MOLECULAR MODELS FOR PHARMACOLOGICAL TOLERANCE AND ADDICTION*

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INTRODUCTION

General

In this paper, I shall summarize a series of experiments that measure the effect of several inhibitors of uridine-5′-monophosphate (UMP) synthesis on the cellular level of activity for enzymes in the synthetic sequence. The inhibitors were administered to human diploid cell strains, growing *in vitro*, and to whole rats. In both instances, the change in specific activity of the cell or tissue protein for enzymes in the UMP pathway was measured. At the conclusion of the paper, I shall try to relate some of the observations made to a molecular model, first proposed by Goldstein and Goldstein in 1961,[10] for pharmacologic tolerance and addiction.

THE SYNTHETIC SEQUENCE LEADING TO UMP IN MAMMALIAN CELLS

FIGURE 1 summarizes the synthetic sequence whereby human cells synthesize uridine-5′-monophosphate.[13,27,28] The pathway is identical to the one employed in *Escherichia coli*,[2] yeast,[20] rodents,[22] and cows.[1,14] In man there are at least two mutations that are known to affect enzymes in the sequence. Each causes a disease.

Type I hereditary orotic aciduria[25]† is characterized by failure to grow, pancytopenia, and the excretion of large quantities of orotic acid. All these features of the disease respond dramatically to dietary supplementation with uridine. Diploid cell strains from affected persons have about one percent of normal activity for the final two enzymes in the UMP sequence.[13,18] The enzyme (FIGURE 1) are orotidine-5′-monophosphate (OMP) pyrophosphorylase and OMP decarboxylase. At least two other enzymes in the pathway (dihydroorotase and dihydroorotic acid dehydrogenase) are not affected in cultured cells.[13,28]‡ Type II hereditary orotic aciduria[8] is clinically indistinguishable from the type I disease, but in type II orotic aciduria the level of OMP pyrophosphorylase appears to be normal while OMP decarboxylase is grossly de-

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†The nomenclature for these diseases was suggested by Dr. Lloyd H. Smith, Jr., of the Department of Medicine of the University of California Medical Center, San Francisco.

‡The first two enzymes of the sequence (aspartate transcarbamylase and dihydroorotase) have been shown to be unaffected by the gene for type I hereditary orotic aciduria in erythrocytes.[26]

ficient. Type I hereditary orotic aciduria is due to an autosomal recessive gene. The genetics of the type II disease are not yet known for certain, but it seems likely that this condition also is caused by a recessive Mendelian mutation.

The final three enzymes of the pathway can be specifically and competitively inhibited by analogues of their respective natural substrates. Dihydroorotic acid dehydrogenase is inhibited by barbituric acid, OMP pyrophosphorylase by 5-azaorotic acid, and OMP decarboxylase by 6-azauridine-5′-monophosphate.[23,24] It seems likely, however, that whole cells—or extracts to

