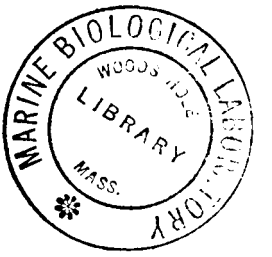

REGULATION OF PYRIMIDINE BIOSYNTHESIS AND ITS STRONG COUPLING TO THE PURINE SYSTEM

VICTOR W. BURNS

From the Department of Physiological Sciences, University of California, Davis

ABSTRACT The control of pyrimidine biosynthesis in a yeast mutant deficient for uracil, adenine, and histidine has been studied in vivo. The uracil mutation causes accumulation of ureidosuccinic acid and dihydroorotic acid in the cells. Accumulation is prevented when the pyrimidine nucleotide level in the cell is raised, apparently owing to feedback inhibition in the pyrimidine system. Investigation of the coupling of purine and pyrimidine systems shows that a high level of purine nucleotides can reverse inhibition in the pyrimidine internal feedback loop. Under certan conditions this reversal may affect only the first step of the pyrimidine system so that ureidosuccinic acid is synthesized and the next element of the pyrimidine pathway, dihydroorotic acid, is not synthesized. Other aspects of coupling between the pyrimidine system and other systems are presented.

INTRODUCTION

This paper[1] presents a study of regulation of pyrimidine synthesis and the relations of this to purine and protein synthesis in a *Saccharomyces cerevisiae* haploid strain with genetic blocks in the purine, pyrimidine, and histidine pathways. The pyrimidine block is the result of a mutation to uracil deficiency and provides two special advantages. First, that part of the pyrimidine biosynthetic pathway preceding the block is intact and the pathway rate of synthesis can be estimated from the rate of accumulation of the terminal products of the intact path, ureidosuccinate and dihydroorotate. Secondly, manipulation and specific labeling of the pyrimidine nucleotides do not depend on maintaining a level of these high enough to cut off or spare internal synthesis of pyrimidines, so that low steady-state nucleotide levels can be maintained, measured, and correlated with other cellular activities.

In the first paper of this series (1) the characteristics of feedback control of purine synthesis in the cell were described. The same type of analysis is now applied to pyrimidine synthesis, with the important addition of a study of *coupling* of the

[1] The second of the series on Regulation and coordination of purine and pyrimidine biosynthesis in yeast.

two pathways. The mutual regulation of the pathways is not symmetrical. The purine system exerts greater control over the pyrimidine system than the latter does on the former, and the details of this will be presented.

METHODS AND MATERIALS

The yeast strain employed in these studies is S1237A, developed and given to us by Dr. R. K. Mortimer (University of California, Berkeley). 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_2his_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 (AICA), or AICA-riboside. The ur_1 allele causes loss of the enzyme dihydroorotate dehydrogenase (2).

Cells were harvested in 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.

Assays of US (ureidosuccinic acid) and DHO (dihydroorotic acid) were made by using the method of Gerhart and Pardee (3). This method allows specific determination of US. DHO is determined by opening the ring with alkaline hydrolysis and assaying the US thus formed. The method is accurate to 5% or better. US and DHO are extracted from cells in hot water (6 min at 90°C) and interfering compounds like citrulline are removed from the extract by passing through a small Dowex 50 column (Dow Chemical Co., Midland, Michigan) before assay.

In studies of the uptake of uracil-C^{14} the compounds receiving the label were separated into the usual two fractions, the acid-soluble pool (SP) and the acid-insoluble pool (IP). The acid-soluble compounds were extracted in cold 5% TCA for 1 hr, followed by TCA wash. Alternatively, this pool was extracted in 95% ethanol for 3 min at 50°C, followed by extraction in 50% ethanol for 3 min 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 substracting the activity of the insoluble fraction from whole cell activity or by direct assay. Details of the membrane filter method have been given (1).

[2] Synthetic medium composition: Vitamins, dextrose, trace elements, and salts, as in Difco (Difco Laboratories, Detroit, Michigan) bacto yeast morphology agar with these exceptions: Inositol, 2 ug/liter; K_2HPO_4, 5.3 gm/liter; KH_2/PO_4, 2.6 gm/liter. Substitutions: Ammonium sulfate, 5 gm/liter; L-tryptophan, 0.2 gm/liter; l-glutamate, 0.2 gm/liter; l-methionine, 0.2 gm/liter; l-tyrosine, 0.25 gm/liter; cysteine, 0.2 gm/liter. Additions: Adenine and uracil, 50 μg/ml; histidine, 200 μg/ml.

