3S γ_1 and the 3S γ_1-globulins fused completely with that formed between antiserum and purified erythrocyte carbonic anhydrase B (Fig. 1b). On immunoelectrophoresis (*10*) with antiserum to 3S γ_1, the mobility of the reacting 3S γ_1-globulin was identical to that for carbonic anhydrase B (Fig. 1c).

Thus, although the plasma 3S γ_1-globulins included a small amount of a protein antigenically related to the immunoglobulins as reported earlier (*2*), most of the γ-globulin in this class of proteins was not related to γG or to γG light chains but was identical or very similar to carbonic anhydrase B, thus explaining the difference in peptide composition noted (*2*) between 3S γ_1 plasma fractions and the immunoglobulins as well as the similarity between an amino-terminal tetrapeptide obtained from the 3S γ_1-globulins and that of carbonic anhydrase B (*11*). In accord with this, the amino acid composition of whole 3S γ_1-globulin preparations is quite unlike that of the immunoglobulin light chains (*1*), but is identical, within the limits of error of the methods, with that reported for carbonic anhydrase B by others (*9*).

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Dihydroorotic Acid Dehydrogenase Activity of Human Diploid Cell Strains

Abstract. *A gene affecting the final two enzymes of uridylic acid biosynthesis does not affect a third, metabolically adjacent enzyme. Similarly, compounds that increase cellular activity for the affected enzymes do not increase activity for the third enzyme. The pyrimidine pathway can be subdivided into groups of concurrently responding enzymes. These groups may be smaller in human cells than they are in microbial cells.*

Dihydroorotic acid dehydrogenase (DAD, E.C. 1.3.3.1), one of the enzymes in the uridine biosynthetic pathway, catalyzes the reversible conversion of dihydroorotic acid to orotic acid (Fig. 1). This enzyme is present in circulating human leukocytes but absent from red blood cells (*1*). Both erythrocytes (*2–4*) and cultured diploid cell strains (*5, 6*) from patients with orotic aciduria, a rare autosomal recessive disorder, are deficient in two sequentially acting enzymes in the biosynthetic pathway (Fig. 1) leading to uridine-5′-monophosphate (UMP). These enzymes are orotidine-5′-monophosphate (OMP) pyrophosphorylase (E.C. 2.-4.2.10) and OMP decarboxylase (E.C. 4.1.1.23); diploid cell strains from mutant homozygous donors show 1 percent of normal activity for both enzymes. When mutant homozygous cultures are grown in a medium containing either 5-azaorotic acid (a competitive inhibitor of OMP pyrophosphorylase) or 6-azauridine (whose ribotide is a competitive inhibitor of OMP decarboxylase), the cells develop nearly normal activity for both enzymes (*7, 8*). Barbituric acid, which is not an inhibitor of either enzyme, also causes mutant homozygous cells to develop nearly normal activity (*8*). The response of normal and heterozygous cells to these agents is similar to that of the mutant homozygous strains, but in both enzymes the proportionate increase is smaller. The other two enzymes peculiar to this pathway (Fig. 1), aspartate transcarbamylase (E.C. 2.1.3.2) and dihydroorotase (E.C. 3.5.2.3), which catalyze consecutive reactions immediately preceding the one which DAD catalyzes, are not deficient in mutant cells (*2, 3, 6*). Moreover, cellular dihydroorotase activity does not rise in response to at least one of the agents which increases OMP pyrophosphorylase and OMP decarboxylase activity (*8*). The effect of the gene for orotic aciduria, or of the compounds mentioned above, on the activity of DAD has not been reported. We have therefore sought answers to the following questions. (i) Does the gene for orotic aciduria affect DAD activity? (ii) Do human cells increase their DAD activity in response to the compounds that

