*STUDIES ON THE CONTROL OF PYRIMIDINE BIOSYNTHESIS IN HUMAN DIPLOID CELL STRAINS, I. EFFECT OF 6-AZAURIDINE ON CELLULAR PHENOTYPE**

By Leonard Pinsky† and Robert S. Krooth

LAWRENCE D. BUHL CENTER FOR HUMAN GENETICS, DEPARTMENT OF HUMAN GENETICS, UNIVERSITY OF MICHIGAN MEDICAL SCHOOL, ANN ARBOR

Communicated by James V. Neel, February 13, 1967

Hereditary orotic aciduria[1, 2] is a rare recessive disease characterized by a megaloblastic anemia of infancy and severe developmental retardation.[1–3] Its clinical and hematologic features respond to supplementation of the diet with uridine.[3] The disorder is associated with deficient activity of two enzymes which catalyze sequential steps in the *de novo* biosynthesis of uridine-5′-monophosphate (UMP) (Fig. 1). The enzymes affected are orotidine-5′-monophosphate (OMP) pyrophosphorylase and OMP decarboxylase.[4–6] Two other enzymes of the pathway (Fig. 1), aspartate transcarbamylase[4] and dihydroorotase (DHOase),[2, 3, 6] are not deficient. It is not yet known whether the gene affects dihydroorotic acid dehydrogenase.

This report is one of a series describing the behavior of cultured diploid cell strains grown in the presence of substances which inhibit specific enzymes in the synthetic

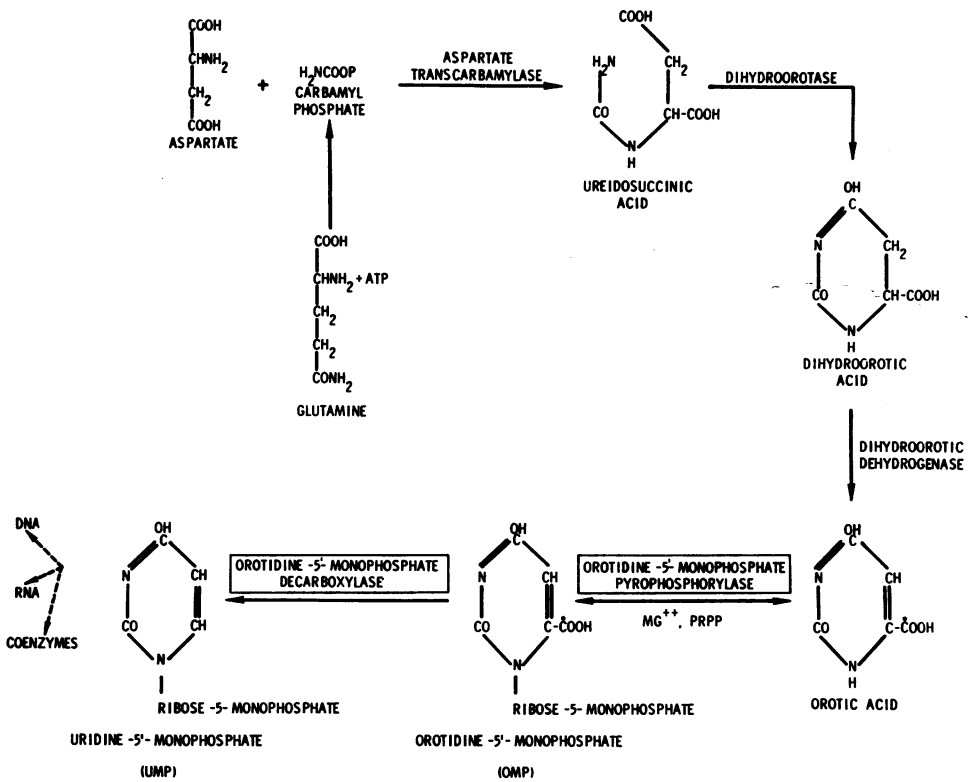

FIG. 1.—The *de novo* pathway of uridine-5′-monophosphate biosynthesis in mammalian cells.

pathway leading to UMP, or in the presence of certain precursors of UMP.[7] The cell strains were derived from individuals with each of the three Mendelian genotypes at the locus for orotic aciduria. The inhibitory substances were 6-azauridine-5'-monophosphate (6-AZUMP), whose riboside, 6-azauridine (6-AZUR), was employed, 5-azaorotic acid, and barbituric acid. After the cells were grown in the presence of each inhibitor, the cell protein was assayed for enzyme activity under conditions where the inhibition was overcome. In this paper we shall be concerned only with the effect of 6-AZUR.