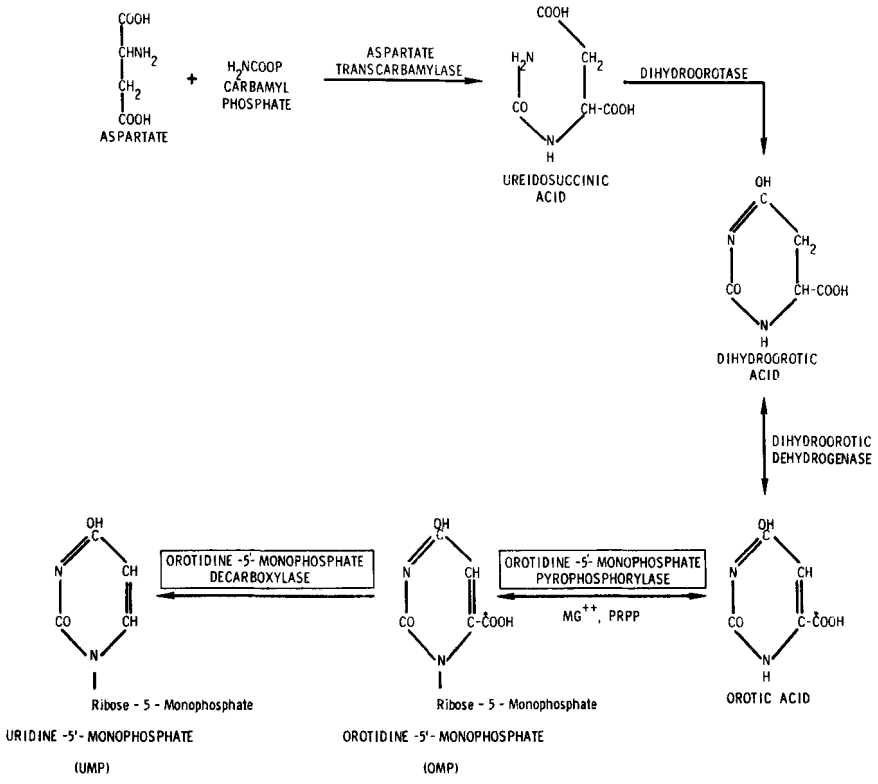

FIGURE 1. The synthetic sequence whereby human cells synthesize uridine-5′-monophosphate.

which PRPP and magensium have been added—convert quite significant amounts of barbituric acid and 5-azaorotic acid to the corresponding ribotides.[4,5,22]§ These ribotides then competitively inhibit OMP decarboxylase. The inhibition of the decarboxylase is probably the main action of all three of these drugs when they are administered to whole cells.

§PRPP refers to 5-phosphorylribose-1-pyrophosphate.

Methods and Materials

The cell strains, enzymatic assays, and tissue culture techniques employed in these studies have been described elsewhere.[19,23,28] In the experiments employing whole animals, adult Sprague-Dawley rats were given daily intraperitoneal injections of 6-azauridine (Sigma Chemical Co., St. Louis, Mo.) dissolved in 0.86% sodium chloride, or of the sodium chloride solution alone, for ten days. On the tenth day, all animals were sacrificed, and the specific OMP decarboxylase activity[28] of several different organs was measured.

Results

Experiments with Human Diploid Cell Strains

When diploid cells of any genotype are grown in the presence of barbituric acid, 5-azaorotic acid, or 6-azauridine, they develop increased levels of activity

TABLE 1

SPECIFIC OROTIDINE-5'-MONOPHOSPHATE DECARBOXYLASE ACTIVITY OF DIPLOID CELL STRAINS GROWN IN THE PRESENCE AND ABSENCE OF 1.2×10^{-3} M BARBITURIC ACID†

Cell Strain	Genotype at the Orotic Aciduria Locus‡	Additive to Reaction Mixture§	Additive to Growth Medium	Specific Orotidine-5'-Monophosphate Decarboxylase Activity¶
RU	RR	—	—	1.088
		Barbituric acid	—	1.052
		—	Barbituric acid	3.191
OFR	Rr*	—	—	0.614
		Barbituric acid	—	0.645
		—	Barbituric acid	1.997
AUC	r*r*	—	—	0.006
		Barbituric acid	—	0.006
		—	Barbituric acid	0.541

† The cell strains and the details of the experiment, except for the enzyme assay, are as previously described.[23] The assay has been modified somewhat, so that activity is now more nearly proportional to protein concentration. (The deviation from proportionality, with the present method, is only about 30% over a 1,000-fold range of protein concentrations.) The cells were grown in experimental media for 11 days prior to harvest. All media contained 6×10^{-5} M cytidine.

‡ R denotes the normal allele at the orotic aciduria locus and r* its mutant allele.

§ Barbituric acid either was not added at all or else was added either to the reaction mixture (at the start of the enzyme assay) or to the growth medium (one day after subculture). The final concentration of added barbituric acid was 1.2×10^{-3} M in either incubation mixture or growth medium.

¶ Millimicromoles orotidine-5'-monophosphate decarboxylated per hour of incubation per milligram cell protein.

