129°C), an indication that the radioactivity was almost entirely associated with squalene.

The composition of the incubation mixture had a profound effect on the pattern of products formed from 2-^{14}C-mevalonate (Fig. 2). In Fig. 2A, the basic incubation mixture is shown giving rise to farnesol and geranylgeraniol. The geranylgeraniol peak yielded 25,100 count/min on liquid-scintillation counting, and the farnesol peak 12,000 count/min. Together they represent a 20 percent conversion of the racemic substrate or 40 percent of the physiologically active isomer. The major product resulting from the addition of pyridine nucleotides to the system is squalene (Fig. 2B) (24,900 count/min), an indication that synthesis of squalene requires pyridine nucleotides. This requirement is approximately equally well satisfied by NAD, NADP, NADH, and NADPH. Formation of geranylgeraniol (18,700 cpm) is less than in the absence of squalene synthesis, and farnesol (870 cpm) is almost absent. Apparently, farnesyl pyrophosphate which is the common precursor of squalene and geranylgeranyl pyrophosphate (Fig. 1) is used up in the presence of pyridine nucleotides, and its utilization for the formation of squalene results in a decreased incorporation into geranylgeraniol. In Fig. 2C, the effect of manganese ion in the absence of pyridine nucleotides is shown. Geranylgeraniol (27,800 count/min) is the dominating peak, farnesol (2100 count/min) is relatively small and there are three new peaks (6800 count/min together) which did not appear in the incubations without Mn++. These peaks have not been identified yet, but they are of special interest, since they are formed under a definite cofactor requirement and because at least the two fastest components have chromatographic properties like C-40 carotene precursors. On silica gel G developed with a petroleum ether-benzene system (95 : 5), they migrate like phytoene (overlapping β-carotene) and phytofluene. On activated alumina developed with the same solvent, they have R_F values of 0.9 and 0.8, respectively (β-carotene, 0.6). The third component remains at the origin of the alumina plate. There is no incorporation into β-carotene. Figure 2D shows incorporation in the presence of both pyridine nucleotides and Mn++. In comparison with Fig. 2B, the incorporation into geranylgeraniol (24,600 count/min) and the material migrating like carotenoid precursors (7600 count/min) is favored over that into squalene (6300 count/min) in the presence of Mn++. Therefore Mn++ exerts a kind of regulation on the system. However, as important as this information is for the characterization of the system in vitro, no conclusions about the role of Mn++ in regulating the pathway in vivo are warranted.

In Fig. 2, A, C, and D, geranylgeraniol was the main product, in Fig. 2B, squalene. In Fig. 2E, farnesol (21,500 count/min) is the main product when the basic incubation mixture contains only 1/10 of the standard amount of P-35 fraction. Apparently this slows down the conversion of farnesyl pyrophosphate to geranylgeranyl pyrophosphate without affecting the production of the former from mevalonate. Geranylgeraniol (12,200 count/min) is still formed in the incubation illustrated, but if even less P-35 is used, farnesol becomes practically the only product. If such an incubation is done in the presence of pyridine nucleotides, squalene becomes the only product. This is also the situation found in insufficiently centrifuged supernatant preparations, which still contain traces of particulate matter. Extensively centrifuged S-200 incorporates only a small fraction of the radioactivity incorporated by the complete system. It is assumed that this activity is due to solubilization of particle-bound enzymes during the preparation. The P-35 fraction alone has no activity.

The formation of geranylgeraniol from mevalonate in cell-free systems from plant shoots has not been reported previously, although the material described by Pollard *et al.* (*8*) as coming off the column after farnesol probably was geranylgeraniol. Its formation as a major product in the present system is of special interest, since geranylgeranyl pyrophosphate is considered a precursor of phytol, gibberellins, carotenoids, and higher isoprenoid alcohols.

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Benzpyrene Hydroxylase Induction by Polycyclic Hydrocarbons in Hamster Embryonic Cells Grown in vitro

Abstract. *Treatment of hamster embryonic cells with 1,2-benzanthracene for 4 to 48 hours induced a three- to tenfold increase in the activity of benzpyrene hydroxylase. That the increase in enzyme activity was completely prevented by puromycin suggested an induction of enzyme synthesis.*

The administration of certain polycyclic aromatic hydrocarbons and drugs to several mammalian species causes a marked increase in the activity of a number of microsomal enzyme systems (*1*). These enzymes generally require reduced nicotinamide-adenine dinucleotide phosphate (NADPH) and O_2 and are involved in the oxidative metabolism of a variety of drugs, carcinogens, and steroids (*2*). The induction of increased enzyme activities has considerable pharmacological significance as manifested by a shorter duration of drug action (*1*) and inhibition of hepatocarcinogenesis (*3*). One of the

Table 1. Benzpyrene hydroxylase activity in hamster embryonic cells treated with 1,2-benzanthracene or 3-methylcholanthrene.