For aspartate-C^{14} uptake studies, separation of the individual C^{14}–labeled compounds is accomplished by chromatography on Gelman ITLC silica gel paper (Gelman Instrument Company, Ann Arbor, Michigan) (4). By using phenol-H_2O as solvent, a clear separation of the main labeled components, asparate, glutamate, US, and DHO, is obtained in 50 min. Identification of these components was made by the aid of two-dimensional chromatography on large sheets of Whatman paper (H. Reeve Angel & Co., Clifton, New Jersey) and by the use of the method of Smith (5). Quantitative measurement of activity of the components was made by using a Vanguard 880 strip scanner and integrator (Vanguard Instrument Corp., Long Island, New York).

RESULTS

Identification and Assay of Pyrimidine Intermediates Accumulating as the Result of the Uracil Deficiency Mutation. In one approach to identification of intermediates, cells grown in synthetic medium were then incubated several hours in the absence of uracil and harvested in gram quantities. Cells were then extracted first in ether and then in 66% ethanol. Extracts were chromatographed by the descending method, using either propanol-water solvent or butanol-ethanol-formic acid-water (BEF) solvent. The extracts were run alone with orotic acid, dihydroorotic acid, and ureidosuccinic acid standards. The dried chromatograms were photographed under ultraviolet light. With this method 5 or 10 μg of orotic acid could be detected as an ultraviolet-absorbing spot. A number of ultraviolet-absorbing spots appeared on chromatography of the extract, and a few of these had R_f's near, but not identical with, that of orotic acid. These spots were eluted, and ultraviolet absorption spectra were obtained from them. Since none of the spectra showed an absorption peak in the neighborhood of 280 mμ (orotic acid has a distinguishing absorption peak at this wavelength), it was concluded that orotic acid does not accumulate. The same amount of extract did, however, give rise to ultraviolet-absorbing spots having the same R_f as DHO and US. These results are in agreement with Lacroute's (2) demonstration that the ur_1 mutation in yeast leads to loss of the enzyme DHO-dehydrogenase which converts DHO to orotic acid.

In order to add to the specificity of our identification of US and DHO and to provide a method of quantitative estimation of these compounds, we adapted a method of carbamylamino analysis, first described by Koritz and Cohen and improved by Gerhart and Pardee (3). This colormetric method is specific for carbamylamino. The proportions of US and DHO in the mixture can be determined without separation of the two because US gives a positive carbamylamino test directly, whereas DHO must be hydrolyzed in 0.5 N alkali before it will give a positive test. Identification and quantitative estimation were combined in the following experiment. Two aliquots of nonproliferating cell suspension were incubated in identical fashion in complete medium, except that aliquot A lacked uracil but had adenine and aliquot B lacked adenine but had uracil. Application of the carbamylamino test to crude extracts from these aliquots showed twice as much carbamy-

lamino per A cell as per B cell. Purified extracts showed five times as much carbamylamino in A as in B. Paper chromatography of the equivalent amounts of extract from each aliquot was accomplished in BEF solvent. All extracts and standards were run in duplicate. One of each pair was saved for later elution, and the other was sprayed with DMAB (*p*-dimethyl–aminobenzaldehyde). DMAB, 2 g in 20 ml concentrated HCl and 200 ml ethanol, gives a yellow color reaction with carbamylamino compounds or indoles. The A aliquot gave strong positive DMAB tests; one spot had the same R_f as US and the other the same R_f as DHO. The B aliquot showed very faint spots slightly behind US and DHO standards. The position of the spots and the sprayed strips of the chromatogram were used as a guide for the elution of adjacent areas in the unsprayed strips. Amounts of US and DHO in the eluants were then estimated (Table I).

TABLE I

Aliquot	DHO/10^8 cell	US/10^8 cell
	ug	ug
A	57	71
B	4.6	4.2

A similar procedure was used to develop chromatograms in phenol-water solvent. In this solvent the relative R_f's of US and DHO are reversed, in contrast to the order in BEF solvent. The results were similar to those obtained in BEF solvents.

The above results confirm that DHO and US accumulate only in the absence of uracil. The identity of US and DHO is strongly supported by the fact that compounds from A aliquot having the appropriate R_f's of US and DHO in two different solvent systems appear. These compounds show a positive reaction in the Koritz and Cohen test specific for carbamylamino.