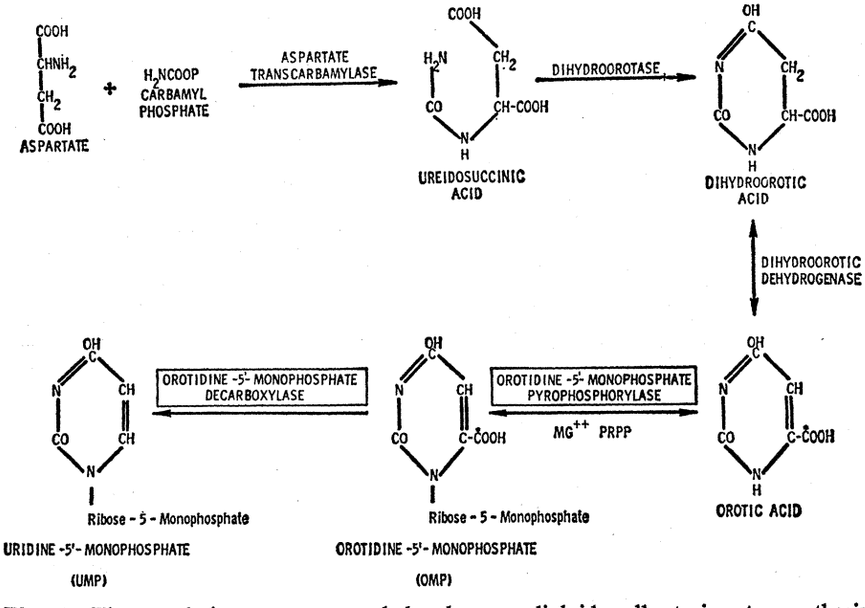

Fig. 1. The catalytic sequence used by human diploid cell strains to synthesize uridine-5′-monophosphate.

Table 1. The inhibition in vitro of the activity (nmole mg^{-1} hr^{-1}) of dihydroorotic acid dehydrogenase by barbituric acid. Cells were grown in nucleomedium. The symbol r^* denotes the gene for orotic aciduria, and R its normal allele. Experiments 1 and 2 were performed on separate sets of replicate cultures and show the variation between experiments.

Cell strain	Specific activity			
	Expt. 1: Barbituric acid		Expt. 2: Barbituric acid	
	None	25 μmole	None	25 μmole
RU(*RR*)	10.5	4.0	11.5	5.9
OFR(*Rr**)	15.7	5.4	17.4	6.1
AUC(*r*r**)	9.3	4.2	11.7	5.2
PIE(*r*r**)	8.1	3.7	10.9	4.1

augment cellular activity for OMP pyrophosphorylase and OMP decarboxylase? (iii) Is barbituric acid a competitive inhibitor of the DAD activity of human cells?

We used a normal strain (RU), a heterozygous strain (OFR), and two strains (AUC and PIE) mutant homozygous at the orotic aciduria locus (*5*, *6*). The cells were routinely propagated in "nucleomedium" (*5*), which contains whole human serums (12 percent) and many different low-molecular-weight nutrients. To measure the effect on cell enzyme activity of additives to the medium, we grew the cultures in automedium (*5*), in which the whole serums are replaced by dialyzed serums, and the number of nutrients is reduced. Automedium is devoid of detectable quantities of pyrimidines and purines and of their derivatives. Both media are based mainly on the one described by Eagle (*9*). All cultures were harvested shortly after the formation of a confluent monolayer; cells of each strain were always grown and processed concurrently (*5*, *6*).

The assay for DAD activity was based on the method described by Beckwith *et al.* (*10*). The reaction mixture contained (in a final volume of 1.0 ml) 0.25 mmole of tris buffer (*p*H 8.6), 1 μmole of 1-dihydroorotic acid, and a quantity of crude cell extract which usually corresponded to about 0.5 mg of cell protein. After 5 hours, the reaction was stopped, and the protein was precipitated by the addition of 0.1 ml of 35 percent perchloric acid (*11*). The precipitate was removed by centrifugation at about 1500 g for 10 minutes, and the optical density, at 280 nm, of the supernatant was measured with a Gilford model-2000 spectrophotometer. The millimolar extinction coefficient of authentic orotic acid was 6.5 for an optical path of 10 mm. Specific activity was expressed as the number of nanomoles of orotic acid synthesized per hour of incubation per milligram of cell protein. The activity of OMP decarboxylase was assayed by a modification of the method described previously (*6*). Dihydroorotase was measured as before (*6*).

The DAD activity, assayed as described above, is associated with a nondialyzable fraction; it is destroyed by boiling the cell extract for 10 minutes. The reciprocal of the reaction velocity is a linear function of the reciprocal of substrate concentration. When substrate is present in excess, the amount of activity is directly proportional to time, for at least 5 hours, and also to the concentration of cell protein in the reaction mixture. Finally, hemolyzates from normal individuals have no significant activity by this assay, although the same hemolyzates yielded high activities for two other enzymes of the UMP pathway (OMP decarboxylase and dihydroorotase). This result confirms (by a different assay method) the report cited earlier. The addition of nicotinamide-adenine dinucleotide or cysteine at concentrations of $10^{-4}M$ and $10^{-3}M$ respectively, did not measurably influence the velocity of the reaction in both crude and dialyzed tissue culture cell extracts.