Materials.—Cell strains: The orotic aciduric strain[5] (r*r*) was developed from the patient described by Becroft and Phillips.[3] The heterozygous strain[5] (Rr*) was derived from the brother of the first patient reported.[1] The RU strain[5] (RR) was used as a normal control.

Media: The composition of the basic cell culture media used in these experiments has been previously described.[5] "Nucleomedium" is an enriched medium containing nucleosides, whole sera, and a number of other ingredients. "Automedium" is the basic medium. It consists of Eagle's[8] minimum essential medium, supplemented with the nonessential amino acids and sodium pyruvate at the concentrations used by Eagle.[8] The solvent for the nutrients consists of 12% dialyzed pooled human sera in triply distilled water. The conditions for dialysis of the serum have been described elsewhere.[9]

Supplements added to automedium in specific experiments: Cytidine and uridine were obtained from Calbiochem (Los Angeles). 6-AZUR was obtained from the Sigma Chemical Co. (St. Louis). Tritiated uridine and thymidine were obtained from the Nuclear Chicago Co., and tritiated 6-AZUR from Schwarz BioResearch, Inc. (Orangeburg, N. Y.). 5-fluoroorotic acid was obtained from Hoffman-La Roche (Nutley, N. J.).

Other compounds: 6-AZUMP was obtained from Calbiochem (Los Angeles). The source of the reagents used in the enzyme assays is, with one exception, given elsewhere.[6] The orotidine-5'-monophosphate-7-C^{14} was obtained from the New England Nuclear Corp. (Boston). The specific radioactivity was 19 mc/mmole. The evidence of chemical and radiochemical purity was the same as previously described.[6]

Methods.—Cell culture: The methods employed for cell culture are given elsewhere.[5]

Preparation of cells for assay: As previously described[6] the cells were removed from the glass floor of the culture flask with trypsin, washed, and disrupted by ultrasonic energy. An aliquot of the sonic extract was used for determination[10] of cell protein. In experiments in which growth, but not enzyme activity, was measured, a slightly different method[11] was used for the estimation of cell protein.

Enzyme assays: The methods have been described in detail elsewhere,[6] except for the technique[12] used to collect $C^{14}O_2$. The assay for OMP decarboxylase measures the rate at which a cell extract releases $C^{14}O_2$ from OMP-7-C^{14}, while the assay for OMP pyrophosphorylase measures the release of $C^{14}O_2$ from orotic-7-C^{14} acid in the presence of excess purified yeast OMP decarboxylase, magnesium ions, and excess 5 phosphorylribose-1-pyrophosphate. Both assays were adapted to extracts of cultured cells from methods[4] originally developed for human hemolysates.

In each case the amount of substrate used was sufficient to saturate the enzyme: All reactions followed zero order kinetics, and the use of greater concentrations of substrate did not lead to higher estimates of reaction velocity. Even when the cells had been grown in the presence of 6-AZUR, the velocity of the decarboxylase reaction was not accelerated by the use of larger quantities of substrate. For this reason, it is believed that the concentration of substrate employed was sufficient to overcome the effects of any competitive inhibitor trapped within the cell at the time of extraction.