Accumulation of US and DHO in Cells and Inhibition by Uracil. Log phase cells grown in presence of uracil and adenine were put in media containing varying proportions of uracil and adenine and the US-DHO of the cells was determined every 10 or 15 min for the 1st hr. Results are as follows:

(*a*) In zero uracil with or without adenine, DHO and US begin to accumulate immediately, i.e. time lag less than 10 min, and the amount per cell increases approximately *linearly* with time for the 1st hr.

(*b*) In 2 ug/ml uracil or more (less was not tried at this time) DHO and US do not accumulate.

It is noteworthy that detectable quantities of US and DHO do not appear in the medium during the first couple hours of incubation in the absence of uracil. This, coupled with the observation that the rate of accumulation is constant during this

time, suggests that rate of accumulation is equivalent to rate of production. Fig. 1 shows the results of an experiment in which cells at time zero were transferred from log phase growth in medium containing adenine and uracil to medium containing no adenine and either 10 μg/ml uracil or none. In the absence of uracil, US and DHO accumulate at constant but different rates. In the presence of uracil the production of US and DHO is inhibited. The effects are readily reversible: production of US and DHO is rapidly resumed when uracil is removed, and stopped when uracil

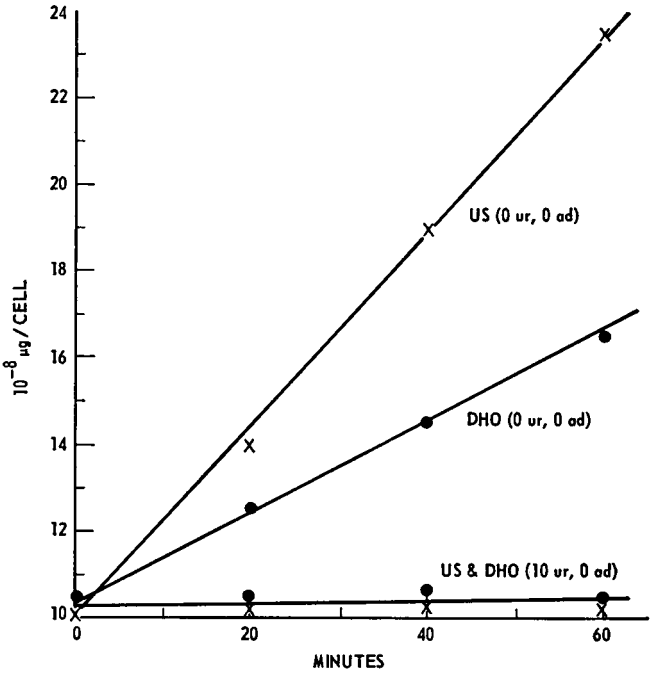

FIGURE 1 Accumulation of ureidosuccinic acid (US) and dihydroorotic acid (DHO) in cells exposed to concentrations of uracil (ur) and adenine (ad) as marked on the graph. The figures in parentheses give concentration in micrograms per milliliter.

is added. Under the conditions of growth employed here the uracil moiety is metabolized to pyrimidine nucleotides and thence to RNA. Inhibition is presumably caused by one or more of these compounds. This has been established for *Escherichia coli* (6). Experiments to establish this in vivo and to determine the effects of coupling to the purine pathway on control in the pyrimidine pathway were next undertaken.

Dependence of Synthesis of the Pyrimidine Intermediates US and DHO on Pyrimidine and Purine Pools. In the yeast S1237 the pyrimidine ring of pyrimidine nucleotides is derived only from the uracil supplied in the medium. If C^{14}-uracil is supplied, all pyrimidine nucleotides will be labeled and if this labeling

is continued until constant or steady-state activity is obtained in the pyrimidine pool, then each pyrimidine will have the same specific activity as the applied uracil (7). Under these conditions the total amount of pyrimidine nucleotides is proportional to the activity of the pool and only activity measurements need be made to determine amount.

