finding indicates that the treated group did not become anemic as a result of blood lost by way of the urinary route. However, the incidence of acute anemia in the chronic human alcoholic is not uncommon (*5*). It may be added that, in our study, three male human patients with a history of chronic alcoholism also showed hematuria. Causes of hematuria other than alcoholism were not ruled out, however. Two of these patients eventually developed other clinical complications and died. Further studies on human alcoholics are indicated.

The cause of the hematuria following administration of ethanol is not apparent at present. In a study on infectious mononucleosis in man, Custer and Smith (*6*) attributed the hematuria observed to infiltration of the kidney by lymphocytes. A possible explanation of our findings may be that ethanol induces lysis of the "old" erythrocytes by subjecting them to gross osmotic stress (*7*). Hemoglobin thus released could pass through the glomerulus and into the urine. Pertinent in this connection is a current report (*8*) that the administration of ethanol to rats leads to an increase in certain phospholipids in the liver, notably lysolecithin. Since this substance is known to lyse membranes, it seems that the hematuria herein reported could be related to an increase in the amount of lysolecithin in the liver, or perhaps in other tissues, as a consequence of ethanol ingestion.

Another possible explanation for the hematuria is that a "relative choline deficiency" develops following the prolonged administration of ethanol (*9*). Due to lack of sufficient choline, the animal may suffer renal damage and eventually pass blood into the urine. Supporting this hypothesis is the work of Valaitis *et al.* (*10*) who observed myoglobinuric nephrosis in human patients with a history of alcoholism. In a parallel study on osteomyelitis produced by *Salmonella*, hemoglobinuria was observed after the resorptive capacity of the proximal renal tubules was exceeded (*11*).

Indeed, in our study, considerable damage to the epithelial cells of the renal proximal tubule was observed microscopically in the treated group of animals. Statistically significant renal enlargement was also found in the treated rats.

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Isoprenoid Biosynthesis in a Cell-Free System from Pea Shoots

Abstract. *A cell-free system consisting of a soluble fraction and plastid fragments from pea shoots incorporates 2-^{14}C-mevalonate very actively into farnesol, squalene, geranylgeraniol, and other isoprenoids of different carbon-chain lengths. The products have different cofactor requirements, which makes it possible to channel the pathway into different products by varying the incubation mixture.*

The metabolism of 2-^{14}C-mevalonate in a cell-free system from pea shoots was studied. Such a system might be used to detect differences in the pathways of related intermediates and end products during the development of the plants. Several lines of evidence indicate a correlation between regulation of isoprenoid biosynthesis and development of plants. While seedlings are growing in the dark, cyclic triterpenoids and steroids are formed with squalene as an intermediate; but on transfer to light, the pathway is channeled into the formation of carotenoids, and the side chains of chlorophyll (phytol) and quinones instead (*1*). Inhibitors of steroid biosynthesis affect the development of plants only if they are applied at the right stage of development (*2*), a fact which indicates a different significance of the pathways at different times. Gibberellins, which are diterpenoid growth regulators with fundamental roles in plant development, accumulate preferentially during long-day conditions or at specific stages in the life cycle (*3*). Abscisin II (dormin), a sesquiterpenoid plant regulator which induces dormancy in a number of species and promotes abscission, accumulates mainly during short-day conditions (*4*).

The biosynthesis of these compounds from mevalonic acid initially proceeds along a common pathway (Fig. 1) (*1*, *5*).

The isoprenoid pathways in higher plants have been studied mainly in whole plants, excised seedlings, and tissue slices (*1*, *6*). Successful systems which incorporated substantial amounts of mevalonic acid beyond the stage of isopentenyl pyrophosphate have been derived from fruits, roots, or seeds (*7*). Some studies of mevalonic acid incorporation in cell-free systems from shoots have recently appeared (*8*).