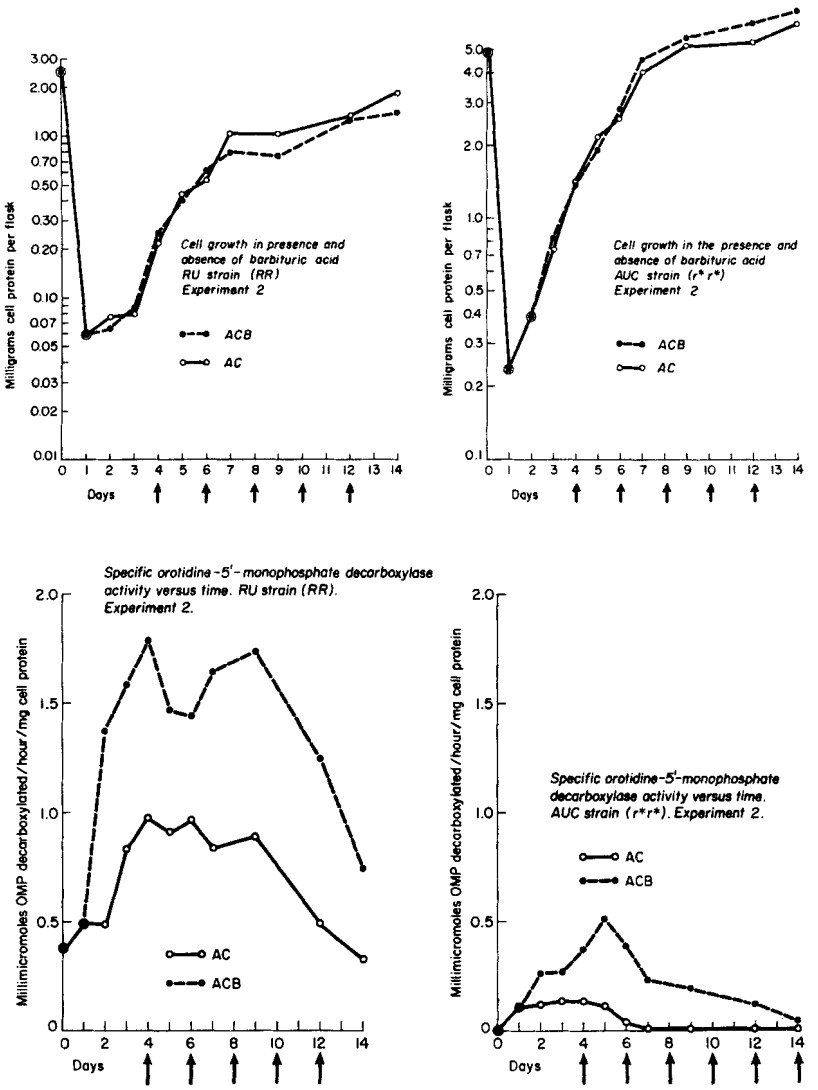

FIGURE 2. The change in specific OMP decarboxylase activity during the growth of two human diploid cell strains. The cells were subcultured on day zero. The cell protein and the OMP decarboxylase activity were measured in the parental flask on this day. On subsequent days, cell protein and OMP decarboxylase activity were measured in the daughter cultures. The arrows beneath the abscissa denote days on which the medium was changed. The concentration of barbituric acid and cytidine were, respectively, 1.2×10^{-3} M and 6×10^{-5} M. R denotes the normal allele at the type I orotic aciduria locus and r* the mutant allele. Cells of both genotypes were grown, harvested, and assayed concurrently. Top describes the growth of the cell strains, and bottom the change in specific OMP decarboxylase activity.

for the final two enzymes of the UMP sequence.[17,23,24] The cellular level of at least two other enzymes in the sequence are, under precisely the same conditions, unaffected by these drugs. The affected enzymes (FIGURE 1) are OMP pyrophosphorylase and OMP decarboxylase. TABLE 1 shows an experiment in which cell strains of each of the three Mendelian genotypes at the type I hereditary orotic aciduria locus were grown in the presence of barbituric acid. At the conclusion of the experiment, the specific OMP decarboxylase activity of the cell protein was measured. Note that although barbituric acid stimulates cells of all three genotypes to develop increased levels of enzyme activity, the *proportionate* effect is much greater in the case of the mutant strains. The mutant homozygous cells develop levels of activity equal to those of heterozygous cells grown in the absence of barbituric acid. In some experiments, the orotic aciduria cells develop nearly normal levels of activity. Similar data have been obtained for the second enzyme responsive to these inhibitors—OMP pyrophosphorylase. The effect of growth in 5-azaorotic acid or 6-azauridine is essentially the same as the effect of growth in the presence of barbituric acid.

