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COORDINATE REPRESSION OF THE SYNTHESIS OF FOUR HISTIDINE BIOSYNTHETIC ENZYMES BY HISTIDINE

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Introduction.—In several instances, the rate of synthesis of the enzymes of a biosynthetic pathway appears to be regulated by the size of the pool of the end product of that pathway.[1-4] This phenomenon has been called enzyme repression.[2]

We have observed repression by histidine during the investigation of the histidine biosynthetic enzymes in histidine-requiring mutants of *Salmonella typhimurium*.[5] The cellular concentration of the four histidine biosynthetic enzymes which were examined were increased about 15-fold by limiting the amount of histidine available to a mutant during growth. A simple method for limiting the availability of histidine in the cell during the entire growth period, and thus increasing the enzyme concentrations, was realized by growing histidine-requiring mutants on a derivative of histidine, formylhistidine, as a source of this amino acid. It appeared, therefore, that the normal low level of the biosynthetic enzymes in wild-type cells grown on minimal medium was probably due to the repression by the normal pool of histidine of most of the cellular capacity for synthesizing these proteins.

This investigation is concerned with utilizing the histidine biosynthetic system from *Salmonella* to answer the following question: Does the histidine repression of enzyme synthesis affect each of the enzymes of the pathway to the same extent, i.e., is there a parallel increase in specific activity of all four enzymes on lowering the internal histidine pool and a constant ratio of the activity of one histidine enzyme to another independent of the concentration of the enzymes in the cell? A secondary point of interest is whether there is any influence of the concentration of the enzyme substrates on the enzyme synthesis control mechanism.

The strains used in this study were some of the several hundred histidine-requiring mutants of *Salmonella* that have been mapped by Hartman, *et al.*[6, 7] They were able to demonstrate that each of the mutants could be classified into one of the seven genetic loci, *E*, *F*, *A*, *B*, *C*, *D*, and *G*, which were closely linked on the chromosome in the above sequence. These same mutants were analyzed by us for presence or absence of various enzymes of the histidine biosynthetic pathway.[5] The genetic data taken in conjunction with the biochemical analysis gave more direct support for Hartman's original suggestion[8] that the sequence of the genes in the linkage map corresponds to the sequence of the enzymes they control in the biosynthetic pathway.

The pathway of histidine synthesis seems to involve six enzymes, the last four of which have been studied in this investigation.

(1) The first enzyme catalyzes the condensation of phosphoribosyl pyrophosphate (PRPP) with AMP to give an as yet uncharacterized compound called Compound III (Moyed and Magasanik[9]). This enzyme appears to be lacking in the histidine *F* mutants in *Salmonella*.[9, 10]

(2) The second enzyme converts Compound III to imidazoleglycerol phosphate (IGP) and appears to be controlled by the *A* gene.[9, 10]

(3) The third enzyme is IGP dehydrase which catalyzes the conversion of IGP to imidazoleacetol phosphate (IAP), and is missing in mutants of the *B* class.[5, 11]

(4) The fourth enzyme, IAP transaminase, catalyzes the transamination of IAP to histidinol phosphate and is missing in mutants of the *C* class.[5, 12]

(5) The fifth enzyme histidinol phosphate phosphatase hydrolyzes histidinol phosphate to histidinol.[5, 13] No mutants have as yet been found in *Salmonella* missing only this enzyme (cf. *Discussion* in Ames, *et al.*[5]). The gene for the phosphatase does appear to be linked to the rest of the histidine genes, however, as certain multisite mutants are missing the phosphatase in addition to the other histidine enzymes. This enzymatic step appears to be experimentally irreversible.

(6) The last enzyme in the pathway is histidinol dehydrogenase which oxidizes histidinol to histidine (Adams[14]), and which is associated with the *D* gene.[5] This enzymatic step also appears to be irreversible.