Addition to culture medium	Duration of treatment with hydrocarbon (hr)	Enzyme activity [$\mu\mu$mole (hydroxylated product)/mg (protein)]
Experiment 1		
None	0	20
1,2-Benzanthracene	4	78
1,2-Benzanthracene	24	199
1,2-Benzanthracene	48	194
Experiment 2		
None	0	36
3-Methylcholanthrene	24	56
Experiment 3		
None	0	22
1,2-Benzanthracene	4	66
1,2-Benzanthracene + puromycin ($4 \times 10^{-4}M$)	4	18

enzymes markedly increased in several tissues from rats previously treated with polycyclic hydrocarbons is benzpyrene hydroxylase (*1, 4*). An increased activity has also been reported to occur in perfused rat liver (*5*) and in organ cultures of lung tissue (*6*). The induced increase in benzpyrene hydroxylase in rat tissues is inhibited by puromycin and by actinomycin D (*7*), an indication that the higher activities are the result of an induced enzyme synthesis. The increased activity in rat liver is also accompanied by enhanced synthesis of protein and RNA (*8*). We now report the induction of benzpyrene hydroxylase activity in hamster embryonic cells grown in vitro and describe some of the characteristics of the induced enzyme system.

Primary cultures of hamster embryonic cells (H.E.), derived from 10- to 12-day-old fetuses, were treated with 0.25 percent trypsin, and the cells were seeded in 150-mm plastic petri dishes at a density of 10^7 cells per dish in Eagle's minimal essential medium (*9*) containing 10 percent calf serum (complete medium). The secondary cultures were incubated in a humidified atmosphere of 5 percent CO_2 in air at 37°C for 24 to 48 hours. The growth medium was then replaced with fresh complete medium (controls) or complete medium containing either 1.0 μg 1,2-benzanthracene or 1.0 μg of 3-methylcholanthrene per milliliter (*10*), and the cultures were again incubated. After incubation, the cells were washed three or four times with cold Dulbecco's salt solution, *p*H 7.2, collected by scraping and centrifugation, and broken by homogenization in 0.25*M* sucrose in a tight fitting Potter-Elvehjem all-glass homogenizer. Benzpyrene hydroxylase was determined by a modified method of Wattenberg (*11*) described previously (*7*). Each assay flask contained, in a final volume of 3.0 ml, 50 μmole of sodium phosphate, *p*H 7.4; 12 μmole of glucose-6-phosphate; 2.4 μmole of NADPH; 0.2 ml of a supernatant fluid from a homogenate of rat liver centrifuged at 100,000*g* (approximately 2.4 mg of protein); 0.2 μmole of 3,4-benzpyrene; 0.1 ml of methanol; and 0.05 to 0.4 ml of H.E. cell homogenates (5 to 20 mg of protein per milliliter of 0.25*M* sucrose). The flasks were incubated for 14 minutes at 37°C, and the reaction was stopped by the addition of 3.0 ml of acetone. The hydroxylated benzpyrene was extracted, and its fluorescence was determined in an Aminco-Bowman spectrofluorometer with activation at 400 mμ and fluorescence at 522 mμ. A sample of 3-hydroxybenzpyrene was used as a standard, and the enzyme activity was expessed as micromicromoles of hydroxylated product, equivalent to 3-hydroxybenzpyrene, formed per milligram of H.E. cell protein.

Table 2. Requirements for hydroxylation in the enzyme system. The complete system contained cell homogenates from cultures treated with 1,2-benzanthracene for 24 hours. Abbreviations: NADPH, reduced nicotinamide-adenine dinucleotide; G-6-P, glucose-6-phosphate.

Alteration of the complete system	Enzyme activity [$\mu\mu$mole (hydroxylated product)/mg (protein)]
None	154
Minus NADPH and G-6-P	5
Minus NADPH and G-6-P + rat liver supernatant	4
Heated at 90° for 15 min., then incubation	5
Incubation under N_2	36

Table 1 shows the effect of incubation of H.E. cells in monolayer culture with 1,2-benzanthracene or 3-methylcholanthrene. Incubation in the presence of 1,2-benzanthracene for 4 hours increased benzpyrene hydroxylase activity about fourfold, and incubation for 24 or 48 hours increased activity tenfold (experiment 1). Treatment with 3-methylcholanthrene was less effective, increasing the enzyme activity by about 60 percent. The presence of puromycin completely prevented the increase in enzyme activity, an indication that this increase is dependent on protein synthesis (Table 1, experiment 3). Table 2 shows some of the requirements of the enzyme system for hydroxylation. There is essentially no activity in the absence of NADPH and glucose-6-phosphate with or without the presence of the supernatant fraction of rat liver homogenate centrifuged at 100,000*g*. Incubation in a nitrogen atmosphere decreased the activity fourfold, and heated preparations were inactive. The amount of hydroxylated product formed with different amounts of cell homogenates from control cells and those treated with 1,2-benzanthracene is linearly related to the enzyme content (Fig. 1). The capability of inducing microsomal enzyme systems in cells cultured in vitro offers obvious advantages for studies on the controlling mechanisms in the regulation of enzyme synthesis as well as in drug and carcinogen metabolism.

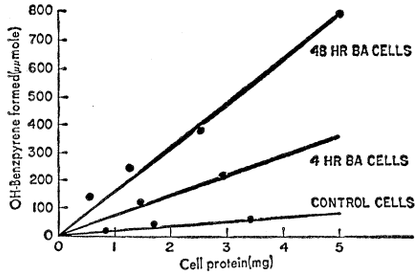

Fig. 1. Benzpyrene hydroxylase activity in total homogenates of control cells and hamster embryonic cells treated with 1,2-benzanthracene from secondary culture.

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