For cells grown in nucleomedium, the mutant gene for orotic aciduria has little, if any, effect on the cellular specific activity for DAD (Table 1). In each strain, activity is inhibited by the addition of 25 μmole of barbituric acid to the reaction mixture. We have observed, at least in the case of extracts of normal cells, that the inhibition is competitive with substrate and that it obeys the Lineweaver-Burk relation (*12*). Since this same kind of inhibition has been noted with purified bacterial enzyme and barbituric acid (*13*), our observation is further evidence that the assay we have used measures authentic enzyme activity. In addition, it shows that barbituric acid, like the other compounds that cause human cells to increase their specific activity for OMP pyrophosphorylase and OMP decarboxylase, is a presumptive inhibitor of UMP synthesis.

For cells growing in automedium supplemented with cytidine, the activity of only the decarboxylase is correlated with genotype (Table 2). In this experiment the mutant homozygous AUC strain had, if anything, elevated activities of DAD, but this result has not been observed in other experiments with AUC cells (Table 1). The three enzyme inhibitors which cause cells of all genotypes to develop increased OMP decarboxylase activity had little or no effect on specific DAD activity: 6-azauridine apparently caused a fall in DAD activity; 5-azaorotic acid had no consistent effect on DAD; barbituric acid may have caused a slight increase in DAD activity, but, at least in the mutant strains, the increase was negligible compared to the increase in OMP decarboxylase activity (*14*).

The chemical sequence by which the human cell synthesizes UMP appears identical to the one used by other microorganisms in which pyrimidine synthesis *de novo* has been studied. In

Table 2. The effect of barbituric acid, 5-azaorotic acid, and 6-azauridine in vivo on the specific activity of dihydroorotic acid dehydrogenase and OMP decarboxylase in diploid human cell strains. Cells were grown in the experimental media for 8 days. A denotes automedium. It consists of Eagle's minimal essential medium with dialyzed human serums (12-percent), nonessential amino acids, and sodium pyruvate. AC is automedium supplemented with 0.06 m*M* cytidine. The results for cells grown in this medium are in italics. ACB is AC medium supplemented with 1.2 m*M* barbituric acid; ACO is AC medium supplemented with 0.07 m*M* 5-azaorotic acid; ACU is AC medium supplemented with 1.2 m*M* 6-azauridine.

Medium	Specific activity	
	Dihydroorotic dehydrogenase†	OMP decarboxylase‡
	RU(*RR*)	
AC	*9.9*	*2.697*
ACB	14.1	4.662
ACO	12.3	3.987
ACU	5.1	4.173
	OFR(*Rr**)	
AC	*12.4*	*0.911*
ACB	13.6	2.573
ACO	10.4	2.436
ACU	8.8	4.208
	AUC(*r*r**)	
AC	*20.5*	*0.012*
ACB	26.8	1.209
ACO	15.4	1.877
ACU	12.0	1.524
	PIE(*r*r**)	
AC	*9.0*	*0.008*
ACB	10.9	1.011
ACO	10.7	1.148
ACU	8.5	1.440

† Units as in Table 1. ‡ Nanomoles of OMP decarboxylated/hr incubation/mg cell protein.

Escherichia coli, all five enzymes of the pathway are repressed by uracil, but the repression is coordinate only in the case of the last four (*10*). In yeast, the first enzyme of the pathway is repressed by uracil, while the second is induced by its own substrate, and the final three are believed to be induced by dihydroorotic acid (*15*). Our data suggest that, for cultured human cells, the final two enzymes are affected by a Mendelian mutation and respond concurrently to several inhibitors of the UMP pathway. Neither the gene nor the inhibitors significantly affect the activity of DAD. Hence in the human cell the last two enzymes of the pathway may respond separately from the enzyme that immediately precedes them.

However, we must emphasize that: (i) Because of imprecision in the assays we have not shown that OMP decarboxylase and OMP pyrophosphorylase are exactly coordinate. (ii) The molecular mode of action of the gene for orotic aciduria is not known. (iii) We have not shown that the increase in OMP pyrophosphorylase and OMP decarboxylase activity, in response to the inhibitors, is due to enzyme induction. We do not know whether it reflects a preferential stimulation enzyme synthesis, or, say, a decelerated rate of enzyme destruction. In cultured human cells these two possibilities are particularly difficult to distinguish (*16*).

