REGULATION AND COORDINATION OF PURINE AND PYRIMIDINE BIOSYNTHESES IN YEAST

I. Regulation of Purine Biosynthesis and Its Relation to Transient Changes in Intracellular Nucleotide Levels

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ABSTRACT The control of purine biosynthesis in a yeast mutant deficient for uracil, adenine, and histidine has been studied *in vivo*. The adenine mutation causes accumulation of aminoimidazole ribotide in the cells. The control curve relating steady-state purine nucleotide level in the cell to rate of synthesis in the *de novo* purine synthetic pathway has been determined. Control in the cell depends on a feedback mechanism involving end-product inhibition. The transient responses of the purine nucleotide pool to changes in adenine input have been studied. Under certain conditions the pool overshoots when shifting from one steady-state to another. Transient changes in nucleotide levels are followed by inverse changes in the rate of attempted *de novo* purine synthesis. A study of the transient responses of specific intracellular nucleotides suggests that inosinic acid controls the rate of attempted purine synthesis. The transient response of nucleic acid synthesis rate to changes in nucleotide levels was studied and the implications for regulation of nucleic acid synthesis discussed.

INTRODUCTION

The purpose of the general investigation of which this paper represents the first part is to utilize the technical advantages of a yeast mutant with multiple independent biochemical deficiencies in the investigation of the internal regulation of systhesis in each of several pathways and the coordination or mutual regulation among pathways. The investigation is designed to yield data for the formulation of a mathematical model of the functional characteristics of the system of several pathways. The system *as a whole* can be studied effectively only *in vivo* and this is what is done. Interpretation of the *in vivo* studies is facilitated by the accumulation of knowledge of biochemical pathways and mechanisms of regulation by others studying enzymes *in vitro*.

This paper presents a study of regulation of purine and purine neucleotide synthesis and the relations of these to nucleic acid synthesis in a *Saccharomyces cerevisae* haploid strain with genetic blocks in the purine, pyrimidine, and histidine pathways. The adenineless mutant used in these investigations provided two special advantages. First, that part of the purine biosynthetic pathway preceding the block is intact and the pathway rate of synthesis can be estimated from the initial rate of accumulation of the terminal product of the intact path-AIR.[1] Secondly, manipulation and specific labeling of the purine nucleotides do not depend on maintaining a level of these high enough to cut off or spare internal synthesis of purines, so that low steady-state nucleotide levels can be maintained, measured, and correlated with the rate of attempted *de novo* purine synthesis.

The studies of many workers have shown that biosynthetic pathways in the cell are generally regulated by a form of rapid control (end-product inhibition) and a slower control which involves removal or manufacture of the synthesizing machinery of the cell (enzyme induction and repression). It is to the rapid form of control that attention is directed in this study.

Several investigations especially relevant to the present study have appeared in the literature. Identification of AIR as an intermediate accumulating in another adenineless yeast has been made by Friedman and Moat (4) and in bacteria by Love and Levenberg (11). Gots (5) and Gots and Goldstein (6) have studied certain aspects of purine regulation in bacteria. Incorporation of adenine into wild type *Saccharomyces* has been studied by Halvorson (7) and Harris and Neal (8) and in *Torulopsis* by Cowie and Bolton (3). The genetics of adenineless strains of yeast has been described by Roman (14). The reaction chains and cycles used as a basis for interpretation in the paper have been described by Buchanan (2) and Magasanik and Karibian (12), among others.

METHODS AND MATERIALS

The yeast strain employed in these studies is S1237A, developed and given to us by Dr. R. K. Mortimer. It is a haploid *Saccharomyces cerevisiae* containing three separately induced and independent biochemical deficiency mutations combined into the one strain. The genotype is ur_1 ad_2 his_8; the colonies become red in the absence of adenine. The uracil requirement is satisfied by uracil or uridine but not by uridylate, UDP, cytidine, cytidylate, thymine, thymidine, or orotate. The adenine requirement is satisfied by adenine or hypoxanthine but not by adenosine, adenylate, ADP, guanine, guanosine, deoxy derivatives of the above, aminoimidazole carboxamide, or AICA-riboside. Of these only the auxotrophic compounds, adenine and hypoxanthine, prevent formation of the red pigment. The histidine requirement is satisfied by none of the compounds above.

Except for the preliminary work reported in the first section, cells were harvested in

[1] Abbreviations used throughout text: AIR, 5-aminoimidazole ribotide; PRPP, 5-phosphoribosyl-1-pyrophosphate; SP, TCA-soluble pool; IP, TCA-insoluble pool; SP* or IP* represents the C^{14}-labeled pool; IMP, inosinic acid.

log phase (10^7 cells/ml) after hundredfold growth in synthetic medium[2] capable of supporting growth to 2×10^8 cells/ml. Only at concentrations above 10^8 cells/ml were protein and RNA synthetic rates per cell decreased. These rates were constant in the range 10^7 to 10^8 cells/ml, indicating that cells in this range were not adapting to stationary phase. In experiments on regulation where growth was permitted the cell concentration never exceeded 2×10^7 cells/ml. Cells were harvested by centrifugation or membrane filter and immediately resuspended in preheated and preaerated synthetic medium. No difference in effect of fresh or used synthetic medium was found with respect to purine and pyrimidine synthesis, so fresh medium was used ordinarily. Incubation was always at 30°C with aeration

Aliquots for assay of radioactivity were taken as described later; the larger quantitites of cells required for AIR assay were obtained by filtration through a 50 mm membrane filter, a process requiring about 2 minutes. Cells were then immediately extracted in 5 per cent TCA at room temperature, and the extract assayed for AIR with the Bratton-Marshall test (16) immediately. TCA extraction gives reproducible recovery of AIR, and better or equal yield than ethanol, toluene, heating in water at 85-100° for 3 to 30 minutes, or sonic disruption.

