Table 1. Effect of gonadotropin treatment on the zona pellucida.

Animal No.	Ovary	Ovariectomy (day after treatment)	Dose schedule	Zona pellucida		
				Normal (%)	Defective (%)	Absent (%)
C-1	Left	0	Untreated	96		4
C-2	Left	0	Untreated	100		
C-3	Left	0	Untreated	100		
C-4	Left	0	Untreated	100		
C-5	Left	0	Untreated	96		4
C-6	Left	0	Untreated	96	4	
C-7	Left	0	Untreated	96	4	
X-1	Left	3	Group 1	0	72	28
	Right	9	Group 1	0	60	40
X-2	Left	6	Group 1	12	88	0
	Right	9	Group 1	24	76	0
X-3	Left	13	Group 1	12	68	20
X-4	Left	9	Group 2	16	48	36
	Right	13	Group 2	12	76	12
X-5	Left	12	Group 2	4	76	20
	Right	12	Group 2	0	76	24

experimental group were studied without reference to the animal records. There is an appreciable agreement on tabulation of the results obtained from any one pair of ovaries even though the ovariectomies were performed after two different periods of time after treatment. There are some individual differences in response to these compounds among the several treated animals, as demonstrated by animal X-2, which appears to be refractile.

Treatment with compounds such as Pergonal may incite a number of mechanisms and produce a variety of factors that may lead to the observed defects of the zona pellucida. These factors, obviously, are biochemical in nature and their action and interaction produce morphological discrepancies that in turn may greatly modify the normal physiological function of the ovum.

Since the zona pellucida is believed to function as a protective membrane, its absence may facilitate the transport of nutritive materials to the developing ovum, thus accelerating its maturation. Also, since it is a barrier that spermatozoa must penetrate to accomplish fertilization, its absence removes any possible function of sperm selectivity that may be exercised by the zona pellucida. Either of these factors may in part account for the high fertility encountered in women treated with such compounds. Whether this could connote undesirable situations that may express themselves genetically cannot be immediately ascertained.

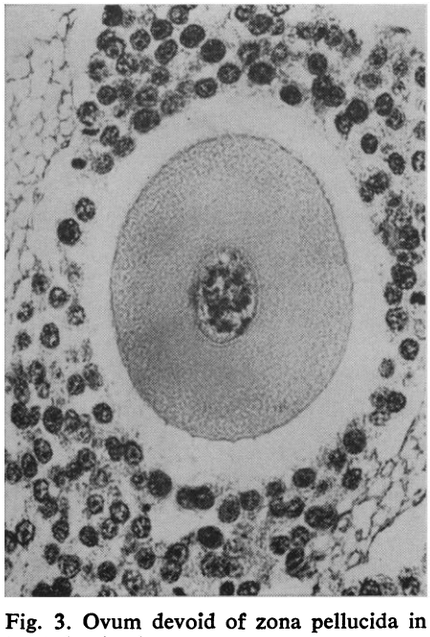

Fig. 3. Ovum devoid of zona pellucida in treated animal.

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Osteolytic Sterol in Human Breast Cancer

Abstract. *Eleven of twelve human breast cancers contained a lipid which increased urinary ^{45}Ca and ^{40}Ca excretion of ^{45}Ca-labeled, parathyroidectomized rats receiving a low Ca diet. The lipid has mobility on thin-layer chromatography and gas-liquid chromatography close to, but not identical with, that of 7-dehydrocholesterol. Authentic 7-dehydrocholesterol has osteolytic activity similar to that of the extracted sterol. Fluorescence and Lieberman-Burchard reactions of the extracted sterol are similar to those of 7-dehydrocholesterol. The lipid was found by thin-layer chromatography in the extracts which had osteolytic activity. Neither the lipid nor osteolytic activity was found in extracts of tissue from two normal human breasts.*

A number of host responses to many kinds of tumors of nonendocrine tissues result from ectopic elaboration of humors by the cancers. All of those so far identified are peptides: ACTH, parathyroid hormone, chorionic gonadotrophin, erythropoietin, and substances with the activities of antidiuretic hormone or thyrotrophin (*1*). In some cases of carcinoma of the lung, kidney, pancreas, and colon (*2*), parathyroid hormone has been identified immunologically; it accounts for the chemical syndrome of hypercalcemia and hypophosphatemia. In most cases, however, the hypercalcemia of malignancy is not accompanied by hypophosphatemia, but by normal or slightly elevated concentrations of phosphate in serum (*3*). Since breast cancer is the most common malignancy producing hypercalcemia (*4*) and since it is not associated with hypophosphatemia (*3*), we studied extracts of 12 human breast cancers, removed at operation (*5*), for osteolytic activity. We now report the presence of an osteolytic sterol in 11 of these tumors.