Possible reasons for the histidine requirement of *E* and *G* mutants, which seem to have all these enzymes, have been briefly discussed.[5]

Methods.—The conditions for growing the various mutants, method of making extracts, and assays for the last four histidine biosynthetic enzymes have been described in detail.[5]

Glutamic dehydrogenase assay: Glutamic dehydrogenase, an enzyme involved in a different biosynthetic pathway, has been assayed as a control in some of the experiments. The reaction catalyzed by the enzyme is:

$$\text{L-glutamate} + \text{DPN} \rightleftharpoons \alpha\text{-ketoglutarate} + \text{DPNH} + \text{NH}_3 + \text{H}^+.$$

In the assay for this enzyme the DPNH formed in the oxidation of glutamate was coupled, with diphorase, to the reduction of the blue dye dichlorophenolindophenol. The assay was performed in a Cary recording spectrophotometer at 25° by following the disappearance of the blue color of the dye at 600 mμ. The assay cuvettes contained: 0.5 ml. of 0.4 mM sodium dichlorophenolindophenol, 0.4 ml of 0.2 *M* triethanolamine-HCl buffer (pH 8.4), 0.005 ml of 50 mM TPN (brought to pH 5 with KOH), an excess of diaphorase (0.01 ml[15]), and 0.005 to 0.03 ml of dialyzed extract. After the small endogenous oxidation had ceased and the dye was not reduced further, 0.05 ml. of 0.5 M L-glutamate (brought to pH 8.5 with KOH) was added and the rate of dye bleaching determined. The oxidation of 0.01 μmole of glutamate results in a decrease in optical density of 0.3 under these conditions. One unit of enzyme activity is defined as the quantity required for the oxidation of one μmole of glutamate per hour under the assay conditions.

Results.—The enzyme levels of hisE-11 during growth on limiting histidine: A "leaky" mutant *hisE-11*, which can make some histidine and grow slowly on minimal medium[6] has been found to produce large quantities of the histidine enzymes