The relationship between the OMP decarboxylase activity of cultured cells and the protein concentration in the reaction mixture is not linear.[6] This phenomenon had been observed earlier by other workers when low levels of activity of the enzyme were measured in human erythrocyte lysates.[4] The linearity of the assay does not, however, appear to be influenced by either cellular genotype at the orotic aciduria locus or growth of the cells in the metabolic inhibitors we have employed; nor is it affected by dialysis or a variety of other treatments. Moreover, the departure from linearity, though significant, is small compared to the effect on enzyme activity of the mutant genotype and of growth in 6-AZUR. The effects on specific OMP decarboxylase

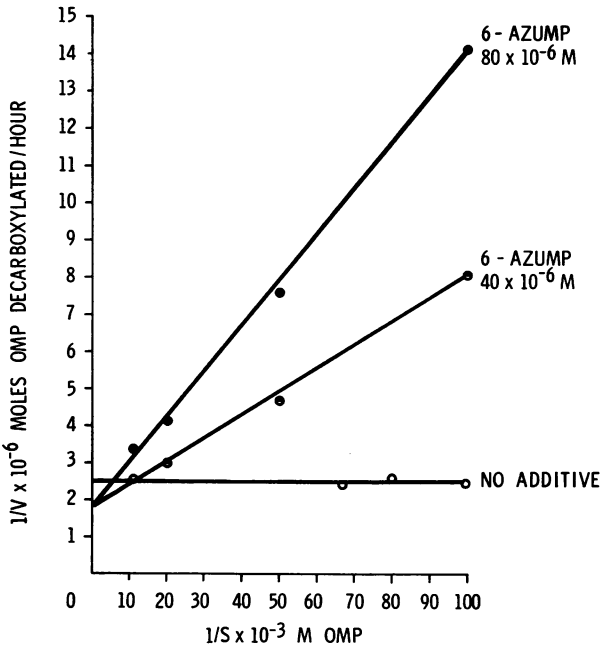

FIG. 2.—Lineweaver-Burk plot of the inhibition by 6-AZUMP of the OMP decarboxylase activity in an extract of cell strain RU(RR). Plot of the reciprocal of reaction velocity versus the reciprocal of substrate concentration in the presence of two concentrations of 6-AZUMP and in its absence.

activity of both cellular genotype and growth in 6-AZUR have been confirmed using a recently developed assay for OMP decarboxylase,[16] in which activity is a linear function of protein concentration.

It was noted previously[5] that specific OMP decarboxylase activity is positively correlated with the population density of the culture at the time of harvest. (It is still not known whether the correlation is causal.) This variable has been largely controlled by harvesting cultures at similar population densities.

Cultures of a single strain harvested at about the same population density but in different experiments showed considerable variation in specific activity (up to a factor of three)—enough to interfere with discrimination between normal and heterozygous cells. However, replicate flasks of cells inoculated from a single parent culture, fed on the same schedule and with the same lot of medium, and harvested simultaneously showed less than 10% variation in specific activity between flasks. Hence all experiments on the effects of metabolic analogues were performed on replicate flasks handled in this fashion. When two or more strains were used, the cells of each strain were simultaneously inoculated in the same lot of culture medium and at about the same initial population density. Thereafter the cells were fed on a common schedule with identical media, and were harvested at the same time. Under these conditions it has been possible to discriminate between normal and heterozygous cells on the basis of specific activity in all experiments thus far performed.

Starch gel electrophoresis: The methods used for electrophoresis and histochemical development of glucose-6-phosphate dehydrogenase,[13] phosphoglucomutase,[14] and the nonspecific esterases[15] are given in publications by other authors.

Incorporations of nucleosides into nucleic acids of mutant homozygous cells: Replicate cultures of mutant homozygous cells in 30-cm^2 culture flasks were placed in automedium containing 6×10^{-6} *M* cytidine, and, respectively, 6×10^{-6} *M* 6-AZUR-H^3, thymidine-H^3, or uridine-H^3, each

at 22 μc/μmole. After 3–4 generations of growth, the monolayers were washed three times with serum-free automedium, harvested, and centrifuged. The cell pellet was resuspended in cold 10% trichloracetic acid (TCA), and then stored at 4°C for 30 min. The acid-insoluble material was centrifuged, washed twice with 5% TCA, and collected on a Millipore membrane filter (mean pore size, 0.45 μ). The filter was washed with five 3-ml volumes of cold 5% TCA containing 1.5 mg/ml of the appropriate unlabeled nucleoside. The filters were dried, placed in 10 ml of counting medium, and radioactivity was measured in a liquid scintillation spectrometer.

