small doses of the latter have an opposite effect.

No vitamin D activity was found in the most osteolytic cancer extract by bioassay (*12*). Vitamin D could not be detected by thin-layer chromatography in any of the 12 cancer extracts.

These data indicate that human breast cancers contain an osteolytic sterol chemically close to, but not identical with, 7-dehydrocholesterol (pro-vitamin D_3). The osteolytic action of this sterol probably accounts for the rise of serum calcium concentration in patients with breast cancer. The fact that this activity is present in 11 of 12 tumors in patients without hypercalcemia (*13*) suggests that the body's numerous efficient homeostatic mechanisms prevent clinical hypercalcemia despite the presence of the stimulus in most cases. It is also in harmony with the fact that 40 percent of patients with disseminated breast cancer have hypercalcemia sometime in the course of their disease (*14*), despite the mechanisms which normally operate to combat hypercalcemia.

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Fumarate Reductase in the Control of Heme Biosynthesis

Abstract. *A drug-induced stimulation of heme biosynthesis in mouse liver was accompanied by altered fumarate metabolism. In liver homogenate, fumarate-1,4-C^{14} was incorporated, via succinate and succinyl coenzyme A, into heme at an accelerated rate. This pathway of fumarate utilization was inhibited by acetoacetate but not by β-hydroxybutyrate. Fumarate reduction to succinate required reduced nicotinamide adenine dinucleotide. The enzyme fumarate reductase is suggested as a link between terminal oxidation and cellular control of the heme biosynthetic pathway.*

In the metabolic control of heme biosynthesis an important function is performed by the first enzyme in the pathway, namely, δ-aminolevulinate synthetase, an enzyme whose activity may be modified by induction or by feedback inhibition (*1*). But, in addition, there are other essential metabolic changes which accompany, or indeed precede, altered heme formation. One such change is in the synthesis of succinyl coenzyme A (succinyl CoA), which, together with glycine-pyridoxal phosphate, forms the substrates for δ-aminolevulinate synthetase.

An inducible form of the mitochondrial enzyme succinyl CoA synthetase has been described (*2*). This enzyme, which differs from the constitutive enzyme in several properties, forms *de novo* in liver when mice are treated with porphyria-inducing drugs as a means of stimulating heme biosynthesis. Stimulated heme synthesis is now shown to be accompanied also by increased fumarate utilization. Essentially the same experimental approach and procedures as those already described (*2*) have been employed here. Mice were treated with allylisopropylacetamide (AIA) in order to induce experimental porphyria and accelerated tetrapyrrole biosynthesis in the liver.

The intraperitoneal injection into mice of a single dose of AIA increased by several-fold the incorporation of both succinate-1-C^{14} and fumarate-1,4-C^{14} into heme by liver homogenate (Table 1). The largest increases occurred in the 30- to 60-minute interval following drug administration. The labeled precursors must have reached heme through succinyl CoA synthetase, since carboxyl-labeled succinate or fumarate can be incorporated into heme only via direct conversion to succinyl CoA; their metabolism via the tricarboxylic acid cycle results in complete loss of radioactivity through decarboxylations (*2*, *3*). The results reported here, obtained with an in vitro system, confirm the previous observation that AIA stimulates the incorporation of carboxyl-labeled succinate into heme in vivo (*2*); they also indicate that fumarate is utilized through the same pathway as succinate.

The incorporation of succinate-1-C^{14} into heme had been observed to be unaffected by acetoacetate (*4*). Fumarate-1,4-C^{14}, however, behaved quite differently (Table 2). Thus, $2 \times 10^{-3}M$ acetoacetate decreased fumarate incorporation into heme by 80 percent, whereas β-hydroxybutyrate had little or no effect on fumarate incorporation. Although there was considerable variation of data between different experiments, the effects of AIA, acetoacetate, and β-hydroxybutyrate within any given experiment were consistent. The behavior of fumarate in the presence of acetoacetate and β-hydroxybutyrate substantiates the hypothesis that fumarate was being reduced to succinate on the route to heme. More direct evidence of fumarate reduction was obtained by measuring its conversion to succinyl CoA, observed as the hydroxamate, in isolated liver mitochondria. Fumarate reduction is rate limiting in this coupled system (*2*). As shown in Table 3, a small amount of hydroxamate formed in both mitochondrial preparations without added fumarate. In the presence of fumarate, there was no change in the controls but a marked increase in hydroxamate formation,

Table 1. Incorporation of succinate-1-C^{14} and fumarate-1,4-C^{14} into heme by mouse liver homogenate. Each of ten mice received a single dose of allylisopropylacetamide (400 mg/kg) and two were killed at each time interval. Liver homogenates (20 percent) were prepared in .067*M* phosphate buffer (*p*H 6.8). Five milliliters of homogenate, 100 μmole of glycine, and C^{14}-precursor in a total volume of 5.5 ml were incubated for 30 minutes at 37°C in air, with shaking. Subsequently, hemin in the incubation mixture was isolated with the aid of carrier, and counted.