Fig. 2 shows the uptake of labeled uracil in the presence of adenine. The cells were not depleted of uracil beforehand. SP and IP have similar meanings as in the adenine work (1): SP is the cold TCA-soluble pool of uracil derivatives; IP is the insoluble or nucleic acid pool. The SP uptake curve is quite different from that of adenine C.[14] Although the SP must contain the precursors of the IP, the rate of turnover of the SP is apparently so high that the IP already has much more activity at 30 min than the SP. The pyrimidine SP continues to expand in the presence of adenine long after the purine SP in the presence of uracil would reach steady state. Another difference is that the rate of uptake of uracil into the IP increases as external uracil concentration is increased in the range of 2 to 10 μg/ml and that the

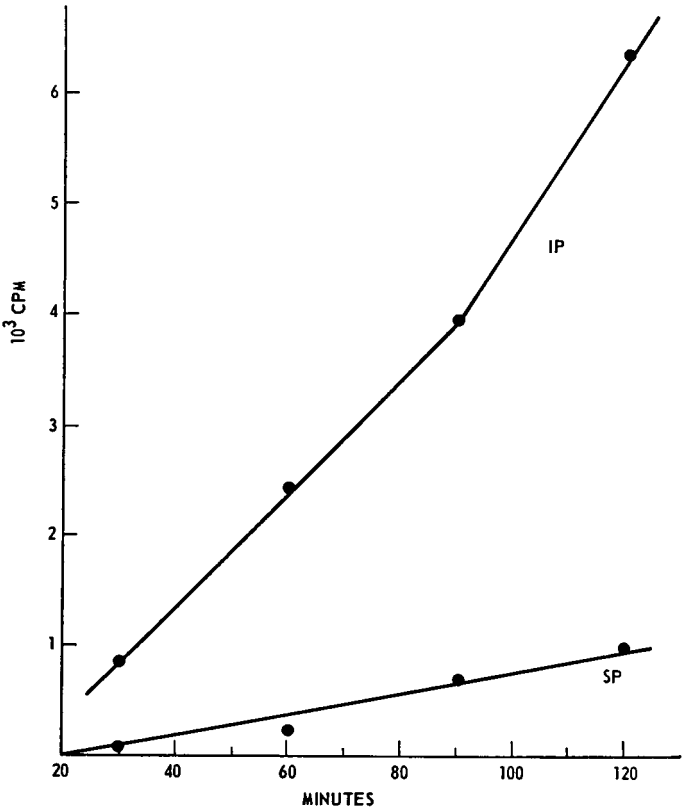

FIGURE 2 Incorporation of uracil-C^{14} into soluble (SP) and insoluble (IP) pools in the cells. Incubation with 5 μg/ml uracil-C^{14} and 5 μg/ml adenine.

SP also expands with uracil concentration as well as with time. The level of the pyrimidine SP when fed with uracil in the presence of adenine is apparently not sharply limited, in contrast to the purine case.

In later work it was found possible to obtain a steady-state pyrimidine SP by incubating cells with 20 μg/ml uracil-C^{14} and zero adenine. Fig. 3 shows the latter part of the SP uptake curve under these conditions. The steady state in 0 adenine is reached in about 90 min. If adenine is added (1.5 or 5 μg/ml) at 90 min, the pyrimidine SP is depressed and comes to a lower steady-state level which persists. The depression of the pyrimidine SP is more rapid the higher the external concentrations of adenine, corresponding inversely to the increase in purine SP (1). With 1.5 μg/ml adenine added, the steady state is terminated at 140 min owing to the exhaustion of adenine from the medium, and the SP rises. It rises well beyond the value it would have if the cells had been in zero adenine uninterruptedly (overshoot). Exposure to adenine apparently increases the capacity of the pyrimidine SP or temporarily decreases self-regulation, although as long as the adenine SP is high (until 140 min), this regulates the pyrimidine SP level.