The genetic block responsible for the adenine requirement of S1237A is in the step succeeding AIR production in the purine biosynthetic pathway. This was deduced from the facts that AIR accumulates immediately in the cells in the absence of adenine and that the steps succeeding AIR are effectively irreversible. AIR does not appear in the growth medium until several hours after the start of accumulation in the cells. It is relatively stable inside the cell. That AIR is stored in the cells is consistent with the known presence of large pools of free amino acids in yeast.

The spectrum of the chromophore resulting from application of the Bratton-Marshall test to AIR is sufficiently unique to identify the compound accumulating in S1237A as an aminoimidazole. Further identification was obtained using the methods Friedman and Moat (4) employed to identify aminoimidazole riboside in the media of purine- and biotin-deficient yeasts and the methods of Love and Gots (5) and Levenberg and Buchanan (9). This involved fractionation of the yeast extract on Dowex 50 or Dowex 1, followed by paper chromatography of the fractions in butanol–acetic acid–water. Extracts of cells grown with and without adenine were run for comparison. Detection and identification on the chromatogram were made by means of the Ames and Mitchell spray for imidazoles (17), determination of R_F, elution of spots for Bratton-Marshall testing, and pentose determination. Only one pentose imidazole appeared in the extracts of cells incubated without adenine that did not appear in cells incubated with adenine. This compound had the characteristics of AIR on the basis of the above tests. The behavior of the compound on the ion exchange resins indicates that it is largely the ribotide rather than the riboside which is extracted from the cells. Comparison with authentic AIR was made possible by a gift of the latter from Dr. J. Buchanan.

It was desired to label cells and then determine the radioactivity of whole cells, acid-soluble and acid-insoluble fractions. It was found that collection of whole or TCA-extracted cells on 0.45 micron membrane filters was not only expeditious but allowed

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[2] Synthetic medium composition: Vitamins, dextrose, trace elements, and salts as in Difco bacto yeast morphology agar with these exceptions: Inositol 2 μg/liter, K_2HPO_4 5.3 gm/liter, KH_2PO_4 2.6 gm/liter. Substitutions: Ammonium sulfate 5 gm/liter, L-tryptophan 0.2 gm/liter, 1-glutamate 0.2 gm/liter, 1-methionine 0.2 gm/liter, 1-tyrosine 0.25 gm/liter, cysteine 0.2 gm/liter. Additions: Adenine and uracil 50 μg/ml, histidine 200 μg/ml.

recovery of virtually all radioactivity (1). Collection and washing on the filter of whole cells required less than a minute. If no more than 5×10^7 cells are collected per 25 mm filter, the layer that results is "infinitely thin" with respect to C^{14} beta emission. The per cent standard deviation of a series of membrane filter samples was close to 1 per cent. The labeled compound used was adenine-8-C^{14}, from Calbiochem Co., Los Angeles. Radioactive compounds of about 0.1 $\mu c/\mu g$ were mixed with sufficient carrier to give 7.5×10^{-6} $\mu c/\mu g$ for tracer experiments.

In studies of the uptake of adenine C^{14} the compounds receiving the label were separated into the usual two fractions—the acid-soluble pool (SP) and acid-insoluble pool (IP). The acid-soluble compounds were extracted in cold 5 per cent TCA for 1 hour, followed by TCA wash. Alternatively, this pool was extracted in 95 per cent ethanol for 3 minutes at 50°C, followed by extraction in 50 per cent ethanol, 3 minutes at 50°C. The two methods give identical results. The insoluble residues of extraction were collected on membrane filters. The activity of the soluble fraction was calculated by subtracting the activity of the insoluble fraction from whole cell activity or by direct assay.

For direct assay an aliquot of the extract is evaporated on acid-washed stainless steel planchets. By careful adjustment of temperature it is possible to drive off TCA as well as water, leaving the soluble compounds deposited in a thin layer for direct counting.

Separation and quantitative measurement of the individual C^{14}-labeled soluble compounds were accomplished with high voltage electrophoresis. The ethanol extract is evaporated quickly under vacuum, water-insoluble lipids removed, and the residue applied to 50 cm strips of Gelman sepraphore membrane paper. By the use of filter paper wicks, about 45 cm of the membrane are available for electrophoretic separation. Citrate buffer, pH 2.7, is used for the electrophoresis which is carried out in a tank containing Varsol as coolant. A field of 50 v/cm was applied for 1½ to 2 hours. The purine nucleotides IMP, AMP, ADP, ATP, GMP, GDP, and GTP are clearly resolved by this method. The per cent recovery of radioactive standards of AMP, ADP, GDP, and ATP run through the entire process of extraction and electrophoresis ranged from 85 to 95 per cent. In the experiments to be described here the cells in log phase were exposed to adenine C^{14} before the start of experiment long enough for the soluble-labeled components to reach steady-state activity, at which time the activity of each component is a measure of its amount. The activities of the nucleotides separated by electrophoresis were measured with a Vanguard scanner and integrator.