Site of action of 6-AZUR: 6-AZUMP is known to be a competitive inhibitor of yeast OMP decarboxylase,[17] and has the same action on the OMP decarboxylase activity recovered from cultured human diploid cells,[18] as shown in Figure 2.

Results.—Growth experiments: 6-AZUR imposes on cells of all three genotypes a new nutritional requirement which is satisfied by either cytidine or uridine. A growth experiment on the two homozygous cell strains is summarized in Table 1. In the presence of 6-AZUR, and in the absence of a pyrimidine nucleoside, growth is not sustained.

Effect of growth in 6-AZUR on the specific OMP decarboxylase activity of the cell protein: Table 2 gives the results of an experiment in which cells of all three genotypes were grown for six days in the presence of 6-AZUR. As can be seen, each of the cell strains developed markedly increased OMP decarboxylase activity. The mutant homozygous cells developed levels of activity almost equal to those of normal cells growing in the absence of 6-AZUR.

The mechanism whereby 6-AZUR produces its effect was studied next. The first question was whether the augmented levels of catalytic activity reflected increased enzyme within the cell, or rather some enhancement of the catalytic activity of preformed enzyme. When 6-AZUR was added directly to cell extracts at a final concentration of 6×10^{-5} M, no effect on catalytic activity was observed. It will be recalled that 6×10^{-5} M was the concentration of 6-AZUR in the medium in

TABLE 1

EFFECT OF 6-AZUR ON THE GROWTH OF MUTANT AND NORMAL HOMOZYGOUS CELLS

	AUC Strain (r*r*), 6-Day Expt.		RU Strain (RR), 10-Day Expt.	
	Mg protein per 15-cm² flask	Growth factor[c]	Mg protein per 15-cm² flask	Growth factor[c]
Inoculum	0.05		0.01[b]	
Medium supplement:[a]				
None	0.315	6.30	0.452	45.2
Cytidine	0.415	8.30	0.402	40.2
6-AZUR	0.093	1.86	0.067	6.7
6-AZUR plus cytidine	0.440	8.80	0.432	43.2

[a] Each of the specified supplements was used at a final concentration of 6×10^{-5} molar.
[b] Because of the small size of the inoculum, we can only specify its order of magnitude.
[c] The growth factor is the final cell protein divided by the inoculum.

TABLE 2

EFFECT OF GROWTH IN 6-AZUR (0.06 mM) FOR SIX DAYS ON THE OMP DECARBOXYLASE ACTIVITY OF CELL STRAINS OF EACH GENOTYPE

Cell strain	Genotype	AC medium[a] supplement	Specific OMP decarboxylase activity[b]
RU	RR	None	15.8
	RR	6-AZUR	25.8
OFR	Rr*	None	2.2
	Rr*	6-AZUR	7.0
AUC	r*r*	None	0.4
	r*r*	6-AZUR	13.2

[a] AC medium is automedium plus 6×10^{-5} M cytidine.
[b] Millimicromoles of OMP decarboxylated per hour per mg cell protein.

experiments employing live cells. 6-AZUMP, as noted earlier, *inhibited* catalytic activity when added to the cell extract. When the inhibition was overcome by the addition of excess substrate, the activity obtained was equal to that of control extracts containing no 6-AZUMP. Activity beyond this level could not be recovered. Hence direct augmentation of preformed enzyme by 6-AZUR or 6-AZUMP could not be demonstrated in cell-free extracts. Neither 6-AZUR nor 6-AZUMP significantly affected the linearity of the assay for OMP decarboxylase, as noted previously.

Mixtures of extracts from cells grown, respectively, in the presence and absence of 6-AZUR yielded levels of activity which were approximately the arithmetic sum of the activities measured for each of the two extracts singly. Similarly, dialysis against 0.5 *N* NaCl in Tris buffer (pH 7.0) of extracts from cells grown, respectively, in the presence and absence of 6-AZUR did not lead to appreciable changes in specific activity in either case. We interpret these results as suggesting that the augmented OMP decarboxylase activity found in cells grown in the presence of 6-AZUR is due to increased enzyme within the cell.