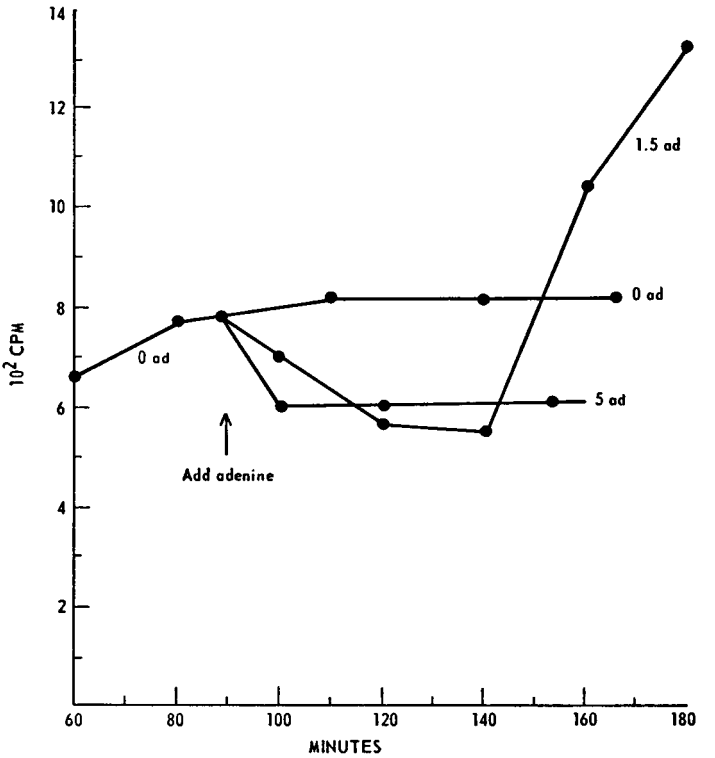

FIGURE 3 Intracellular pyrimidine soluble pool, measured in counts per minute vs. time. Cells were incubated with 20 μg/ml uracil-C^{14} without adenine until 90 min; then 0, 1.5, or 5 μg/ml adenine was provided, as indicated on the graph.

Absence of uracil, which leads to depression of pyrimidine SP, decreases feedback inhibition of attempted pyrimidine synthesis and leads to accumulation of DHO and US. We now ask: Does lowering the pyrimidine SP by purine manipulation in the presence of *excess uracil* also release feedback inhibition? Fig. 4 shows the behavior of US synthesis in an experimental situation exactly the same as in Fig. 3. The lower curve shows the results of incubation in 20 μg/ml uracil and zero adenine. In zero uracil and zero adenine there is no feedback inhibition of US synthesis (Fig. 1). In 20 μg/ml uracil and zero adenine, US synthesis is completely suppressed; the corresponding curve of Fig. 3 shows the pyrimidine SP to be high and constant after 90 min. The upper curve shows what happens when 5 μg/ml adenine is added at 90 min to cells prepared in 20 μg/ml uracil and zero adenine. First there is a lag of 35 min during which no effect of adenine on US synthesis is seen. During this time pyrimidine SP drops (Fig. 3) and purine SP rises (1) concurrently. At 125 min, US begins to accumulate at a rate close to that of the zero uracil control (Fig. 1). Control of pyrimidine synthesis by uracil has been counteracted

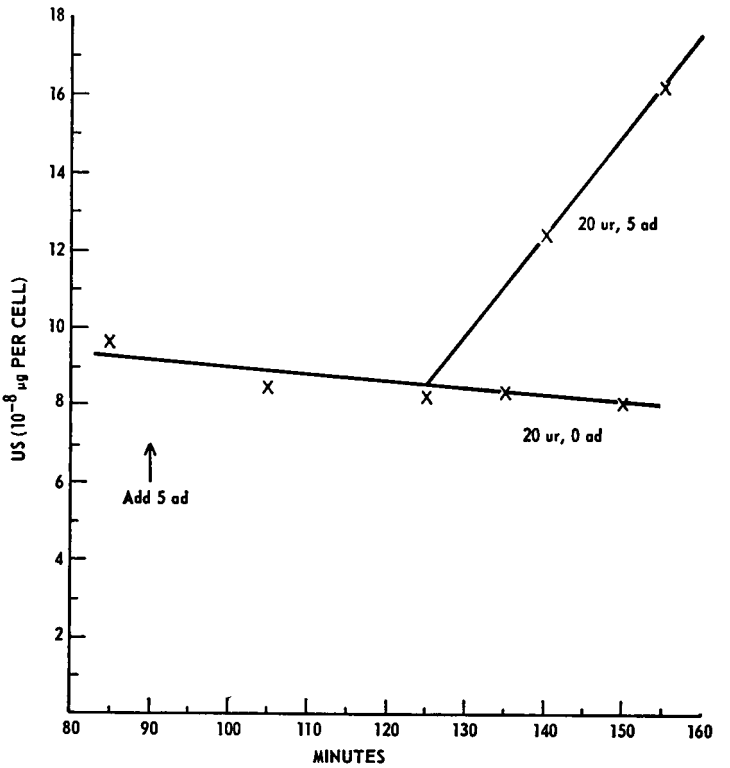

FIGURE 4 Ureidosuccinic acid (US) level in cells incubated with 20 μg/ml uracil (no adenine) until 90 min, after which 0 or 5 μg/ml adenine was added as indicated on the graph. Uracil and adenine concentrations in the medium are not substantially altered by cellular uptake during the time period covered in the graph.

although excess uracil is present and the rate of transfer of uracil into the cells, through the SP, and into the nucleic acids has not been decreased.