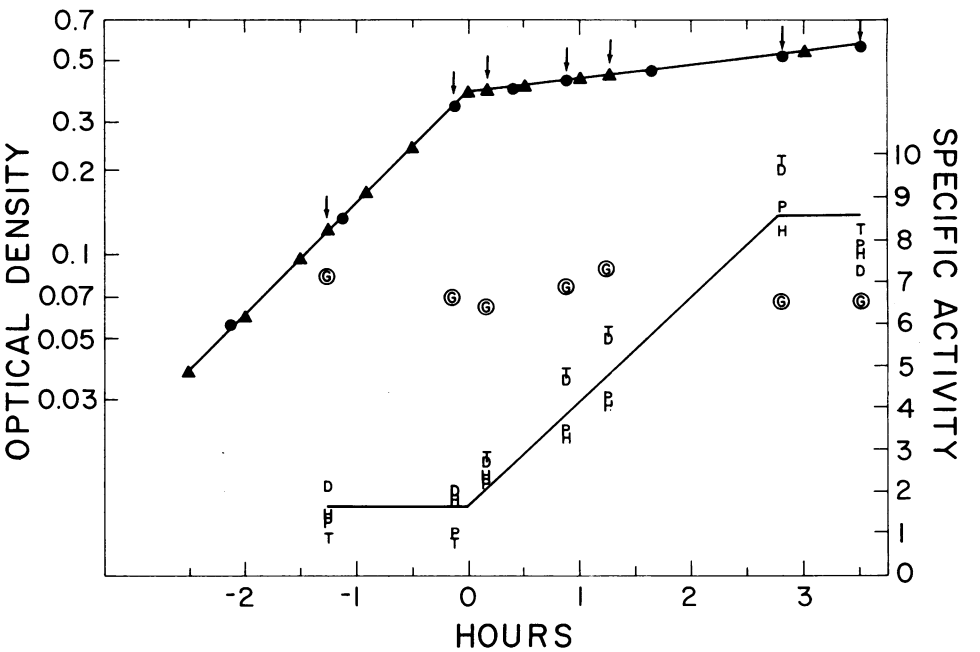


FIG. 1.—The specific activities of four histidine biosynthetic enzymes and glutamic dehydrogenase during the growth of the "leaky" histidine-requiring mutant *hisE-11*. The amount of histidine which was added (0.03 mM) was exhausted midway during the growth period (zero time) and the mutant then grew at a lower rate which was limited by the small amount of histidine it was capable of making. A liter culture was grown in a two-liter flask, shaken at 37° in a rotary shaker,[16] and the optical density readings were determined at various times. The -▲- and -●- symbols on the growth curves represent two separate experiments, run under identical conditions, which are both plotted. At the times indicated by the arrows, 100-ml aliquots of the bacterial culture were harvested, cooled rapidly, and centrifuged in the cold. Extracts were prepared from the bacteria, and protein and enzyme were assayed as previously described[5] (cf. Methods). The specific activities of the phosphatase, P, and glutamic dehydrogenase, Ⓖ, have been plotted in the units (μmoles/mg protein/hour) previously described.[5] The dehydrase, *D*, the transaminase, *T*, and the histidinol dehydrogenase, *H*, specific activities have been normalized by multiplying by the factors 1.1, 3.5, and 7.5 respectively. These factors were determined from the slopes of the lines in Figures 3, 4, and 5, and make the specific activities of each of the histidine enzymes approximately equal.

during the period of slow growth on minimal medium. The kinetics of enzyme formation were examined and are shown in Figure 1. The culture was analyzed for four histidine biosynthetic enzymes during the period of growth on histidine (0.03 mM) as well as during the period after the exhaustion of the exogenous histidine supply when the mutant was growing at its slow "leaky" rate. A control enzyme, glutamic dehydrogenase, which is involved in another amino acid biosynthetic pathway was also assayed in the extracts. Low, wild-type levels of the four histidine enzymes were found during the exponential phase of growth, when histidine was present in excess. When the histidine was exhausted and the growth rate decreased, the specific activity of the histidine enzymes in the cells started to increase rapidly. For mutant *hisE-11* the concentration of the enzymes finally approaches a level that corresponds to an eight-fold increase over that found in the wild-type strain. The kinetic data of Figure 1 indicate that, within the limits of accuracy of the assays on crude extracts, the specific activity of each of the histidine enzymes begins to increase at the same time and increases in parallel with the other histidine