FIGURE 2 shows the kinetics of development of specific OMP decarboxylase activity in a normal and in a mutant (at the type I locus) homozygous strain. Note that as the cells form a confluent monolayer, the specific OMP decarboxylase activity tends to fall. The increase in enzyme activity due to the drug is, for some reason, not sustained. Rather, the drug causes an increase in the amplitude of the spike in enzyme activity that occurs spontaneously during growth.

TABLE 2 contrasts the response of two cell strains to barbituric acid. One of the strains is from a patient with type I hereditary orotic aciduria and the other is from a patient with type II disease. Both cell strains develop increased levels of OMP decarboxylase activity when barbituric acid is present in the medium. The effect is noticeably smaller in the case of the strain from the patient with type II disease, but insufficient experiments have been done to determine whether this difference will prove consistent. It is not yet known how barbituric acid influences the OMP pyrophosphorylase activity of the strain from the patient with type II disease.

TABLE 2

EFFECT OF GROWTH IN BARBITURIC ACID ON THE SPECIFIC OMP DECARBOXYLASE ACTIVITY OF TWO MUTANT HUMAN DIPLOID CELL STRAINS†

Cell Strain	Genotype	Medium Supplement	Specific OMP Decarboxylase Activity
AUC	$r_1^*r_1^*$	—	0.01
AUC	$r_1^*r_1^*$	Barbituric acid	0.50
SAV	$r_2^*r_2^*$	—	0.02
SAV	$r_2^*r_2^*$	Barbituric acid	0.204

† The conditions of this experiment are identical to those for the experiment in TABLE 1, except that in this case the cells were grown in the specified media for 7 rather than 11 days and the media contained fetal calf rather than human sera. In this Table, r_1^* denotes the gene for type I hereditary orotic aciduria and r_2^* denotes the gene for the type II disease.

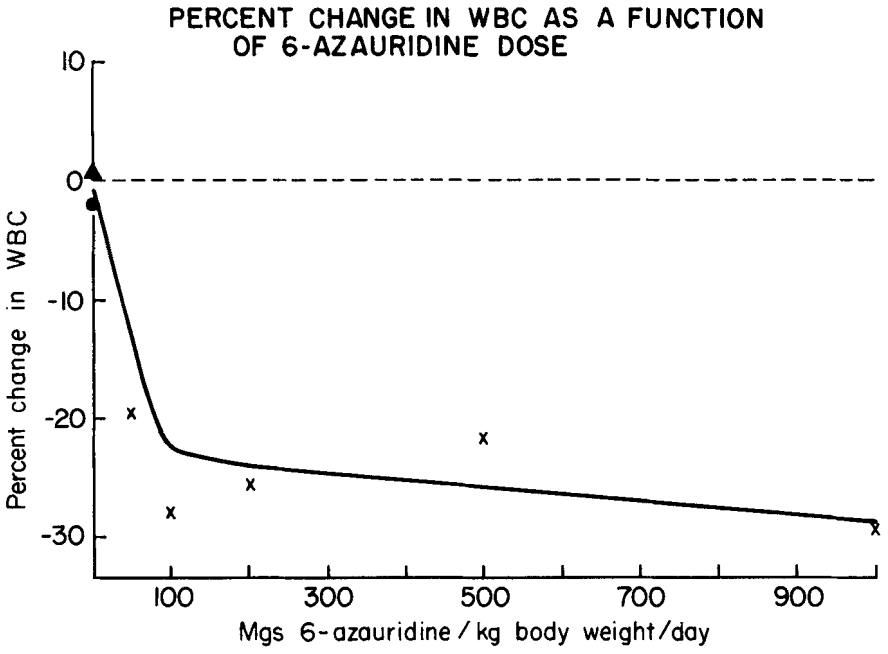

FIGURE 3. Adult male Sprague-Dawley rats were given daily intraperitoneal injections of the specified dose of 6-azaurdine for ten days. The percent change in the white blood is shown on the ordinate, and is defined as:

$$\frac{\text{WBC on day 10} - \text{WBC prior to initial dose of drug}}{\text{WBC prior to initial dose of drug}} \times 100.$$