To identify extracts containing osteolytic activity, we measured their effect on the urinary ^{45}Ca and ^{40}Ca excretion of parathyroidectomized, labeled female rats. Female Sprague-Dawley rats were parathyroidectomized by electrocautery at body weight 60 to 75 g and injected subcutaneously with 10 μc of $^{45}CaCl_2$ (*6*). Starting 1 week after operation the labeled animals were fed a diet low in calcium and low in vitamin D (*7*). Each gram of diet contains 0.067 mg of Ca, 2.2 mg of phosphate, and 1.5 ng (0.06

IU) of vitamin D_2. After 24 hours or more of low Ca intake, the rats' plasma Ca concentration was measured by a microadaptation of the flame spectrophotometric method of Loken *et al.* (*8*). Rats were considered parathyroidectomized if plasma Ca concentrations were 0.9 to 3.8 milliequivalents per liter. (In sham-operated rats maintained on this diet, the plasma Ca ranges from 4.1 to 5.2 meq/liter.) Thirty-five or more days after parathyroidectomy, when urinary Ca excretion had fallen to a low, constant value, and specific activity in urine, plasma, and bone had equilibrated at 2.1 to 2.6 $\times$ 10^7 disintegrations per minute per gram, animals were placed in metabolic cages and urine was collected for 24-hour periods. Each 24-hour sample was diluted to 50 ml, and a 1.0 ml portion was dried and counted for 10 minutes in a Beckman-Sharp low beta counter. Urinary ^{45}Ca excretion was calculated as the number of disintegrations per minute in 24 hours by correction for dilution, self-absorption, physical decay, and counting efficiency. Another 1.0-ml portion was analyzed for stable Ca by the method of Loken *et al.* (*8*)

Twelve human breast cancers were ground and washed with cold acetone; both the acetone washings and the dry powder were saved. The powder was taken up in a mixture of 8*M* urea, 0.2*N* HCl, and 0.1*M* cysteine; then it was washed with ether. The ether-dried powder was taken up in 0.01*M* cysteine in 20 percent acetic acid and precipitated with 7.5 percent trichloroacetic acid. A similar extraction of 1 kg of bovine parathyroid glands yields approximately 1 g of peptide fraction containing approximately 385 units of parathyroid hormone activity per milligram. The human breast cancer trichloroacetic acid precipitate, however, contained no osteolytic activity; radioimmunoassay also showed that it contained no parathyroid activity (*9*).

The acetone-soluble fraction of all but one of the 12 human breast cancers, when evaporated to constant volume and injected subcutaneously in doses of 0.1 ml per day for 2 days in seven or more rats, significantly increased the urinary ^{45}Ca and ^{40}Ca excretion of the test animals. Specific activity did not change. Hexane extracts of the acetone-soluble material yielded a pale yellow oil which also promoted calcium-excretion. The hexane extract was subjected to thin-layer chromatography on silicic acid gel by a Mangold system (petroleum ether, ether, and acetic acid in the proportions 80 : 20 : 1) (*10*) or a mixture of CCl_4 and ethyl acetate (4 : 1). Four bands were identified by ultraviolet fluorescence after the plates were sprayed with 0.01 percent rhodamine,