Another question was whether 6-AZUR becomes incorporated (as 6-AZUMP) into a species of polynucleotide which, because of the abnormal base, in some fashion increases the OMP decarboxylase activity of the cell protein. This possibility was especially suggested by earlier work[19] showing that the wild-type phenotype could be restored to certain mutants of *E. coli* when they were grown in a medium containing 5-fluorouracil—a pyrimidine analogue known to be extensively assimilated into bacterial RNA. However, the following lines of evidence argue against the view that 6-AZUR must be incorporated into a nucleic acid in order to augment OMP decarboxylase activity: (1) Only a minute amount of the label from 6-AZUR-H^3 was incorporated into the TCA-insoluble precipitate of mutant homozygous cells (Table 3); a similar observation has been made by other workers on a heteroploid line of mammalian cells;[20] (2) the specific activity of catalase and DHOase, and the electrophoretic mobility of a number of other enzymes,[21] unrelated to pyrimidine biosynthesis, were unaltered by growth of the cells in 6-AZUR; (3) if 6-AZUR produced both the arrest of growth and augmentation of enzyme activity by virtue of its incorporation into polynucleotides, any factor which eliminated one of its actions might be expected to eliminate the other. However, it will be shown below that the addition of cytidine to the medium nullifies the arrest of growth produced by 6-AZUR without abolishing the ability of 6-AZUR to increase OMP decarboxylase activity.

Another line of evidence involves the use of 5-fluoroorotic acid as a pyrimidine analogue thought to act, partly, by being incorporated into RNA.[22] It is not known to be an inhibitor of an enzyme responsible for UMP synthesis. 5-Fluoroorotic acid did not confer increased OMP decarboxylase activity on cells grown in its presence, and equimolar cytidine could not prevent the arrest of growth it caused.

One of the simplest explanations for the augmented levels of OMP decarboxylase obtained following growth of the cells in 6-AZUR is that 6-AZUR inhibited UMP biosynthesis, and the consequent depletion of the cellular UMP then "derepressed" OMP decarboxylase. This argument has been suggested by other workers[23] to explain the augmentation of aspartate transcarbamylase in a heteroploid mouse

TABLE 3

INCORPORATION OF THYMIDINE-H^3, URIDINE-H^3, AND 6-AZUR-H^3 INTO NUCLEIC ACIDS OF MUTANT HOMOZYGOUS CELLS (AUC STRAIN, r*r*)

	Cpm/mg cell protein
Thymidine-H^3	1012
Uridine-H^3	5936
6-AZUR-H^3	95

Cells grown for 3 days in automedium plus 6×10^{-6} *M* cytidine supplemented with the specified nucleoside at a final concentration of 6×10^{-5} *M*. Specific radioactivity was 22 μc per μmole in each case.

TABLE 4

EFFECTS OF EQUIMOLAR CONCENTRATIONS (0.06 mM) OF CYTIDINE AND 6-AZUR ON THE SPECIFIC OMP DECARBOXYLASE ACTIVITY OF MUTANT HOMOZYGOUS CELLS (AUC STRAIN, r*r*)

Automedium supplement	Specific OMP decarboxylase activity[a]
None	0.32
6-AZUR	6.90
Cytidine	0.21
6-AZUR plus cytidine	7.34

[a] Millimicromoles of OMP decarboxylated per hour per mg cell protein.

line cultured in the presence of 6-AZUR. There are reasons[24] for believing that, in the case of aspartate transcarbamylase, their interpretation is correct. However, it should be noted that the medium (Table 2) in which our cells developed augmented levels of OMP decarboxylase contained 6×10^{-5} *M* cytidine as well as 6-AZUR. Cytidine at this concentration completely satisfies the nutritional requirement for a pyrimidine ribonucleoside imposed on cells of all genotypes by 6-AZUR (Table 1), and is also sufficient to stimulate maximal growth of mutant homozygous cells in medium which does not contain 6-AZUR.[15]

Hence we suspected that cellular depletion of the product of the pathway was not the mechanism by which cells grown in 6-AZUR developed augmented OMP decarboxylase activity. To test this suspicion further, replicate cultures of mutant homozygous cells were grown in 6-AZUR and in the presence and absence of cytidine. The results, shown in Table 4, indicate that cells grown in 6-AZUR developed about the same level of OMP decarboxylase activity, whether cytidine was present or not.

