

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