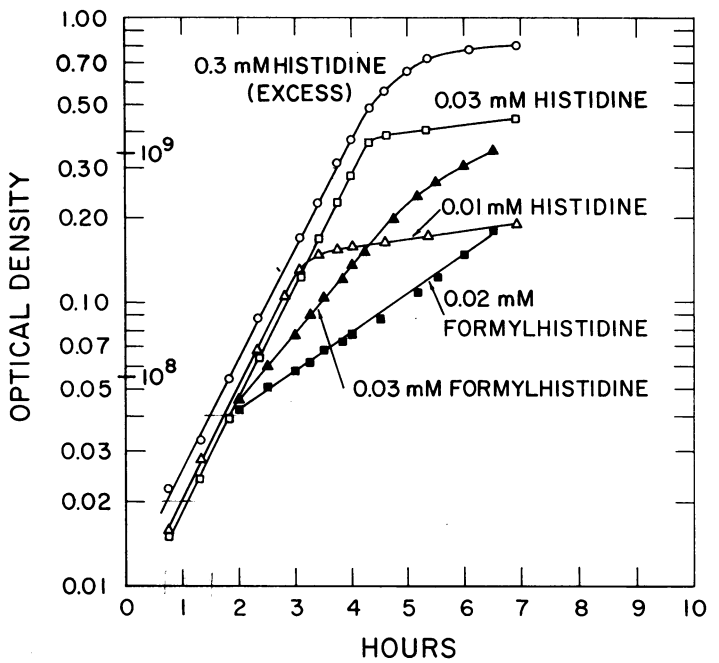


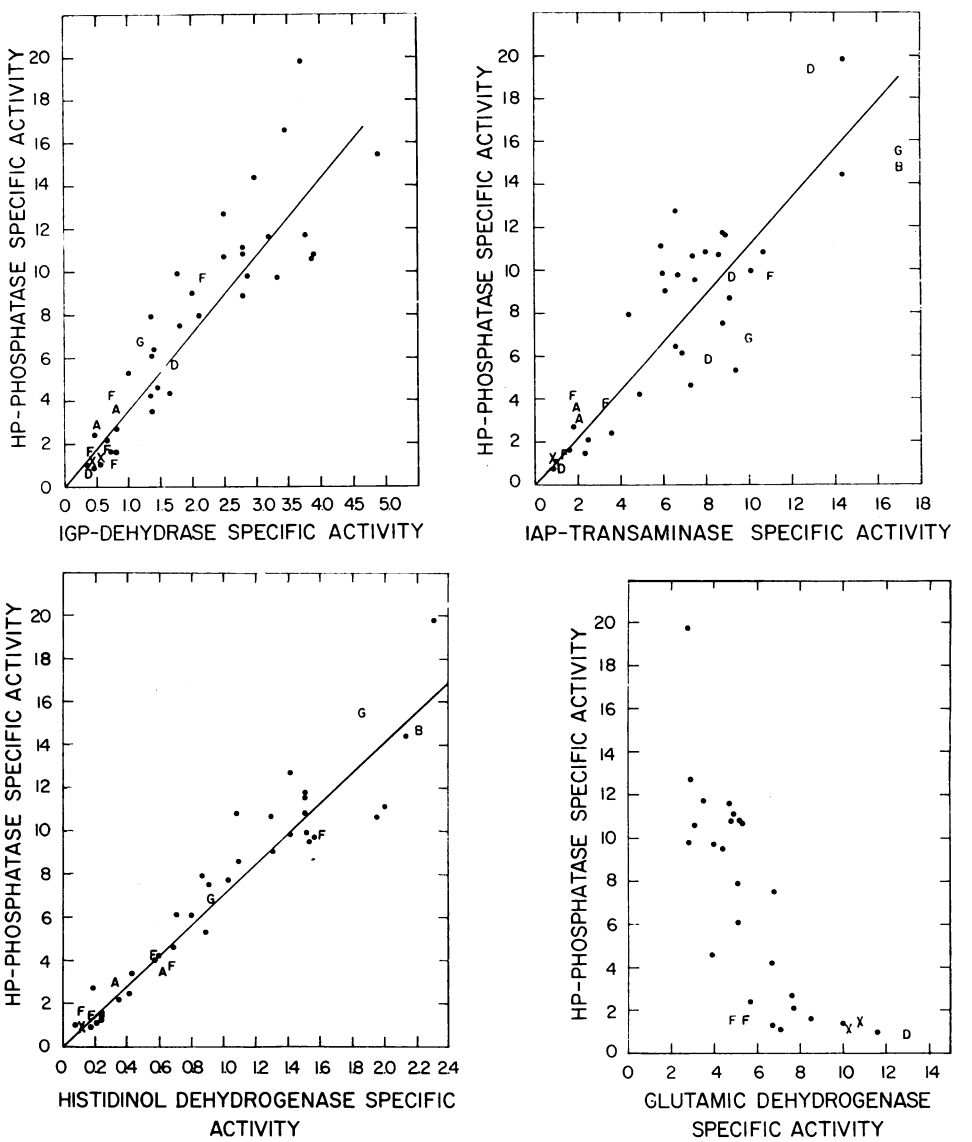
FIG. 2.—*Expt. A*—(L-histidine: Δ, 0.01 mM; □, 0.03 mM; ○, 0.3 mM). The optical density of growing cultures of mutant *hisF-41* on differing amounts of histidine were determined at various times in a Beckman DU spectrophotometer at 650 mμ. The inoculum was grown on 0.2 mM L-histidine. The culture was grown at 37° in a rotary shaker; the minimal medium used and procedures have been previously described.

Expt. B—(Nα-formyl-L-histidine: ■, 0.02 mM; ▲, 0.03 mM). The growth rates of mutant *hisF-41* on differing amounts of formylhistidine were determined in a separate experiment. The conditions were the same as in Figure 1a. Data from a histidine control culture and a culture which was 0.04 mM in formylhistidine, both of which gave the same growth rate as was obtained in Figure 1a, are not presented. No growth was obtained with a culture which was 0.01 mM in formylhistidine.

enzymes. The glutamic dehydrogenase specific activity on the other hand does not increase, but remains relatively constant.