▲ denotes an animal that received no injections and • an animal who received injections of a saline solution (0.86% NaCl in distilled water) free of drug.

Experiments with Whole Rats

Rats develop a variety of abnormalities in response to 6-azauridine. The symptoms include weight loss, depilation, anemia, and leukopenia. FIGURE 3 shows the white blood count as a function of the dose of 6-azauridine administered. FIGURE 4 describes the increase in specific OMP decarboxylase activity, ascribable to 6-azauridine, in a variety of tissues obtained from an animal that received a daily dose of 1,000 mg/kg of the drug for ten days. The increase varied from 1.2-fold for skeletal muscle (quadriceps femoris) to slightly over 3-fold for cardiac muscle. The low level of response of skeletal muscle, compared with the other tissues, has been observed in several experiments.¶ FIGURE 5 is a plot of specific spleen OMP decarboxylase activity as a function of the quantity of 6-azauridine administered. Very similar curves were obtained for the other tissues studied except skeletal muscle.

Thus, the tissues of the rat, like human diploid cell strains, appear to de-

¶The response of skeletal muscle is of the same order of magnitude as the variation between different animals and is not significantly different from no response.

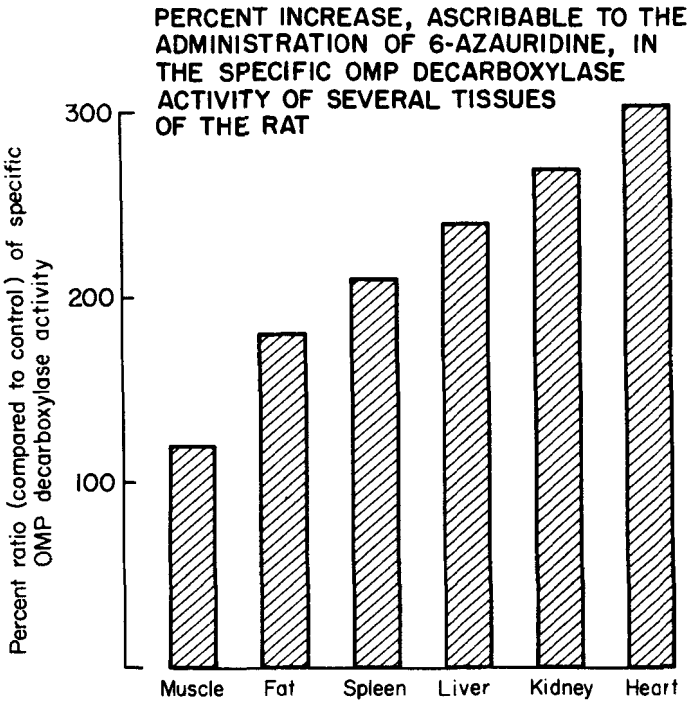

FIGURE 4. The experimental conditions are identical to those described in FIGURE 3. The same animals were used. The percent ratio of specific OMP decarboxylase activity is defined as:

$$\frac{\text{OMP decarboxylase activity on day 10 of specified tissue from animal receiving 100 mg/kg of 6-azauridine}}{\text{OMP decarboxylase activity on day 10 of corresponding tissue from animal receiving saline injections}} \times 100.$$