In my work, peas (*Pisum sativum* L, "Grosse Schnabel") were grown for 10 days at 25°C under long-day conditions. The shoot tips from 1150 plants were cut off just under the third, still very small, leaf and ground with sand in 60 ml of a medium consisting of 0.45*M* sucrose, 0.1*M* tris-HCl buffer (*p*H 8.0), 0.01*M* KCl, 0.005*M* $MgCl_2$, 0.005*M* 2-mercaptoethanol, and 0.001*M* ethylenediamintetraacetic acid (EDTA). The extract was filtered and centrifuged successively at 500*g*, 1500*g*, 35,000*g*, and twice at 200,000*g*. The 35,000*g* sediment, which consisted of mitochondria and large amounts of chloroplast fragments, was washed twice in the homogenization medium and then suspended in 3 ml of a solution of 5 m*M* 2-mercaptoethanol and 1 m*M* EDTA to rupture remaining structures osmotically. This fraction is referred to as the P-35 fraction. The 200,000*g* supernatant was concentrated from 50 ml to 3 ml in a series of steps with the use of dry Sephadex G-25 to take up excess water (*9*). The concentrated solution was then purified over a column of Sephadex G-25 equilibrated with a solution of 0.01*M* tris-HCl, *p*H 7.3, with KCl, mercaptoethanol, and EDTA in the same concentrations as the homogenization mixture. The yel-

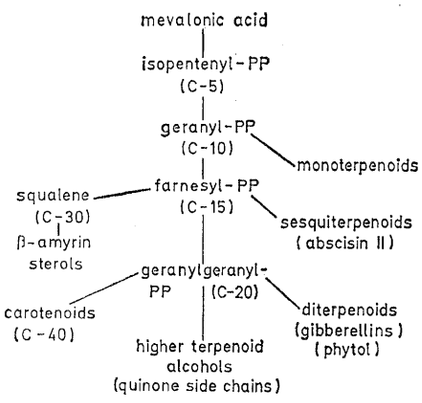

Fig. 1. Simplified scheme of the biogenetic relationship of the isoprenoids.

low large-molecule fraction that is collected immediately after the void volume was collected and clarified by centrifugation, the supernatant being designated S-200.

The reactions were carried out in open centrifuge tubes for 3 hours at 30°C. The basic incubation mixture contained the following ingredients in a total of 0.1 ml: 5 μmole of tris-maleate buffer, *p*H 7.3; 2.5 μmole of 2-mercaptoethanol; 0.8 μmole of $MgCl_2$; 0.6 μmole of ATP; 2.5 μmole of phosphoenol pyruvate; 5 μg of pyruvate kinase; 0.02 μmole of 2-^{14}C-DL-mevalonate (181,200 count/min); 0.4 mg of S-200 protein; and 0.014 mg of P-35 chlorophyll. At the end of the incubation, the mixtures were acidified and left at room temperature for 15 minutes to effect hydrolysis of any remaining prenol pyrophosphates. Acetone was added to extract the lipids, and the phases were separated with benzene. The extracted lipids were saponified, and the unsaponifiable lipids were extracted with a benzene-acetone system (2 : 1).

Five micrograms each of kaurene, squalene, lycopersene, phytol, geranylgeraniol, farnesol, and geraniol were added as markers. Each extract was spotted for two-dimensional chromatography at the corner of a thin-layer plate (20 by 20 cm) with a 0.25-mm layer of activated silica gel G. The spotting, as well as all evaporations, and the saponification were done under nitrogen at room temperature. The plates were developed in the first direction with petroleum ether (b.p. 40° to 60°C) containing 5 percent (by volume) liquid paraffin. The petroleum ether separated the hydrocarbons, whereas the isoprenoid alcohols remained at origin. The paraffin reversed the phases for the separation of the alcohols. The second development, at right angles to the first, could then be accomplished with a mixture of methanol and water (4 : 1) saturated with paraffin. The separated hydrocarbons were not moved out of the first lane by the second development. This method gave an excellent separation of the components and permitted the direct observation of the relations between several products in a single incubation mixture. Radioactivity was located by scanning, and was correlated with the reference compounds which were made visible by exposing the plates to iodine vapor. The radioactivity was then measured more exactly by liquid-scintillation counting.