When *hisE-11* or any of the other mutants was grown on excess histidine, the final concentration of the histidine enzymes was the same as in the wild-type cells.[5] The behavior of this mutant is contrasted with other histidine mutants which stop growing soon after histidine becomes exhausted and which do not show an increase in the specific activity of the histidine biosynthetic enzymes when examined several hours after growth has stopped.

The effect of formylhistidine on growth rate and enzyme level: The growth curves for a typical histidine-requiring mutant, *hisF-41*, grown on differing amounts of histidine is shown in Figure 2. It is apparent that the leveling off point is determined by this histidine concentration, while the growth rate is not. The histidine pool does not limit the growth rate under these conditions. The growth rate was limited by histidine deficiency, however, when the mutant was grown on a derivative of histidine, N-α-formyl-L-histidine,[17] Figure 2. The long generation time of the



FIGS. 3, 4, 5, 6.—The specific activities of the histidine enzymes and glutamic dehydrogenase in various extracts. Each point represents the specific activities of two of the enzymes in an extract of a mutant or wild-type. The wild-type extracts are symbolized by (X), the *E* class of mutants by (●) and the other classes by their letters: F, A, B, C, D, G. The growth conditions are the same as previously described[5] and as discussed in the text.

formylhistidine-grown cells was presumably the result of either slow uptake or slow hydrolysis of formylhistidine by the cells. The growth rate of the wild-type cells on 1.0 mM histidine or 0.03 mM formylhistidine was the same as that of a mutant grown on histidine.

The concentration of enzymes in the wild-type cells grown on minimal medium, a concentration which is relatively constant throughout the growth cycle, has been used as a point of reference in these studies. Mutants that have been grown on

excess histidine have the wild-type specific activity for the histidine biosynthetic enzymes, while mutants grown on formylhistidine have up to fifteen times the wild-type level.[5] A similar effect with two other derivatives of histidine, carnosine and histidylhistidine, has been observed in preliminary experiments.

Specific activity changes of the histidine enzymes in various mutants: It became of interest to examine a variety of different mutants for parallel changes in histidine biosynthetic enzymes, i.e., to see whether there was a constant ratio of one histidine enzyme activity to another independent of the concentration of the enzymes in the cell and independent of which enzyme the histidine mutant was missing. A variety of different histidine mutants from the seven classes, *E*, *F*, *A*, *B*, *C*, *D*, and *G* have been grown under different conditions with respect to the histidine content of the medium and then assayed for the last four enzymes of histidine biosynthesis as well as for glutamic dehydrogenase. In general, the changes in concentrations of the enzymes were as follows: When wild-type cells were grown on minimal medium or when the histidine mutants were grown on excess histidine low levels of the histidine enzymes were found in the extracts.[5] Extracts of *hisE-11* grown on 0.03 mM histidine (Fig. 1), and harvested several hours after the exhaustion of the histidine, contained intermediate levels of the histidine enzymes. High concentrations of the enzymes were found in extracts of mutants grown on 0.03 mM formylhistidine.[5] The ratio of the specific activity of the dehydrase (Fig. 3), transaminase (Fig. 4), histidinol dehydrogenase (Fig. 5), and glutamic dehydrogenase (Fig. 6) to the specific activity of the phosphatase has been plotted for each of these extracts.[18] In cases where a mutant was missing one of the four enzymes examined, the specific activity of that enzyme was not plotted. Figures 3, 4, and 5 show that the ratio of one histidine biosynthetic enzyme activity to another is constant no matter what the mutant or the growth conditions. Figure 6 shows that such a relation is not true for the glutamic dehydrogenase-histidinol phosphate phosphatase ratio.

Separation of the histidine biosynthetic enzymes: Because of the constant ratio of one enzyme activity to another, it was of interest to see whether the four enzyme activities were associated with separate proteins. Figure 7 shows the fractionation of a crude extract of mutant *hisE-11* obtained on a diethylaminoethylcellulose (DEAE)[19] column and the assay of the four enzyme activities in the column fractions.[20] The IAP-transaminase and histidinol dehydrogenase activities were eluted as discrete peaks while the histidinol phosphate phosphatase and IGP-dehydrase activities came off together in a third peak. It is not clear whether this association of the phosphatase and dehydrase is fortuitous or represents some actual physical association. The fact that the four enzymes are associated with a minimum of three distinct proteins does indicate, however, that the parallel enzyme changes cannot be explained by the association of all the enzyme activities on the same protein molecule.