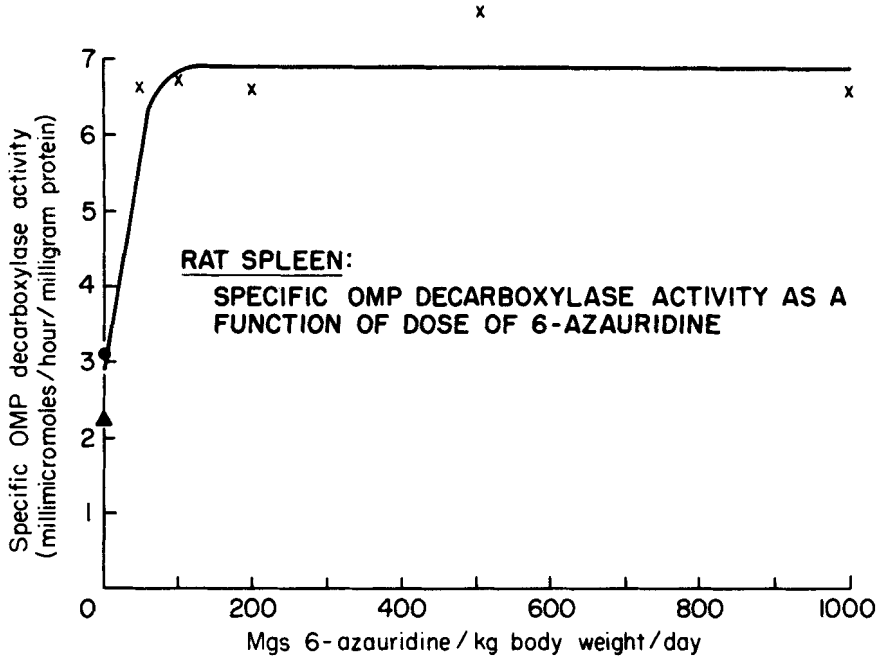

FIGURE 5. Experimental conditions are identical to those described for FIGURE 3.

velop increased levels of OMP decarboxylase activity in response to 6-azauridine—a compound whose immediate metabolite (6-azauridine-5′-monophosphate) inhibits OMP decarboxylase. Preliminary experiments strongly suggest that the increase in enzyme activity can be demonstrated even when the animals are given concurrently both 6-azauridine and the product of the UMP pathway (as cytidine) in amounts sufficient to prevent measurable leukopenia.

DISCUSSION

Mode of Action of the Inhibitors

The mechanism whereby inhibitors of UMP synthesis stimulate diploid cell strains to develop increased levels of OMP pyrophosphorylase and OMP decarboxylase activity is not known.

When 6-azauridine, barbituric acid, or 5-azaorotic acid, is added to cell-free extracts, the agent has no effect on OMP decarboxylase activity.[23,24] Of course, 6-azauridine-5′-monophosphate competitively inhibits the decarboxylase, but when the inhibition is overcome with excess substrate, the level of activity is the same as when 6-azauridine-5′-monophosphate is not present.[24] It seems likely, therefore, that the increase in cellular enzyme activity in response to these compounds is not due to their effect on the catalytic properties of preformed enzyme molecules. The amount of enzyme in the cell

is probably increased. If that is indeed true, the next question is whether the increased quantity of enzyme reflects accelerated synthesis or decelerated breakdown. It should be emphasized that both possibilities are admissible. The protein of cultured mammalian cells turns over quite rapidly.[21] Moreover, at least some of the inhibitors employed tend to stabilize OMP decarboxylase at high temperatures[1] or in the presence of high concentrations of urea.[15] However, for the argument to be described in the next section of this paper, it does not really matter whether accelerated enzyme synthesis or decelerated breakdown is responsible for the augmented levels of activity caused by the drug.

Another question concerns the chemical stimulus to which the cells are responding when they develop increased levels of activity in the presence of the drug. One possibility, already mentioned, is that the response is due to a stabilization of the enzymes by the drugs themselves. At the moment, it does not seem likely that such a mechanism is the sole factor accounting for the increase in the level of enzyme activity caused by the drugs. For one thing, the drugs are so dissimilar: 6-azauridine-5′-monophosphate differs from the product of the final enzyme of the sequence by a 6-aza-substitution; 5-aza-orotic acid differs from the *substrate* of the final enzyme of the sequence by a 5-aza-substitution. Barbituric acid resembles orotic acid, the substrate of the penultimate enzyme of the sequence; it has *no* aza-substitution. Hence, it seems a little more likely that the three drugs have similar effects on the enzyme activity of whole cells because they each inhibit the synthesis of UMP. This surmise is reinforced by the observations, to be discussed later, that when natural substrates and products, such as orotic acid or uridine, are added to the medium, they have only a very small effect on the cellular level of enzyme activity.