Fig. 5 shows the behavior of DHO in the same experimental situation as in Figs. 3 and 4. In 20 μg/ml uracil and zero adenine, DHO synthesis is completely suppressed; in zero uracil and zero adenine it is synthesized at a constant high rate (Fig. 1). Thus far the control of DHO is like that of US. However, when 5 μg/ml adenine is added at 90 min (Fig. 5), it can be seen that DHO does not begin to accumulate again. This is in contrast to the US case. Hence, adenine, through the influence of purine derivatives formed from it (1), can counteract the feedback inhibition of US synthesis but it does not at the same time allow the conversion of US to DHO to resume. In cells not having a mutant block in the pyrimidine pathway this control mechanism would allow storage of the specific pyrimidine precursor US under certain conditions of excess purine and pyrimidine nucleotide supply.

If the cells undergo preliminary incubation in complete medium containing 50 μg/ml adenine and uracil and are transferred to 10 μg/ml adenine and uracil or 10 μg/ml uracil and 3 μg/ml adenine, a different result is obtained. In this case both US and DHO begin to accumulate immediately. US continues to accumulate at least for 1 hr, but DHO accumulation may stop after 30 min or so. Hence the type of response the pyrimidine system makes to shifts in the purine system clearly depends on the states of both systems at the time of shift.

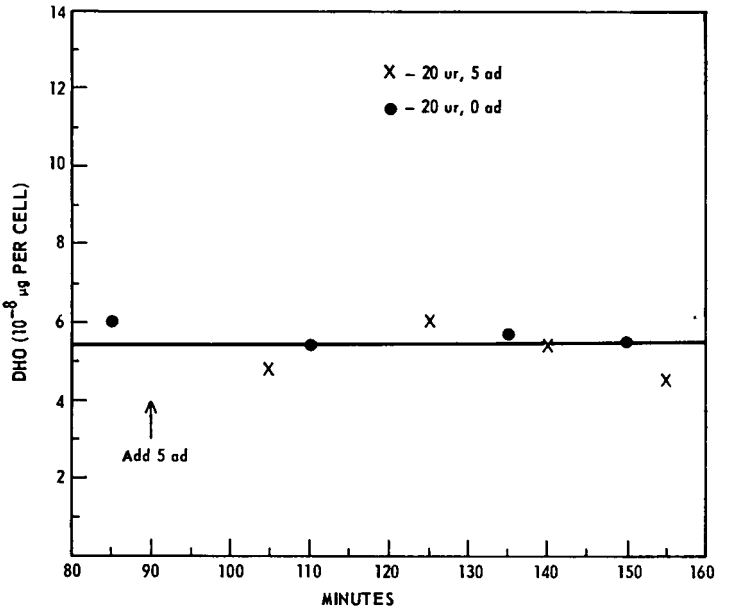

FIGURE 5 Dihydroorotic acid (DHO) level in cells incubated with 20 μg/ml uracil without adenine until 90 min; then 0 or 5 μg/ml adenine was added as indicated on the graph.

Since the pyrimidine pathway is coupled to other biosynthetic pathways, it becomes of interest to ask what indirect effects may be produced by turning pyrimidine synthesis off and on. A direct and immedate precursor of US is aspartate which is also a precursor of various amino acids such as threonine, methionine, lysine, arginine, etc., and of protein. Since aspartate enters the pyrimidine pathway slowly or not at all when the pathway is inhibited by supplying uracil to the cells, it might accumulate or be diverted to other syntheses in greater quantity. To facilitate determination of the fate of aspartate carbon a tracer method employing uniformly labeled C^{14}-asparatate was used. The cells take up aspartate readily although they do not require it. The cold TCA or 50% ethanol extract of aspartate-C^{14}–labeled cells contains the small molecules for which aspartate is a precursor. The only ones exhibiting substantial incorporation of activity have been identified by means of silica gel–glass fiber chromatography as aspartate, glutamate, US, and DHO. The activities of these in samples taken at various times can be measured in a radioactivity scanning and measuring device. The ethanol insoluble activity is contained in protein which has incorporated C^{14}-aspartate, C^{14}-glutamate, and perhaps other indirectly labeled amino acids.