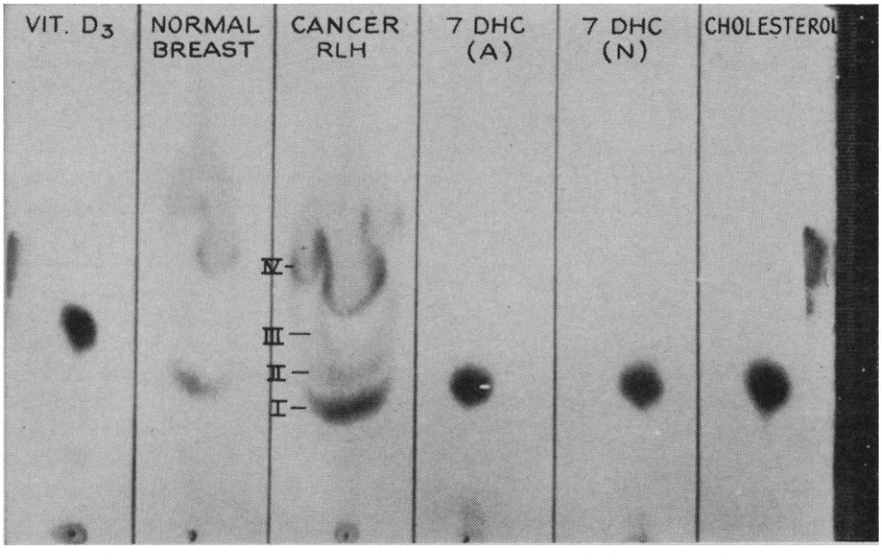

Fig. 1. Thin-layer chromatogram of hexane extracts derived from normal and neoplastic human breast with reference sterols (Mangold system on silicic acid gel sprayed with concentrated sulfuric acid. Each sterol was applied as 20 μl of $CHCl_3$ solution, 5 mg/ml; each tissue extract was applied as 20 μl of extract diluted 1 : 1 with hexane). Abbreviations: 7 DHC, 7-dehydrocholesterol; A, from Ayerst Laboratories; N, from Nutritional Biochemicals Corp.; RLH, patient. Band II is seen in cancer extract but not in extract of normal breast. It has the bluish color of 7-dehydrocholesterol but not the reddish color of cholesterol.

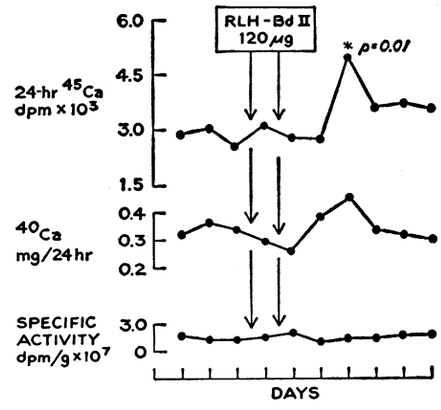

Fig. 2. Effect of methanol eluate of band II on urinary excretion of calcium in seven partially parathyroidectomized rats.

or by the development of color after they were sprayed with concentrated sulfuric acid (Fig. 1). The bands were eluted with hexane or methanol; after the solvent was removed from the eluate, the residue was dried and injected into test animals. No osteolytic activity was found in the eluates from bands I, III, or IV. The methanol eluate from band II, injected in doses of 120 μg daily for 2 days, significantly increased the urinary ^{45}Ca and ^{40}Ca excretion of the test animals (Fig. 2). This material from band II had the Lieberman-Burchard color and fluorescence of 7-dehydrocholesterol but migrated slightly farther on thin-layer chomatography and is retained slightly longer on gas-liquid chromatography.

Authentic 7-dehydrocholesterol (*7*, *11*) was recrystalized from a mixture of methanol and ethyl acetate; it yielded a single component on the two thin-layer chromatography systems. This material, when injected in doses of 50 or 250 μg daily for 2 days, similarly produced an increase in urinary excretion of stable Ca and of radiocalcium. Band II was found in 11 of 12 cancer extracts but was not found by thin-layer chromatography of hexane extracts of two specimens of noncancerous breast tissue obtained at operation. These extracts also lacked osteolytic activity.

The system used to assay osteolytic activity also demonstrates increased Ca excretion without change of specific activity after administration of vitamin D_2 or D_3, parathyroid peptide, and some glucocorticoids. Conversely, Ca excretion is decreased by estrogens, androgens, pituitary and chorionic gonadotrophins, thyrocalcitonin, prolactin, and growth hormone, though

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,