RESULTS

AIR Production as a Function of External Adenine and Uracil Concentrations. Since yeast S1237 cannot produce its own purine end-product nucleotides the level of these can be manipulated in response to the external level of adenine. Although internal nucleotide levels are not necessarily proportional to external adenine concentration, the effect of manipulation of the latter on feedback inhibition of the blocked purine pathway was studied preliminary to study of the former. For this study *stationary phase* cells adapted to the synthetic medium in the presence of adenine, uracil, and histidine were transfered to medium containing no uracil, zero or 20 μg/ml histidine, and various concentrations of adenine. The amount of AIR per cell was determined as a function of time. The presence or absence of histidine had no significant effect on AIR production. Fig. 1 shows ac-

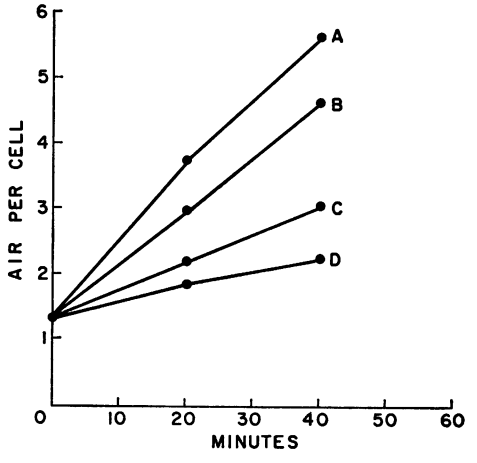

FIGURE 1 Intracellular accumulation of AIR *versus* time in a stationary phase population. AIR per cell is plotted here in arbitrary units. Cells grown in 20 μg/ml adenine were transferred at time zero to the following concentrations of adenine in synthetic medium: *A*, 0 μg/ml; *B*, 1.5 μg/ml; *C*, 3.0 μg/ml; *D*, 5.0 μg/ml.

cumulation of AIR in the presence of histidine and O, 1.5, 3, and 5 μg/ml adenine. The rate of AIR accumulation is nearly constant during the first 40 minutes at all adenine concentrations, indicating that during this initial period the accumulated AIR does not inhibit its own production nor is it transformed or degraded, because either of these processes would tend to make the curve level off. As little as 1.5 μg/ml adenine inhibits AIR production appreciably and 5 μg/ml reduces the rate to about 20 per cent of its value in the absence of adenine.

The inhibition is not due to a general toxic effect of adenine because the over-all growth rate of the cells is maximal at levels of adenine above about 2 μg/ml and is not lowered even at adenine levels of 100 μg/ml. AIR production can be switched on or off with less than a 10 minute lag in response to changes in external adenine level from 5 μg/ml to zero and back to 5 μg/ml.

A similar type of experiment was performed to determine the effect of various levels of uracil on feedback inhibition of the purine pathway in combination with various levels of adenine. All combinations of 0, 2, 5, and 20 μg/ml uracil with 0, 2, 4, and 7.5 μg/ml adenine were tried and AIR assays made at 30 minutes' incubation and later. In all cases uracil had no significant effect, the accumulation of AIR was affected only by the level of adenine.

The data described in this section provided a basis for more detailed investigation of regulation of purine synthesis. In particular, the following hypotheses are supported: (*a*) Adenine and/or its derivatives inhibit purine synthesis at a step preceding the synthesis of AIR. (*b*) The degree of inhibition can be varied over a wide range in this system. (*c*) The rapid, reversible response indicates that in-

hibition, as distinguished from repression and induction of enzymes, is the primary mechanism involved in controlling purine synthesis in these short term experiments. (*d*) The initial rate of accumulation of AIR after synthesis is turned on provides a valid index of the rate of synthesis of that part of the purine pathway preceding the genetic block.

Characteristics of the Purine End-Product Pool and Its Relation to Feedback Inhibition of AIR Production in Log Phase Cells. Figs. 2 and 3 present uptake curves for adenine C^{14} at various concentrations in complete medium, with 20 μg/ml uracil and histidine. For these experiments log phase cells were first incubated for 2 hours in full medium lacking adenine to reduce the size of the TCA-soluble pool (SP). This reduced the time required for the purine components of the SP to exchange with adenine C^{14}. The SP* curve for cells not first depleted of adenine (not shown here) was similar but an additional 20 to 30 minutes was required before the SP* curve leveled off. To prevent significant change of concentration of adenine in the medium during uptake at the lower adenine concentrations it was found necessary to reduce cell concentration to 6×10^5 cells/ml. In each of the experiments described in this section adenine concentration was essentially constant during the experiment.

Fig. 2 shows the incorporation of radioactivity into the SP* and into the cells as a whole (total) as functions of time when adenine C^{14} at levels of 2, 3, or 4 μg/ml is added at time zero. The activities plotted have been corrected for growth of the population during the experiment. It is seen that the SP* approaches a steady-state or dynamic equilibrium level after 15 minutes which is independent of external adenine concentration. 1 or 20 μg/ml adenine C^{14} give curves similar to those of Fig. 2. There is apparently a storage pool which exchanges slowly with the direct precursors of nucleic acid (more evidence for this will be pointed out later) and this accounts for the slow increase of SP* after 15 minutes. Note that the SP* fills faster in the first 15 minutes the higher the adenine C^{14} concentration but reaches the same steady-state level in each case. The total rate of incorporation reaches and maintains approximate constancy after 15 minutes, and is not dependent on adenine concentration so long as the SP* is not; *i.e.*, in the range 1 to 20 μg/ml. The TCA-insoluble labeled pool IP*, which consists of labeled nucleic acids, is the difference between total and SP*. In Fig. 2 the IP* after 15 minutes is a straight line nearly parallel to the curve of total incorporation. (See also Fig. 3.) The rate of transfer of adenine through the precursor pool has reached steady-state and is independent of external adenine concentration in the range 1 to 20 μg/ml. Further evidence that the rapidly labeled part of the SP* consists of direct precursors of IP* will be presented later.