The most illuminating experiments of many performed involved the incubation of cells with C^{14}-aspartate and 50 μg/ml adenine for 150 min without uracil, at which time 20 μg/ml uracil is added. Fig. 6 shows the behavior of the labeled aspartate and glutamate pools. These pools increase slowly in the absence of uracil but the rates of increase nearly double soon after uracil is added, presumably reflecting decreased consumption of aspartate in attempted pyrimidine synthesis.

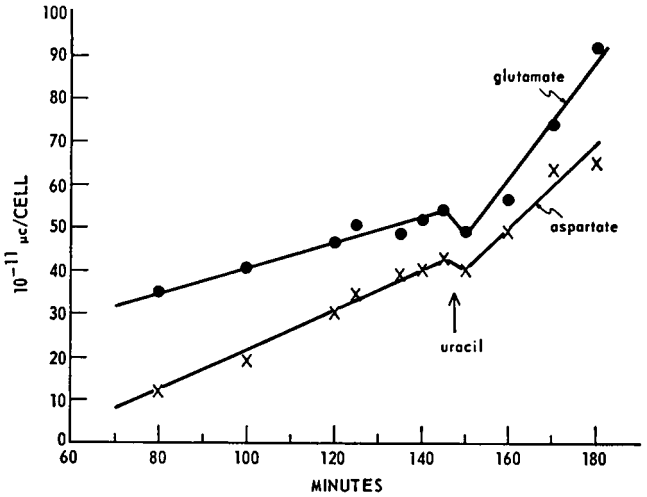

FIGURE 6 Changes in labeled free aspartate and glutamate pools with time. Cells were incubated with C^{14}-aspartate and adenine at essentially constant levels until 150 min, after which 20 μg/ml uracil was supplied. Ordinate: 10^{-11} μc per cell.

Since more aspartate would then be available for protein synthesis and the provision of uracil to cells starved 150 min for pyrimidines might be expected to enhance messenger RNA synthesis, one might expect increased protein synthesis. Measurements of C^{14} incorporation into the insoluble pool indeed showed this. The rate of incorporation was constant at 1200 CPM/hr before uracil addition and for about 10 min after, at which time it increased sharply to a new constant value of 2250 CPM/hr. The introduction of uracil then approximately doubled the incorporation rate and presumably doubled the rate of protein synthesis.

This same experiment allowed an independent check, under somewhat different conditions than those used previously, of the differential feedback control of US and DHO synthesis when both purine and pyrimidine nucleotides are present in the cells. Fig. 7 shows the activities of US and DHO as a function of time. These increase steeply before uracil addition as a result of the rapid synthesis and accumulation

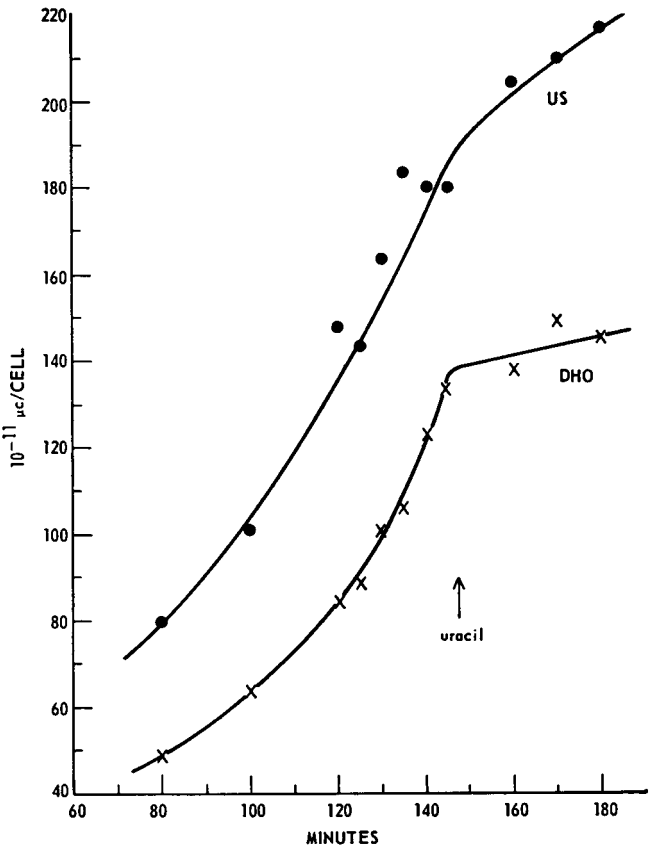

FIGURE 7 Changes in labeled US and DHO pools with time. Cells were incubated with C^{14}-aspartate and adenine until 150 min, whereupon 20 μg/ml uracil was added. Ordinate: 10^{-11} μc per cell.