Effect of growth in 6-AZUR on the specific OMP pyrophosphorylase activity: Table 5 describes an experiment in which replicate cultures of cells of each of the three genotypes were simultaneously grown in the presence and absence of 6-AZUR. The cellular extracts were then assayed for both OMP pyrophosphorylase and OMP decarboxylase. Cells of each of the genotypes developed augmented levels of OMP pyrophosphorylase as well as of OMP decarboxylase. In this experiment, like the one described in Table 2, all cultures were grown in the presence of cytidine.

Discussion.—The fact that cells grown in 6-AZUR develop augmented levels of activity whether cytidine is present or not suggests that depletion of product is not the mechanism primarily responsible for augmentation. It does not prove this hypothesis, however. It could be that in the presence of 6-AZUR exogenous cytidine, though sufficient to satisfy the cell's nutritional requirements, leaves the cell with a diminished pool of UMP, or of some compound derived from UMP. Perhaps the cell does not synthesize UMP from cytidine as rapidly as it would normally (in the absence of 6-AZUR) synthesize UMP *de novo*, or perhaps there are several nucleotide compartments within the cell, and the nucleotides derived from exogenous cytidine do not equilibrate with all of them. We cannot directly rule such mechanisms out. Moreover, we have shown,[16] in the case of mutant homozygous cells, that a very high concentration of cytidine in the medium does dampen the level of augmentation caused by growth in 6-AZUR. The effect is not large, however. For example, in one experiment a 20-fold increase in the con-

TABLE 5

SIMULTANEOUS MEASUREMENT OF THE EFFECT OF GROWTH IN 6-AZUR ON THE SPECIFIC OMP DECARBOXYLASE AND OMP PYROPHOSPHORYLASE ACTIVITIES OF CELLS OF EACH GENOTYPE

Strain	Genotype	AC medium supplement	Specific OMP decarboxylase activity[c]	Specific OMP pyrophosphorylase activity[d]
RU	RR	None	10.6	3.65
	RR	6-AZUR[a]	13.9	7.30
OFR	Rr*	None	3.9	1.40
	Rr*	6-AZUR	9.4	8.52
AUC	r*r*	None	0.20	0.013
	r*r*	6-AZUR	3.0	0.305

[a] Concentration of 6-AZUR in the medium was 6×10^{-5} *M*. Cells were grown in experimental media for 6 days.
[b] AC is automedium plus 6×10^{-5} *M* cytidine.
[c] Millimicromoles OMP decarboxylated per hour of incubation per mg cell protein.
[d] Millimicromoles of OMP produced per hour of incubation per mg cell protein per hour.

centration of cytidine (to 1.2 mM) resulted in only about a 50 per cent reduction in the level of augmentation.

If the augmented levels of activity observed in cells grown in 6-AZUR are not due primarily to depletion of the cellular pool of UMP, then an alternative hypothesis is required. One possibility is that the cells are responding instead to an intermediate in the pathway which accumulates following the inhibition of OMP decarboxylase.

Summary.—Human diploid cell strains grown in a medium containing 6-azauridine develop augmented levels of both orotidine-5′-monophosphate (OMP) pyrophosphorylase and OMP decarboxylase. A cell strain from a patient whose genotype caused a deficiency of these two activities developed near normal levels when grown in 6-azauridine.

The data suggest that the augmented activity is due to increased enzyme within the cell. The mechanism of augmentation is probably not associated with the incorporation of an azapyrimidine into cellular nucleic acid. It also seems unlikely that the mechanism involves depletion of the cellular pool of pyrimidine nucleotides, though this possibility is difficult rigorously to rule out.

We are indebted to Dr. R. Rodney Howell, Department of Pediatrics, the Johns Hopkins School of Medicine, for performing the OMP pyrophosphorylase assays. Miss Era Hill assisted in the conduct of these experiments.