If the drugs cause cells to develop increased activity for the final two enzymes of the UMP sequence *because* the drugs inhibit UMP synthesis, another question arises: By what mechanism does this occur?

When an enzyme in a synthetic sequence is inhibited, the cell presumably accumulates substrate and becomes depleted of product. Hence, two possibilities are that the increase in cellular enzyme activity seen in the cultured cells is a response to depletion of product or to the accumulation of an intermediate in the pathway. These possibilities are, of course, not exhaustive or mutually exclusive. Of the two, however, the latter—the response to accumulated intermediate—seems more likely to be correct. Several lines of evidence tend to support this conjecture.[16,17] For one thing, the augmentation of enzyme activity in the presence of the inhibitors is observed even when the medium contains the *product* of the pathway (as cytidine). The amount of cytidine added to the medium is more than sufficient to satisfy the nutritional requirement for product imposed on the cells by the inhibitor.[23] Second, when intermediates, such as orotic acid, in the synthetic sequence are added directly to the medium (which also contains product), a twofold increase in cellular OMP decarboxylase activity is observed. The absolute magnitude of this effect, however, is *much* smaller than the effect of the inhibitors and can be detected only in mutant homozygous cells.[24] Third, when cells are grown in the presence of an inhibitor of one of the early enzymes of the sequence, they develop decreased levels of OMP decarboxylase, suggesting that an intermediate in the pathway is necessary for the maintenance of activity.[17] It should be added, however, that the absolute magnitude of this effect, like the effect of orotic acid, is not large and is observable only in the case of mutant homozygous cells. Fourth, Lacroute[20] has shown in the case of another eucaryotic

cell (*Saccharromyces*) that an intermediate in the sequence—dihydroorotic acid—stabilizes or induces the terminal enzymes of the pathway.

Another question concerns why *two* enzymes respond to these inhibitors and are affected by the gene for type I orotic aciduria. One possibility, of course, is that the two enzymes are encoded for in an operon[7,19] or in loci that are transcribed in a polycistronic message. Still another possibility is that the two catalytic activities reside in a single protein, although this perhaps is unlikely since, after purification, the activities recovered from calf thymus are separable by starch-gel electrophoresis.[14] A third possibility, and one that I favor at present, is that the two activities reside in separate proteins but that the two proteins are normally aggregated in a supramolecular complex inside the cell. In this case, for example, a structural mutation in one of the enzymes might alter the complex in a way that affects the catalytic activity of both enzymes—or perhaps affects their stability inside the cell. There are observations, which I shall not review here, to support such a formulation, and there are numerous precedents for it in the biology of eucaryotic fungi.[6,9,11] In any case, the inhibitors might act by causing an intermediate, which stabilizes the enzyme complex, to accumulate.

Molecular Models for Pharmacologic Tolerance and Addiction

Goldstein and Goldstein[10] proposed the following model for pharmacologic tolerance and addiction: If a drug inhibiting a particular enzyme in a biosynthetic sequence is administered, the subsequent deficiency of product may lead to derepression of the enzymes of the sequence. Assuming the pharmacologic effect of the drug is due to its enzyme-inhibiting action, larger and larger doses may be necessary to obtain a fixed response. It also follows from this model that abrupt withdrawal of the drug may temporarily cause the process to go too fast. At the moment of withdrawal, the cells of the organism may have very high concentrations of the enzymes in the sequence and also of intermediates that have accumulated proximally to the site of inhibition.