Fig. 3 shows the uptake curves for 1 and 0.2 μg/ml adenine C^{14}. Not shown are curves for uptake in the range between 1 and 0.2—these fall between the curves shown. At 0.2 μg/ml adenine the cells are unable to take up adenine fast enough

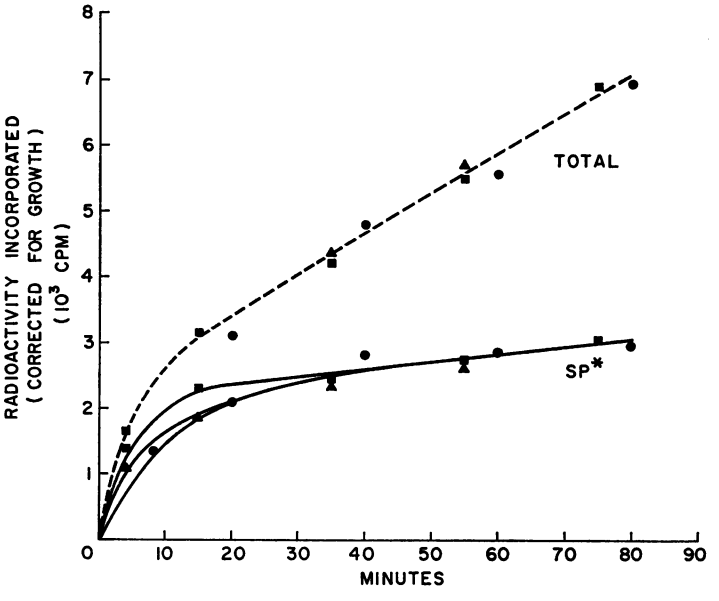

FIGURE 2 Incorporation of adenine-C^{14} into the acid-soluble pool (SP*) and total cellular uptake (total). The symbols are: ■, 4 μg/ml adenine C^{14}; ▲, 3 μg/ml; ●, 2 μg/ml. For simplicity the first points on the total curve for 2 and 3 μg/ml are omitted; these fall below the corresponding points for 4 μg/ml adenine C^{14}.

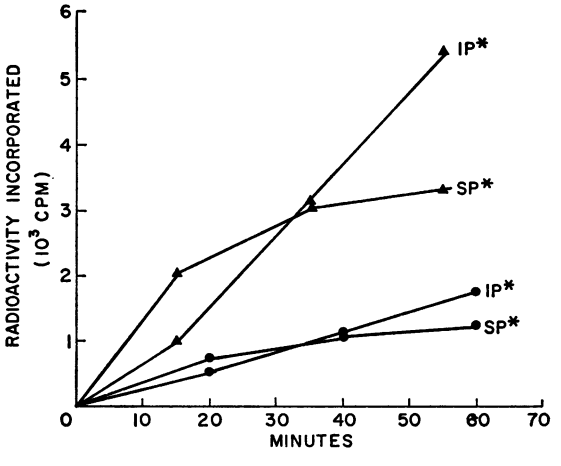

FIGURE 3 Incorporation of adenine C^{14} into the acid-soluble pool (SP*) and acid-insoluble pool (IP*). The symbols are: ▲, 1 μg/ml adenine C^{14}; ●, 0.2 μg/ml adenine C^{14}. These concentrations remain essentially constant during the course of the experiment.

to reach the normal steady-state level. Instead a level about one-third the 1 μg/ml adenine level is attained in the same time, and the SP* then comes into dynamic equilibrium with the rate of entry of adenine into cells equal to the rate of incorporation into the IP*. The rate of nucleic acid synthesis as measured by IP* rate now depends on external adenine concentration. In fact, the rate of incorporation of adenine C^{14} into IP* has decreased to one-third of the rate in 1 μg/ml adenine in direct proportion to the SP* level. This and the achievement of steady-state at the same time in both cases indicate that the transfer rate of an adenine C^{14} molecule along the precursor chain is the same in both cases but the number of molecules entering the cell per unit time is decreased to one-third in 0.2 μg/ml adenine.

If feedback control of purine synthesis involves one or more of the adenine C^{14}-labeled compounds of the SP*, we expect lowering of the SP* to decrease feedback inhibition. An experiment was made with cells treated as described for Fig. 3 but with AIR per cell determined as a function of time. The rates of AIR synthesis in the interval 40 to 60 minutes corresponding to steady-state SPs for 1 and 0.2 μg/ml adenine were determined. The rates in arbitrary units were 84 for 0.2 adenine and 20 for 1 adenine, or a ratio of 4.2 to 1. Comparing with the corresponding SP* ratio of 1 to 3 it is apparent that there is an inverse correlation between SP* level and AIR rate.