Incubation of the S-200 fraction with P-35 fraction in the basic incubation mixture, resulted in the incorporation of mevalonate into two components migrating like geranylgeraniol and farnesol on the chromatogram. Addition of pyridine nucleotides to the incubation mixture resulted in formation of a third component which migrated like squalene. That the label actually was associated with geranylgeraniol and farnesol was ascertained by mixing each with purified carriers, preparation of the 4-(4′-nitrophenylazo)benzoyl esters (*10*), and recrystallization to constant specific activity. When the material from the geranylgeraniol peak (175,000 count/min) generated in a large-scale incubation mixture (1 ml) was mixed with *trans*-geranylgeraniol (3.17 mg) giving a specific activity of 16,000 count/min per micromole, the constant specific activity of the ester after chromatography and four recrystallizations was 14,400 count/min per micromole (melting point 80.5°C). Thus at least 90 percent of the radioactivity of this peak was associated with *trans*-geranylgeraniol.

The material from the farnesol area (23,700 count/min) was mixed with *trans*-farnesol containing a small amount of *cis*-isomer (together 2.98 mg), resulting in a specific activity of 1700 count/min per micromole. The specific activity of the ester was 2000 count/min per micromole which was retained through five recrystallizations (final m.p., 88.5°C). The increased specific activity was due to the loss of the unlabeled *cis*-isomer during purification of the ester. Since the final specific activity was reached immediately and the total yield of radioactivity in the ester was 89 percent of the original, at least 89 percent of the radioactivity was associated with *trans*-farnesol. Radioactive squalene was identified in a mixture of the same composition containing reduced nicotinamide-adenine dinucleotide phosphate (NADPH) (2.5 μmole/ml) in addition. The radioactive material (158,000 count/min) corresponding to squalene on the chromatogram was mixed with purified squalene (100.0 mg) to give a specific activity of 650 count/min per micromole. After preparation of the hexahydrochloride (*11*), several recrystallizations yielded a constant specific activity of 630 count/min per micromole (isomeride mixture melting 124° to

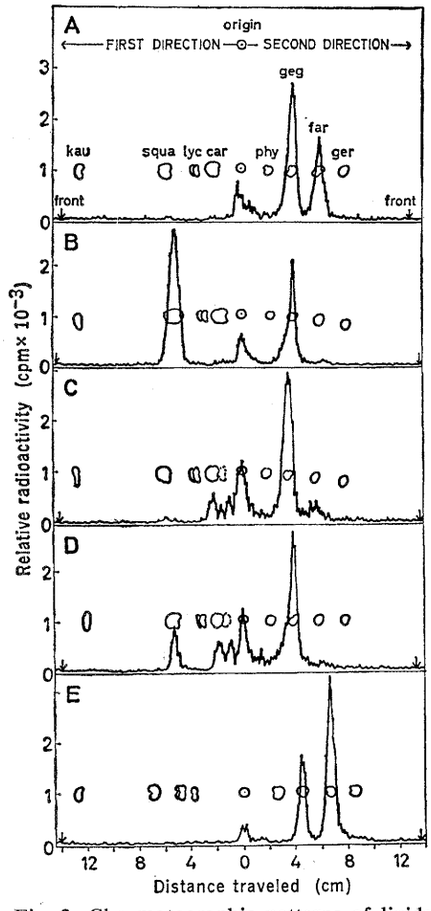

Fig. 2. Chromatographic patterns of lipids formed from 2-^{14}C-mevalonate in a cell-free system from pea shoots. The 0.1-ml incubation mixture containing P-35 (plastid) fraction and S-200 (soluble) fraction is described in the text. (A) Basic incubation mixture. (B) NAD (0.25 μmole) and NADPH (0.25 μmole) added. (C) $MnCl_2$ (0.3 μmole) added. (D) NAD, NADPH, and $MnCl_2$ added. (E) Basic incubation mixture but with only 1/10 the standard amount of P-35 fraction. The reference substances are kaurene, squalene, lycopersene, β-carotene, phytol, geranylgeraniol, farnesol, and geraniol. Scanning was done with a 2-mm slit width and a range setting of 6000 count/min. The two developments at right angles to each other have been laid out in one dimension for the sake of illustration.

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