Goldstein and Goldstein[10] applied their model to a metabolic pathway, the synthesis of whose enzymes was regulated by end-product repression. The basic argument is very general, however, and can be applied equally to other kinds of control of the amount of enzyme inside a cell. It does not matter, for example, whether the quantity of enzyme increases in response to the drug because of end-product derepression or because the enzyme is induced by the accumulation of substrate. The argument holds equally well in either case. It also is irrelevant whether the drug leads to induction of all the enzymes in the metabolic sequence or of just one of them; nor does it matter whether the sequence is a synthetic or a catabolic one. Finally, the logic of the model is independent of whether the increased quantity of enzyme in the cell is due to induction—that is, preferential stimulation of enzyme synthesis—or enzyme stabilization. For example, if the large quantities of accumulated substrate protect the enzyme from denaturation or from the action of cellular proteases, the model can be applied just as logically as if the substrate induces the enzyme. Indeed, if the drug itself induces or stabilizes the enzyme, the model is still applicable, provided only that the increase in the amount of cellular activity caused by the drug is quantitatively greater than the enzyme inhibition effected by the drug. In other words, to provide a model for tolerance, one must assume that the drug eventually causes the cell to contain more catalytic activity than the drug can inhibit.

The experiments reported in this paper suggest that when a compound whose sole action appears to be the inhibition of a known enzyme is administered to human diploid cell strains of several genotypes, the cultures develop increased levels of activity of the inhibited enzyme and of a metabolically adjacent one as well. In the case of one genotype, the increase is over 100-fold. The mechanism responsible for the increase is unknown, but it may well reflect the stabilization of an enzyme complex-containing both catalytic activities—by an accumulated precursor in the pathway. The accumulation would, of course, be secondary to the enzyme inhibition.

When a compound whose immediate metabolite is an inhibitor of the final enzyme of the sequence is administered to whole rats, the specific activity of the enzyme inhibited rises in a variety of tissues.

These experiments, it seems to me, provide some support for the model proposed by Goldstein and Goldstein,[10] for their model requires that mammalian cells respond to an enzyme inhibitor by increasing the amount of activity they contain for the inhibited enzyme. At least in the case considered here, that seems to happen.

The experiments described do not, of course, prove that the model is correct. They merely confirm one prediction. Other mechanisms for tolerance are also likely to be operative in some cases. For example, the administration of barbiturates stimulates the liver to develop greatly increased levels of activity for an enzyme capable of conjugating these drugs.[3] However, this need not be the only basis for the development of tolerance to the barbiturates, nor can such a mechanism easily explain the development of objectively measurable withdrawal symptoms. The fact that the symptoms of drug withdrawal tend in some cases to be the opposite of those ascribable to the drug also, incidentally, lends support to the Goldstein and Goldstein model.

The model may in addition serve to explain, in certain cases, the acquisition by a tumor cell population of resistance to a chemotherapeutic agent. Such resistance need not always reflect mutation and selection. In other words, the escape of a neoplastic population from a chemotherapeutic agent might in some instances reflect the same kind of process as the development of tolerance by a patient who has experienced the unremitting administration of a narcotic or barbiturate.[15,19] If such a mechanism actually operated, it would have two important practical consequences. For one thing, the resistance would presumably be as likely to develop in a normal cell as in a neoplastic one. Hence, the patient might be able to tolerate a dose of drug late in therapy that he could not tolerate initially. Second, the sequential administration of chemotherapeutic agents to which the cell can respond in this fashion would be likely to lead to *longer* clinical remissions than their simultaneous administration. One might expect a priori that a cell could acquire this kind of resistance to several agents concurrently.

Summary

In preparing this paper, I have been chiefly concerned to do four things. First, like other authors in this monograph, I wished to illustrate the principle that the response to drug is influenced by genotype. Second, I wanted to call the Goldstein and Goldstein model for pharmacologic tolerance and addiction to more general attention and to point out that mammalian cells (at least in certain circumstances) appear to behave in a way consistent with the logic of the model. Third, I wished to develop an argument to support the following

recommendation: Whenever a drug known to inhibit a specific enzyme is administered, the level of activity of that enzyme should, when feasible, be followed during the course of therapy.[12] Finally, I wished to emphasize the possible importance for the whole organism of autonomous cellular responses.

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