Rates of AIR synthesis *versus* precursor pool level measured when the SP* has reached *steady-state* between 60 and 70 minutes are plotted in Fig. 4. The precursor pool level is determined by subtracting the activity of the storage pool (curve *A* SP* at 60 minutes (Fig. 5)) from the over-all SP* activity. The shape of the curve is in doubt because of scatter of the points taken from various experiments but it is consistent with a mechanism of regulation involving allosteric competitive inhi-

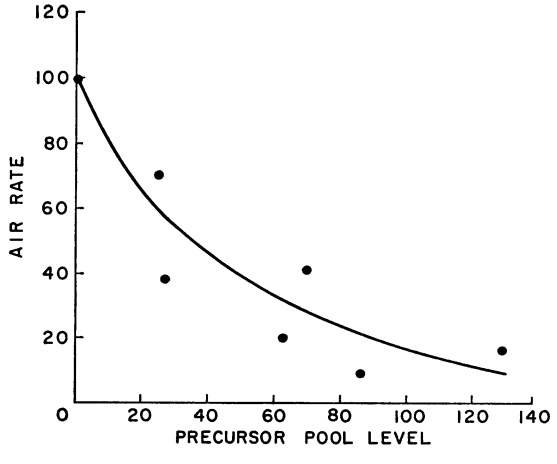

FIGURE 4 Rate of synthesis of AIR *versus* steady-state purine nucleotide precursor pool level. See text for details.

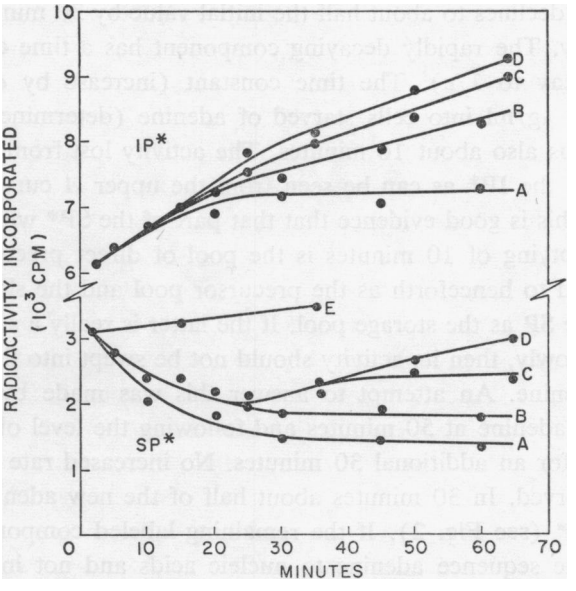

FIGURE 5 Shift down experiment. The cells were incubated in 5 μg/ml adenine C^{14} until steady-state SP* was attained and transferred at time zero to the following adenine concentrations: *A*, O; *B*, 0.15 μg/ml; *C*, 0.25 μg/ml; *D*, 0.35 μg/ml; *E*, 5.0 μg/ml. SP*, acid-soluble pool; IP*, acid-insoluble pool.

bition (13) by inhibitor(s) whose concentrations are proportional to the precursor pool level.

Transient Changes in the Purine End-Product Pool and in the Levels of Specific Nucleotides Related to the Control of de Novo Purine Synthesis. The first series of experiments was designed to reveal the kinetics of shift from high level steady-state SP* to low level steady-state and the relation between AIR rate and SP* in transition and in new steady-state. Although levels of adenine as low as 0.15 μg/ml were used to maintain low SP* levels, the concentration of adenine in the medium was not appreciably reduced during an experiment.

The SP was labeled beforehand by incubating cells in 5 μg/ml adenine C^{14} for several hours to obtain steady-state SP*, then the cells were removed quickly by filtration and immediately resuspended in medium of the same composition (including 20 μg/ml uracil and histidine) except for adenine. Fig. 5 shows a typical experiment where the shifts were to 0, 0.15, 0.25, 0.35, and 5 μg/ml adenine C^{14}. These concentrations were chosen under 1 μg/ml so that the SP* level would be forced to shift down. Shifting down to 1 μg/ml does not shift the SP* level down. The process of changing media does not shift the SP* down, as can be seen from the 5 μg/ml control curve.

Curve *A* shows the course of IP* and SP* on transfer to zero adenine. The ac-

tivity of the SP* declines to about half the initial value by 30 minutes and then declines very slowly. The rapidly decaying component has a time constant of about 10 minutes (decay to $1/e$). The time constant (increase by e) for uptake of adenine C^{14} at 5 μg/ml into cells starved of adenine (determined from data like those of Fig. 2) is also about 10 minutes. The activity lost from the SP* is transferred directly to the IP* as can be seen from the upper *A* curve and none leaks out of the cell. This is good evidence that that part of the SP* with a time constant for filling or emptying of 10 minutes is the pool of direct precursors of the IP*. It will be referred to henceforth as the precursor pool and the slowly turning over component of the SP as the storage pool. If the latter is really a storage pool which fills or empties slowly, then its activity should not be swept into the IP* by a chase of unlabeled adenine. An attempt to answer this was made by administering 5 μg/ml unlabeled adenine at 50 minutes and following the level of labeled material in SP* and IP* for an additional 30 minutes. No increased rate of change in IP* or SP* was observed. In 30 minutes about half of the new adenine taken up will appear in the IP* (see Fig. 2). If the remaining labeled components were all intermediates in the sequence adenine to nucleic acids and not in storage, at least half of them should have appeared in the IP*. Since this did not occur the remaining labeled component(s) are evidently not direct precursors of nucleic acid.