The following abbreviations are used: UMP, uridine-5′-monophosphate; OMP, orotidine-5′-monophosphate; DHOase, dihydroorotase; 6-AZUR, 6-azauridine; 6-AZUMP, 6-azauridine-5′-monophosphate (6-azauridylic acid); "R" denotes the normal allele at the orotic aciduria locus, and "r*" the mutant one.

* This work was supported by USPHS grant GM-09252.

† Holder of a fellowship from the Queen Elizabeth II Canadian Research Fund (1964–65). Present address: Department of Medical Genetics, Jewish General Hospital and Montreal Children's Hospital, Montreal, Quebec, Canada.

[1] Huguley, C. M., Jr., J. A. Bain, S. L. Rivers, and R. B. Scoggins, *Blood*, **14**, 615 (1959).

[2] Smith, L. H., C. M. Huguley, and J. A. Bain, "Hereditary orotic aciduria," in *The Metabolic Basis of Inherited Disease*, ed. J. B. Stanbury, J. B. Wyngaarden, and D. S. Frederickson (New York: McGraw-Hill Book Co., Inc., 1966), pp. 739–758, 2nd ed.

[3] Becroft, D. M. O., and L. I. Phillips, *Brit. Med. J.*, **1**, 547 (1965).

[4] Smith, L. H., Jr., M. Sullivan, and C. M. Huguley, Jr., *J. Clin. Invest.*, **40**, 656 (1961).

[5] Krooth, R. S., in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 29 (1964), p. 189.

[6] Howell, R. R., J. R. Klinenberg, and R. S. Krooth, *Johns Hopkins Med. J.*, **120**, 81 (1967).

[7] Pinsky, L., and R. S. Krooth, *Proc. III Int. Cong. Human Genetics* (Abstract 90, pp. 56–57) (1966).

[8] Eagle, H., *Science*, **130**, 432 (1959).

[9] Krooth, R. S., and A. N. Weinberg, *J. Exptl. Med.*, **113**, 1155 (1961).

[10] Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

[11] Oyama, V. G., and H. Eagle, *Proc. Soc. Exptl. Biol. Med.*, **91**, 305–307 (1956).

[12] Jeffay, H., in *Advances in Tracer Methodology*, ed. S. Rothchild (New York: Plenum Press, 1963), vol. 1, p. 113.

[13] Shows, T. B., R. E. Tashian, and G. Brewer, *Science*, **145**, 1056 (1964).

[14] Spencer, N., D. A. Hopkinson, and H. Harris, *Nature*, **204**, 742 (1964).

[15] Tashian, R. E., and M. W. Shaw, *Am. J. Human Genet.*, **14**, 295 (1962).

[16] Krooth, R. S., and L. Pinsky, unpublished observations (1966).

[17] Handschumacher, R. C., *J. Biol. Chem.*, **235**, 2917 (1960).

[18] The inhibition of human OMP decarboxylase by 6-AZUMP is not an entirely original discovery. It was strongly suggested by the work quoted earlier on yeast[17] and by studies using whole mammals (Pasternak, C. R., and R. E. Handschumacher, *J. Biol. Chem.*, **234**, 2992 (1959)) and whole human beings (Rabkin, M. T., E. W. Frederick, M. Lotz, and L. H. Smith, *J. Clin. Invest.*, **41**, 871 (1962); and Fallon, H. J., M. Lotz, and L. H. Smith, *Blood*, **20**, 700 (1962)).

[19] Champe, S. P., and S. Benzer, these PROCEEDINGS, **48**, 532 (1962).

[20] Schindler, R., and A. D. Welch, *Biochem. Pharmacol.*, **1**, 132 (1958).

[21] The enzymes tested were glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase, phosphoglucomutase, and a series of nonspecific esterases.

[22] Wagner, N. J., and C. Heidelberger, *Biochem. Biophys. Acta*, **68**, 78 (1963).

[23] Ennis, H. L., and M. Lubin, *Biochem. Biophys. Acta*, **68**, 78 (1963).

[24] Lacroute, F., thèse: "Regulation de la Chaine de Biosynthèse de l'Uracile Chez Saccharomyces Cerevisiae," Faculté des Sciences, Université de Paris (Juin 1966).