of these compounds. After uracil addition US continues to accumulate at a slightly reduced rate, but the accumulation of DHO is virtually stopped. US, of course, would also be stopped if no adenine were supplied to the cells. Clearly there is differential control of US and DHO synthesis, similar to that illustrated in Figs. 4 and 5. In that case adenine was added to cells deprived of it but consistently supplied with uracil, whereas in the present case the starvation and addition regime were vice versa.

DISCUSSION

The synthesis of US and DHO in S1237 can be turned off and on rapidly through variation of uracil concentration outside the cells and the resultant change in internal pyrimidine nucleotide levels. Since this is rapid, it must involve control of enzyme activity rather than amount although control through repression and derepression presumably exists. End-product inhibition in the pyrimidine pathway has been demonstrated in other systems, notably in *Escherichia coli* and lettuce (3, 8). In these systems the site of feedback control appears to be the enzyme aspartate transcarbamylase. In *Escherichia coli* the most effective end-product inhibitor is cytidine triphosphate; in lettuce it is uridine monophosphate. Although we have not been able to extract active aspartate transcarbamylase from the yeast for in vitro studies, the data presented from in vivo studies strongly suggest that this enzyme is controlled by feedback inhibition in yeast.

The demonstration that increasing the level of the purine nucleotide pool by applying adenine counteracts feedback inhibition in the pyrimidine pathway raises questions of mechanism. First of all it is noted that the over-all level of the pyrimidine nucleotide pool decreases when the purine pool level is increased. Decreasing the pyrimidine level in this way then might reverse feedback inhibition. However, US and DHO syntheses can be differentially affected by purine counteraction but not by manipulation of the pyrimidine level through adjustment of uracil concentration. If the purine effect takes place through the pyrimidine pool we must then postulate that the composition of the pool is different when lowered by purines than by uracil and that the enzymes aspartate transcarbamylase and dihydroorotase are inhibited by different pyrimidine nucleotides. An alternate explanation can be based on the demonstration (9) that ATP can reverse the CTP inhibition of aspartate transcarbamylase obtained from *Escherichia coli*. If the yeast enzyme responds the same way, then the purine reversal effect may be the result of ATP level increasing when adenine is added and the ATP then acting directly on the transcarbamylase. This explanation requires no action of ATP on dihydroorotase. It is noteworthy that the effect of purines on the pyrimidine pathway is not reciprocal; that is, we cannot counteract purine end-product inhibition of the purine pathway by application of uracil to the cell system (1).

In Fig. 3 it was noted that application of adenine to cells in excess uracil with subsequent exhaustion of adenine produces an expansion of the pyrimidine pool beyond what it would have if adenine had never been applied. This "overshoot" behavior is hard to explain on the basis of our present knowledge of the dynamics of the system. A possibility is that the overshoot is the result of pyrimidines collecting in a storage pool (10) which can hold more pyrimidine after the cells have grown in the presence of adenine.

The demonstration that the synthesis of US can occur under certain conditions of feedback balance between the pyrimidine and purine pools while at the same time synthesis of DHO is inhibited reveals a new type of control mechanism. The utility of this mode of control for the cell would be that under conditions of temporary pyrimidine excess a store of precursor that can only be used for pyrimidine synthesis is built up. Aspartate stores would not guarantee pyrimidine synthesis in times of low aspartate production since this compound is also used for protein synthesis. Lacroute (2) has reported that in yeast US can induce the enzyme dihydroorotase that converts US to DHO. This slow control mechanism (involving enzyme formation) presumably would act to set an upper limit to the pool of US that could be accumulated. Production of US and not DHO when both uracil and adenine are present does not always occur. When the cells undergo no preliminary starvation for either purine or pyrimidines, both US and DHO may be synthesized.

Observation of the metabolism of aspartate as affected by manipulation of the purine and pyrimidine pools reveals some of the characteristics of weak coupling (11) among pools. When there is an ample supply of purines and pyrimidines, which provide raw material for M-RNA synthesis, aspartate is diverted to protein synthesis, and pools of protein raw materials like aspartate itself and glutamate begin to grow.

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