Time after AIA administration (min)	Radioactivity in hemin* (count/min per 6 mg of hemin)	
	Succinate-1-C^{14}	Fumarate-1,4-C^{14}
Control	70	18
30	82	29
60	350	75
120	390	110
180	460	110

* Data for succinate and fumarate were obtained in separate experiments. The counts per minute were corrected to 1 μc per incubated sample.

that is, fumarate reduction, in the AIA-treated preparation. The further addition of nicotinamide adenine dinucleotide (NAD) caused a slight stimulation of fumarate reduction in both types of mitochondria, but reduced nicotinamide adenine dinucleotide ($NADH_2$) greatly increased fumarate reduction, the maximum reductase activity being almost 200 percent higher in the AIA-treated mitochondria than in controls. The considerable fumarate reduction in these mitochondria without added $NADH_2$ indicates that some reduced coenzyme was available.

Several previous findings indirectly support the hypothesis that increased fumarate reduction results from greater activity of a $NADH_2$-dependent fumarate reductase rather than from succinate dehydrogenase: (i) turnover of the tricarboxylic acid cycle in the livers of animals with experimental porphyria is considered to be normal (*2*, *5*); (ii) the respiratory rate of intact tissue culture cells utilizing succinate was unaffected by AIA (*6*); and (iii) succinate dehydrogenase activity remained unchanged in porphyric rat liver (*7*). An increase in fumarate reductase activity has not yet been correlated with an inducible form of the enzyme as has been done for succinyl CoA synthetase. However, the consistent finding of greater fumarate utilization resulting from AIA treatment suggests that this enzyme may likewise be inducible. Three different fumarate reductases have been identified in yeast and two of these appear in anaerobiosis but are suppressed by oxygen (*8*). The decreased fumarate excretion in rats with experimental porphyria and in patients with acute intermittent porphyria (*9*) could possibly be explained by an increased rate of fumarate reduction.

Table 2. Effects of acetoacetate and β-hydroxybutyrate on incorporation of fumarate-1, 4-C^{14} into heme. Experimental details were as described in Table 1 with the exception that all mice were killed 3 hours after administration of allylisopropylacetamide.

Addition	Radioactivity in hemin (count/min per 6 mg of hemin)	
	Control	AIA-treated
Experiment 1		
None	25	66
Acetoacetate (0.002*M*)	4	13
Experiment 2		
None	43	110
β-Hydroxybutyrate (0.0036*M*)	44	120

While a direct effect of porphyria-inducing drugs upon the heme biosynthetic pathway has not been experimentally demonstrated, these compounds do inhibit $NADH_2$ oxidation in purified enzymes or intact cells (*6*, *10*). The many relationships between heme or heme-protein biosynthesis and oxygen tension (*1*) imply some kind of metabolic link between the state of terminal oxidation and the control of heme biosynthesis. Perhaps the variable activities of fumarate reductase and succinyl CoA synthetase provide such a link. Since the amount of succinyl CoA generated by normal turnover of the tricarboxylic acid cycle is far greater than is needed for heme biosynthesis (*11*), the observed metabolism of fumarate and succinate may be explained by postulating an intramitochondrial compartmentation in which the succinyl CoA utilized for heme biosynthesis under stressed conditions arises from a source other than the tricarboxylic acid cycle.

Origin of the dicarboxylic acids for induced heme biosynthesis requires consideration. One possible source of the acids is increased carboxylation of pyruvate, which results from accelerated glycolysis (*6*). Preliminary experiments dealing with pyruvate and CO_2 utilization support this view and are being pursued.

It is interesting to note that there are several metabolic changes which accompany increased succinyl CoA formation, namely, (i) an increase in fumarate reduction to succinate, (ii) the *de novo* formation of succinyl CoA synthetase, and (iii) accumulation of Mg^{++}, an activator of inducible succinyl CoA synthetase, within the mitochondria (*12*). All of these changes precede any chemically detectable increase in tetrapyrrole formation.

Table 3. Fumarate reductase activity. Mice were killed 3 hours after administration of allylisopropylacetamide, and liver mitochondria were isolated from these and from a control group which received no AIA. Enzyme activity was then determined as succinyl-hydroxamate formed in an incubation mixture containing 10 μmole of adenosine triphosphate, 13 mμmole of coenzyme A, 20 μmole of glutathione, 10 μmole of $MgCl_2$, 960 μmole of hydroxylamine, 100 μmole of tris (*p*H 7.4), 20 μmole of NAD or $NADH_2$, 10 μmole of fumarate, and 0.5 ml of mitochondrial suspension in a final volume of 2.0 ml. Incubation was at 37°C for 30 minutes. This assay measures other acyl CoA compounds together with succinyl CoA; however, these contribute little in the absence of succinate or a succinate precursor, especially in the AIA-treated samples (*2*).

Additions	Fumarate reduction in mitochondria (mμmole hydroxamate/hr per mg of protein)	
	Control	AIA-treated
None	11	14
Fumarate	10	60
Fumarate + NAD	15	68
Fumarate + $NADH_2$	40	110

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