Discussion.—The data presented indicate that in acting as a repressor, histidine appears to affect the synthesis of each of the histidine biosynthetic enzymes to the same extent, within the experimental error of the assays. It is proposed that this phenomenon be called coordinate repression.

An indication that the histidine repression is independent of the quantity of the substrate of each enzyme present in the cells (cf. also Yates and Pardee[3]) is given by

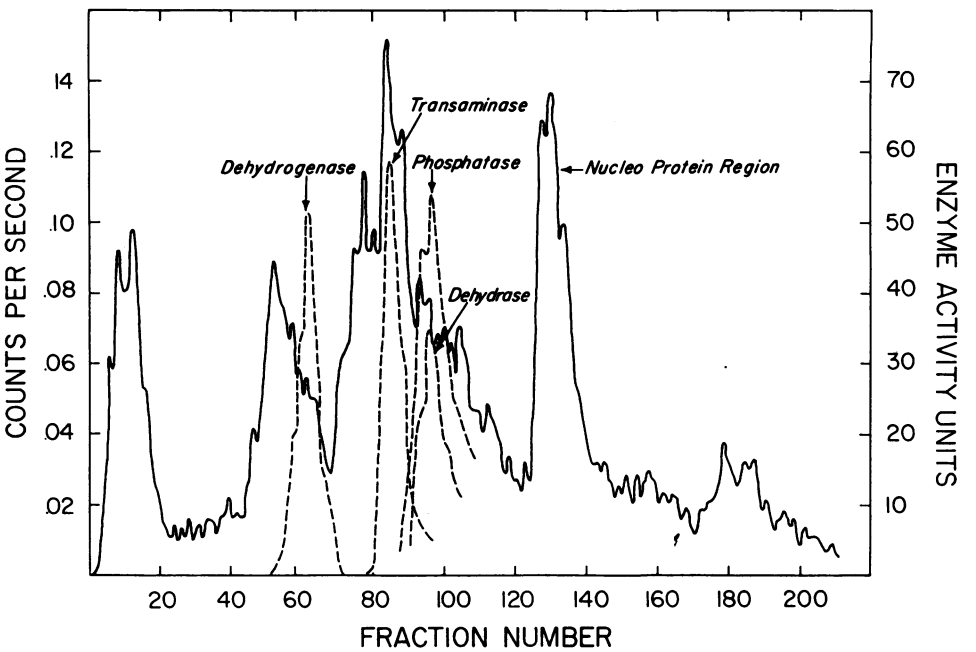


FIG. 7.—The fractionation on a DEAE column of an extract of mutant *hisE-11* grown on formylhistidine. The bacteria were grown as previously described,[5] except that randomly labeled C^{14}-fructose was used as a carbon source instead of glucose. The bacteria were broken in a French pressure cell (cf. Cowie *et al.*[21]). 35 mg. of protein were run on the column[21] by Dr. Dean Cowie who also analyzed the eluate fractions for radioactivity and protein.[22] The protein and radioactivity determinations showed a constant ratio and only the latter has been plotted (——). The activities of the four histidine enzymes(– – –) were determined as previously described.[5] The dehydrogenase units have been multiplied by a factor of 10 and the dehydrase units by a factor of 2 so that the peaks are of approximately the same height. Enzymes were localized by assaying combined aliquots from groups of ten tubes; no enzyme activity was detected for any particular enzyme in fractions other than the peak indicated. Over 90 per cent of the activity of each enzyme put on the column was recovered in the combined fractions from each peak. The specific activities of the enzymes in the crude extract were the same as previously found for mutant *hisE-11* grown on formylhistidine.[5] The specific activities of the pooled fractions for each enzyme represented a five- to 10-fold increase over the crude extract.

the finding that mutants from all genetic classes show these coordinate changes in enzyme levels. It had been previously shown that mutants accumulate intermediates before the enzymatic block in large amounts[6, 8] and since the last steps of the pathway appear to be irreversible the mutants have presumably never contained the histidine precursors after the enzymatic block. Thus, depending on the mutant, an enzyme can be in the presence of no substrate or an abnormally large amount of substrate without influencing the repression of its synthesis by histidine.