Curve *B* SP* shows a "critical damping" approach to a new steady-state in 0.15 μg/ml adenine. Although the new level is not much higher than the level in 0 adenine, the rate of transfer through it to the nucleic acids of labeled adenine is fairly high as indicated by the slope of the IP* curve.

SP* curves *C* (0.25 μg/ml adenine) and *D* (0.35 μg/ml adenine) are interesting cases of overshoot oscillation in approaching new steady-states. Considerable effort has been made to develop a kinetic model of the phenomenon. The most interesting conclusion obtained from this effort is that a rate constant of one or more of the reactions involved must change after a lag. Such a rate constant change would most likely be due to change in level of enzyme. Some insight into the reason for the overshoot can be gained by reference to the IP* curves for *B*, *C*, and *D*. These all follow the same course before 20 minutes, a period during which the precursor pool is reduced because nucleic acid synthetic rate is not reduced fast enough to equalize output to input while the pool level is still high. After 20 minutes the IP* rate is reduced for *B*, *C*, and *D* and to about the same level. The reduced rate of nucleic acid synthesis is high enough to keep *B* SP* low (and hence to increase the rate of attempted purine synthesis, as will be seen next) but lower than the input rate of adenine to the precursor pool for *C* and *D*. Hence the precursor pools of *C* and *D* begin to fill again. The disparity between input and output persists, allowing the pools to continue filling for a time.

In the next series of experiments the synthesis of AIR during the course of the nucleotide pool variations after shift of adenine level was investigated. From

AIR analyses made at 10 minute intervals the rates of AIR synthesis in each interval were calculated and these are plotted in Fig. 6. Curve 1 corresponds to a shift down to zero adenine, curve 2 to a shift to 0.3 μg/ml adenine, and curve 3 to no shift. Within 10 minutes after the shift to zero or 0.3 adenine the rate of AIR synthesis increases more than 20-fold. This is apparently the maximum rate the *de novo* pathway is capable of since it increases no further during 2 hours' incubation of the cells in zero adenine. Curve 3 shows that the rate of AIR synthesis remains

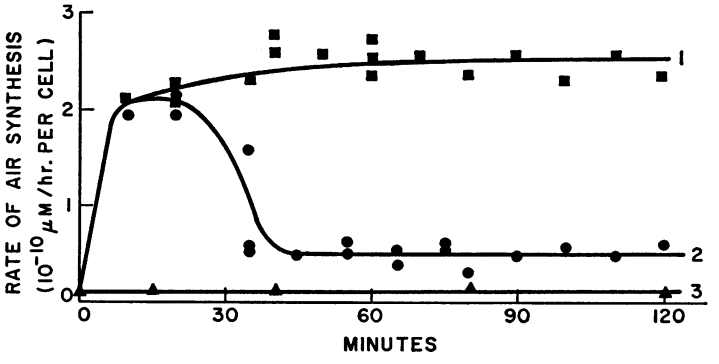

FIGURE 6 Rate of AIR synthesis after shift from 5 μg/ml adenine to lower levels. Curve 1, zero adenine; curve 2, 0.3 μg/ml adenine; curve 3, no shift.

low as long as the purine nucleotide pool level is maintained by external adenine at 1 μg/ml or more. Curve 2 shows that AIR synthesis is switched on immediately to maximum rate after the shift to 0.3 μg/ml adenine, but declines rapidly between 30 and 40 minutes after the shift to a new constant rate which is about 25 per cent of the maximum rate. (It is worth emphasizing again that the adenine in the medium is not exhausted or reduced during the experiment.) By comparison with curves *C* and *D* of Fig. 5 it is seen that the AIR rate goes up when the SP* nucleotide pool level goes down and when the pool level goes up after 30 minutes the AIR rate drops rapidly.

The observation that AIR synthesis is inhibited after shift to 0.3 μg/ml adenine only when the purine nucleotide pool, having reached a minimum earlier, begins to increase, provides an approach to the problem of which nucleotide(s) are effective *in vivo* in inhibiting *de novo* purine synthesis. If the hypothesis that AIR synthesis is shut off by end-product inhibition is accepted, then one or more of those end-products which increase just before shut-off must cause the shut-off. Three experiments were performed in which quantitative analyses of the levels of AMP, ADP, ATP, GMP, GDP, and IMP were made. These and a constant component tentatively identified as a coenzyme account for more than 80 per cent of the activity derived from adenine C^{14} and soluble in ethanol-water. Most of the

remaining ethanol–water-soluble C^{14} is found in adenine or adenosine and a small amount in GTP.

In Fig. 7 a radioactivity profile of the steady-state labeled purine nucleotides extracted from cells growing in 5 μg/ml adenine C^{14} and separated by strip electrophoresis is presented. The areas of the peaks correspond to the amounts of each component. The excellent resolution of components demonstrated in this figure and automatic digital integration of activities during scanning of the strip contributed a great deal to the accuracy and reproducibility of the nucleotide analysis.