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14. In this experiment barbituric acid was used at a concentration of 1.2 millimolar, whereas in the experiment showing inhibition in cell-free extracts the concentration was 25 millimolar. Barbituric acid could not feasibly be applied to whole cells at the higher concentration, because even 10 m*M* barbituric acid will prevent the growth of diploid cell strains, whether cytidine is present or not. The high concentration of barbituric acid used in the cell-free experiments was made necessary by the large excess of substrate present in the reaction mixture. Since the inhibition is competitive, a 50-percent decrease in velocity can theoretically be obtained over a wide range of concentrations of barbituric acid by making an appropriate change in substrate concentration. However, because of the limited sensitivity of the spectrophotometric assay, it is necessary to use high concentrations of substrate (to obtain maximum reaction velocity and thus ensure that catalytic activity can be accurately measured). The concentration of substrate in the cell is almost certain to be much less than the concentration ($10^{-3}M$) used in the reaction mixture.
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Cigarette Smoking: Stimulatory Effect on Metabolism of 3,4-Benzpyrene by Enzymes in Human Placenta

Abstract. *The enzymatic hydroxylation of 3,4-benzpyrene was not detected in human placentas obtained after childbirth from nonsmokers, whereas this enzyme activity was present in placentas obtained from individuals who smoked cigarettes. The degree of induction of benzpyrene hydroxylase caused by cigarette smoking varied in different individuals. Treatment of pregnant rats with benzpyrene increased the activity of this hydroxylase in the placenta.*

3,4-Benzpyrene (BP) and several other polycyclic aromatic hydrocarbons are environmental carcinogens that are present in polluted city air (*1*), certain smoked or cooked foods (*2*) and tobacco smoke (*3*). Studies on the metabolism of BP revealed in the liver microsomes of rats and humans an enzyme system dependent on the reduced form of nicotinamide-adenine dinucleotide phosphate (NADPH) that hydroxylates this carcinogen (*4, 5*). Because the hydroxylated metabolites of BP are less carcinogenic than the parent molecule, the enzymatic hydroxylation of BP is a detoxification mechanism.

The administration of BP or other polycyclic hydrocarbons to rats induces severalfold increases in BP-hydroxylase activity in the liver, lung, gastrointestinal tract, skin, and other tissues (*4, 6*). This adaptive response in rats probably provides protection from the carcinogenic effects of polycyclic aromatic hydrocarbons and other carcinogens. Indeed, treatment of rats with suitable enzyme inducers markedly inhibits the carcinogenic activities of such compounds as BP (*7*), 3′-methyl-4-dimethylaminoazobenzene (*8*), 2-acetylaminofluorene (*8*), and 9,10-dimethyl-1,2-benzanthracene (*9*). Hence, we wanted to evaluate the quantity of carcinogen-metabolizing enzymes in tissues of man and to determine if constant exposure of people to polycyclic hydrocarbons increases the quantity of enzymes that detoxify these compounds. Because BP and other polycyclic hydrocarbons are constituents of cigarette smoke, we now report the effect of cigarette smoking on BP-hydroxylase activity in human placenta —a readily obtainable human tissue.

Placentas were placed in a freezer at −15°C immediately after normal childbirth and were assayed for BP-hydroxylase activity within 48 hours. A pie-shaped section (6 to 10 g) of the placenta was homogenized in 0.25*M* sucrose solution to yield a 10-percent suspension. Homogenates of rat placenta were prepared in the same way, except that all placentas from each rat were pooled and the tissues were assayed immediately after the animal was killed. The homogenate (1 ml) was incubated aerobically with 50 μg of BP in the presence of an NADPH-generating system; the amount of 8-hydroxy-3,4-benzpyrene formed in 15 minutes was measured by the method of Kuntzman *et al.* (*5*). In that the activation and fluorescence spectra of BP metabolites formed by placenta were identical with those of authentic 8-hydroxy-3,4-benzpyrene in sodium hydroxide solution, the hydroxylation of BP was expressed as nanograms of 8-hydroxy-3,4-benzpyrene formed, even though the fluorescence measured may represent a mixture of hydroxylated metabolites. The amount of 8-hydroxy-3,4-benzpyrene formed by human or rat