Many hypotheses would be consistent with the finding that histidine represses the synthesis of all of the biosynthetic enzymes together. One attractive possibility is that the repressor blocks enzyme synthesis at the gene level.[23] The genes for the series of histidine enzymes have been shown by Hartman[6, 8] to be closely linked on the *Salmonella* chromosome and conceivably this length of the chromosome is "functionally turned off" by a histidine-nucleic acid repressor (e.g., histidine-soluble RNA) with a specific affinity for part of the histidine section of the chromosome. In *Neurospora*[24] and yeast,[25] where the histidine genes are scattered on the

chromosomes, it will be of interest to see if enzyme repression is possible. No effect of added histidine has been observed on the synthesis of various histidine biosynthetic enzymes in *Neurospora*.[26] However, this is not conclusive as the system may be maximally repressed when growing on minimal medium as is *Salmonella*.

Yates and Pardee[3] have examined three enzymes of orotic acid biosynthesis in *E. coli*, each of which was repressed by uracil. Their data do not indicate coordinate repression as each enzyme appears to change independently in response to uracil. It is not known whether the genes for these enzymes are linked or not in *E. coli*.

The use in nature of this latent capacity to make histidine enzymes in wild-type *Salmonella* is of interest. The function of the repression is not to decrease the amount of histidine synthesized when histidine is in the medium, since the enzyme levels are not repressed below the minimal medium level even by large amounts of histidine. The work of Yates and Pardee[3] and of Schwartz *et al.*,[27] however, suggests a function for this cellular capacity for making increased biosynthetic enzymes. Yates and Pardee investigated a uracil biosynthetic enzyme in *E. coli* and found that on a complete amino acid medium (which could support very rapid growth) the cells contained over five times as much enzyme as when they were grown on minimal medium. This increased synthesis was repressible by uracil. A similar case has been found in arginine biosynthesis by Schwartz *et al.* who found a six-fold increase in ornithine transcarbamylase in *E. coli* grown on an arginine-free enriched medium as compared to a minimal medium. Thus wild-type bacteria which are capable of growing with a doubling time of close to 15 min. in a highly enriched medium without histidine would presumably need much more of the histidine enzymes under these conditions than they would when growing on minimal medium with a doubling time of close to 40 min. Certain systems cannot be repressed below the minimal medium enzyme level (e.g., histidine, uracil[3]) while others can (e.g., arginine [4, 27]).

Another mechanism, distinct from enzyme repression, appears to regulate histidine synthesis at a substrate level when excess histidine is present in *Salmonella*. This mechanism, the inhibitory effect of the end product of a pathway on the activity of an early enzyme of the pathway, has been called "feedback control" and has been described by a number of investigators[28–34] in a variety of organisms. Moyed and Friedman[35] have presented some evidence that in *E. coli* histidine inhibits the activity of the first enzyme in the biosynthetic pathway and thus presumably prevents the synthesis of histidine when excess histidine is present in the medium. A feedback control in both *Salmonella* and *Neurospora* is indicated by the fact that histidine precursors do not accumulate before a genetic block when excess histidine is added to the minimal medium[26] through the specific activity of the histidine biosynthetic enzymes are not lowered.

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*AMINO ACID ACTIVATION AND TRANSFER TO RIBONUCLEIC ACIDS IN THE CELL NUCLEUS**

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The *in vitro* incorporation of amino acids into the proteins of calf thymus nuclei (isolated in isotonic sucrose) has been shown to be dependent upon the presence of ATP.[1, 2] In isolated nuclei ATP synthesis depends on an aerobic phosphorylating system which differs from that of mitochondria in being insensitive to various mitochondrial inhibitors (carbon monoxide, methylene blue, and calcium ions).