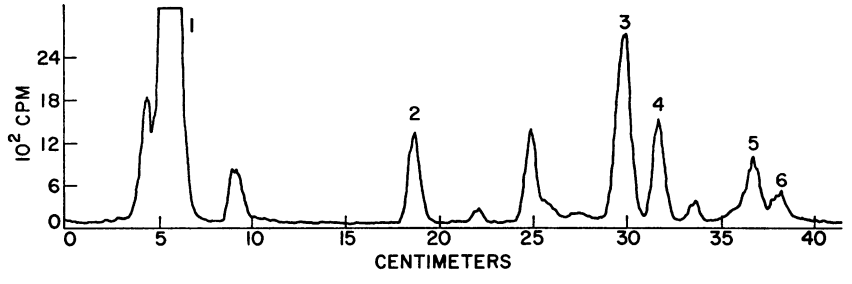

FIGURE 7 Radioactivity profile of the ethanol-water soluble compounds separated by strip electrophoresis from cells labeled in 5 μg/ml adenine C^{14}. The peaks are: 1, AMP; 2, GMP; 3, ADP; 4, IMP; 5, GDP; and 6, ATP. The peak between 2 and 3 is a constant component used for standardization.

Shift experiments were performed as described for Fig. 5, the shift down being to 0.3 μg/ml adenine C^{14}. Analyses of the average amount of each nucleotide per cell were made of samples taken at varying times up to 70 minutes after the shift. Since recovery of nucleotides in duplicate samples varied by as much as 15 per cent use was made of the fact that the coenzyme component previously referred to maintained constant level after a shift, and this component was employed as an internal standard. By normalizing to this standard duplicate analyses agreed to within 5 per cent. Results are presented in Figs. 8 and 9.

It is seen that AMP, which is as abundant as all the other nucleotides taken together, declines quickly after the shift to a new steady-state level about two-thirds of the pre-shift level. Since AMP is the major component and is known to be an effective nucleotide inhibitor *in vitro* of PRPP amidotransferase (15), we might expect it to be dominant in end-product inhibition. Nevertheless, the inhibition is released when AMP is still at high level and there is no increase in AMP at the time inhibition is reestablished. ADP remains relatively constant after the shift with the possible exception of the first 10 minutes. Preliminary experiments without internal standard which suggested that ADP increased at 30 to 40 minutes have not been borne out by more rigorous investigation. ADP is therefore not involved in the cycling of inhibition.

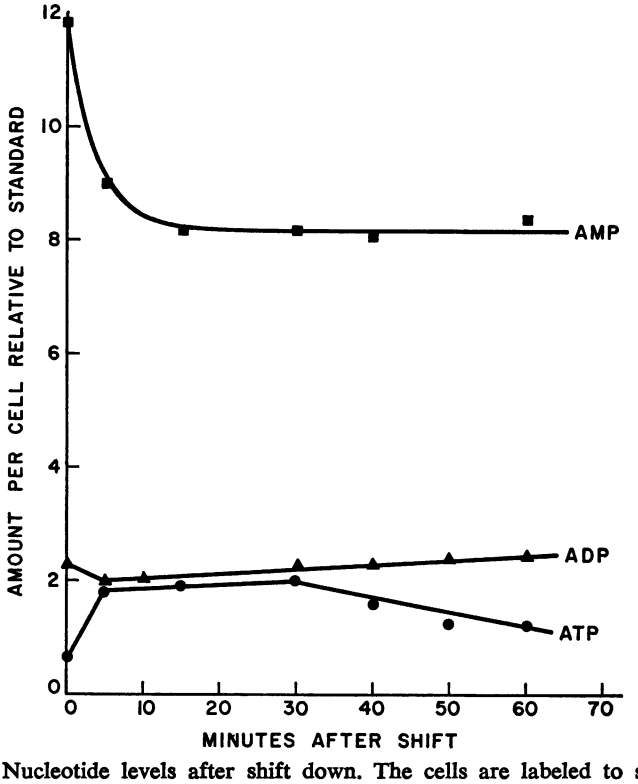

FIGURE 8 Nucleotide levels after shift down. The cells are labeled to steady-state in 5 μg/ml adenine C^{14} and then shifted at time zero to 0.3 μg/ml adenine C^{14}.

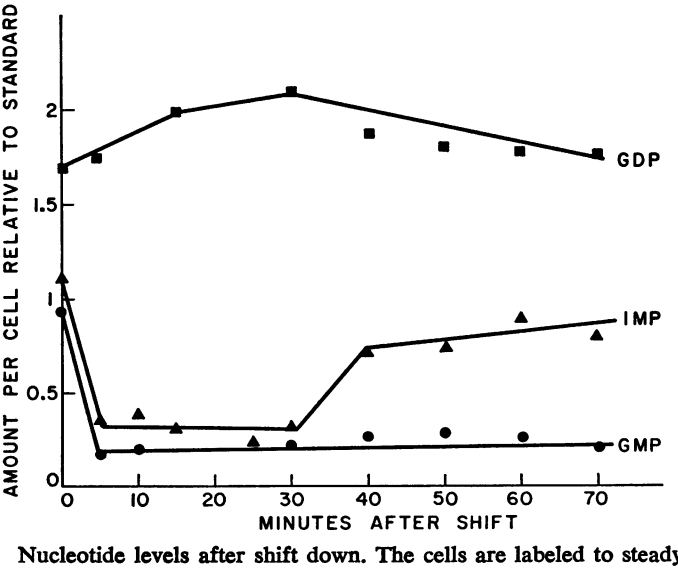

FIGURE 9 Nucleotide levels after shift down. The cells are labeled to steady-state in 5 μg/ml adenine C^{14} and then shifted at time zero to 0.3 μg/ml adenine C^{14}.

ATP level doubles after the shift and then declines gradually starting at about 30 minutes. Thus it is out of phase with the course of inhibition and ATP is therefore not the inhibitor. GMP level drops immediately after the shift to about 20 per cent of its original level and it then stays at this level. It does not rise at the time AIR rate is reduced.

Of the nucleotides tested, IMP is the only one that drops immediately after the shift as AIR rate rises and increases at the time AIR rate decreases. Although it does not regain its original level it comes up to about 75 per cent of it; this may be why the rate of AIR synthesis does not drop all the way to minimum value by 40 minutes—the IMP level may not be high enough to give maximum inhibition. IMP then behaves as expected of a controlling end-product inhibitor.

DISCUSSION

Wyngaarden and Ashton (15) have shown that ATP, ADP, AMP, GDP, GMP, and IMP competitively inhibit *in vitro* the binding of PRPP to the first enzyme unique to the purine pathway, phosphoribosyl–pyrophosphate amidotransferase. According to these data IMP inhibits PRPP amidotransferase effectively, but not as effectively as AMP, ADP, and ATP. Since in our yeast the latter are found in greater amount than IMP (IMP accounts for about 12 per cent of the purine nucleotide pool), one must assume that IMP is either a better inhibitor *in vivo* than Wyngaarden and Ashton's data suggest or that compartmentation of IMP and the enzyme provides a high local concentration of IMP in the vicinity of the enzyme. Another possibility is that IMP acts on another enzyme of the purine path, but this is unlikely because such control subsequent to the first irreversible step of the purine path is uneconomical.

Although the *in vitro* experiments of Wyngaarden and Ashton demonstrate that PRPP amidotransferase is sensitive enough to purine nucleotides to be inhibited at the concentrations of these probably found in the cell, it is possible that other mechanisms of control of purine synthesis by adenine or its derivatives exist. Gots (5) has suggested that the conversion of adenine to AMP might lower the level of PRPP enough to cut off *de novo* purine synthesis. In the present system, attempted *de novo* purine synthesis continues to be inhibited after the rate of uptake of adenine becomes equal to its rate of incorporation into nucleic acids. The rate of use of PRPP at a given rate of nucleic acid synthesis should be the same per AMP molecule formed, whether the ribose phosphate is attached directly to adenine or through the steps of *de novo* purine synthesis. Hence the same level of PRPP should be found in adenineless mutants in steady-state uptake of adenine as in wild type yeast synthesizing nucleotides *de novo*. Hence the exposure of the mutant to adenine is unlikely to be able to reduce PRPP level enough to cut off AIR synthesis. An additional argument against the PRPP "shunt" mechanism is that PRPP is required for tryptophan synthesis and tryptophan for protein synthesis.

Protein synthetic rate for the S1237 mutant is about as high as for the wild type, so PRPP is presumably in sufficient supply to allow tryptophan synthesis.

The transient responses of the purine nucleotides during a shift experiment are much more complicated than would be expected if their kinetic behavior were governed only by mass law. That AMP, GMP, and IMP drop after the shift is expected since these derive directly from the externally supplied adenine.[3] ADP and GDP do not drop, however, and ATP (also GTP in the one experiment in which it was measured) increases after the shift. In Fig. 5 the *C* and *D* IP* curves show that the rate of nucleic acid synthesis does not increase as ATP level increases after the shift but rather it starts to decrease. The rate of nucleic acid synthesis then does not depend on the purine nucleotide triphosphate concentration through mass law kinetics. It seems necessary to assume that regulatory mechanisms exist which adjust the rate of nucleic acid synthesis to the rate of purine synthesis independently of the levels of purine nucleotide diphosphates and triphosphates. The utility of this kind of regulation is evident. For example, ATP and ADP are required for glycolysis and oxidative phosphorylation. If their levels should be lowered due to shortage of the purine base, carbohydrate metabolism would be impaired. By reducing nucleic acid synthetic rate as soon as the precursors of ADP and ATP—presumably AMP and/or IMP—decrease, the cell would be able to continue carbohydrate metabolism instead of shutting it down along with nucleic acid synthesis.

The transient behavior of the IMP also implies more than mass law control. The drop in the intracellular level of IMP when the adenine level is lowered is predictable from mass law but the increase in level after a lag of 30 minutes to a new steady-state level suggests that derepression of an enzyme required for the formation of IMP has occurred during the 30 minutes. The increase in enzyme level would permit increase in IMP level thus permitting feedback inhibition of attempted *de novo* purine synthesis. Although repression control of nucleotide pyrophosphorylase has not to my knowledge been reported, repression of two of the enzymes of IMP metabolism in bacterial mutants has been described by Levin and Magasanik (10). In these mutants the formation of both inosinicase and IMP-dehydrogenase is controlled by guanine derivatives.

With the identification of the controlling element of the feedback loop, IMP, and the kinetic data presented here, a mathematical model of the purine nucleotide system becomes possible. The complicated regulation of the nucleotide levels, however, introduces difficulties which, though not insurmountable, require further data on the characteristics of the nucleotide system.

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[3] The levels of free nucleotides are probably not influenced by turnover of nucleic acid since in growing *Saccharomyces cerevisiae* the maximal breakdown rate is 0.01 per hour (7).

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