tide (DPN) dependent oxidation of semialdehydes.

The dehydrogenase has been purified approximately 150-fold from monkey brain to a specific activity of 3 μmole/mg of protein per hour at 20°C and *p*H 8.1, with succinic semialdehyde as substrate. Maximal initial rates are obtained with $10^{-4}M$ succinic semialdehyde. Triphosphopyridine nucleotide is inactive as a hydrogen acceptor. Aged enzyme preparations require preincubation with cysteine or mercaptoethanol for maximal activity. Glyoxylate and malonic semialdehyde are also oxidized by the preparation, and preliminary experiments indicate that the relative rates with these substrates are species-dependent. 5-Hydroxypentanal is oxidized at a comparatively slow rate, while formaldehyde, acetaldehyde, and glyoxal oxidation was not significant at comparable substrate and enzyme concentrations. A dehydrogenase from *Pseudomonas* exhibiting specificity for succinic semialdehyde is described in an accompanying report (*2*).

The soluble enzyme from monkey brain was obtained by freezing and thawing the particulate fraction R_3 prepared according to the method of Brody and Bain (*3*). Further purification was achieved by absorption and elution from nucleic acid and treatment with protamine and Dowex-1 chloride.

The assay system consisted of $1 \times 10^{-4}M$ aldehyde, $4 \times 10^{-4}M$ DPN, $4 \times 10^{-3}M$ mercaptoethanol, and $0.05M$ tris(hydroxymethyl)aminomethane buffer, at *p*H 8.1. DPNH formation was measured fluorimetrically in whole homogenates (*4*) and spectrophotometrically in the case of the soluble enzyme preparations.

Studies with C^{14}-γ-aminobutyrate have shown that it enters the tricarboxylic cycle rapidly in brain in vivo (*5*). Although evidence for the importance of other oxidative pathways involving semialdehydes in brain is lacking, the possibility cannot be excluded. Malonic semialdehyde presumably arises from the in vitro transamination of β-alanine (*1, 6*).

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Preparation of Cell Suspensions from Insect Tissues for in vitro Cultivation

Previous studies of the cultivation of insect cells in vitro have followed the classical technique of the explanation of tissues and organs. Insect tissue cultures have been maintained for only relatively short periods of time, and serial passage of the cells has not been achieved. The literature in the field has been reviewed by Schmidt and Williams (*1*). Much valuable information is presented in recent papers by Grace (*2*) on culturing techniques, by Loeb and Schneiderman (*3*) and Loeb (*4*) on the use of synthetic medium 199 (*5*), and by Wyatt (*6*) on the development of a medium based on the chemical composition of silkworm hemolymph. The limited success of these studies with explanted tissue has led us to try another approach to the cultivation of insect cells by modifying the technique of Dulbecco and Vogt (*7*) to attain a suspension of cells from disaggregated tissue. We wish to report the results of these preliminary studies (*8*).

The variegated cutworm, *Peridroma margaritosa* (Haworth) (Lepidoptera : Phalaenidae), is readily available in the San Francisco Bay area and is easily raised in the laboratory. The integument of the thoracic segments of the fifth-instar larva was used for the present study. It is comprised of differentiated larval cells and undifferentiated cells in the wing buds and leucopoietic organs.

Prior to dissection, the larvae were anesthetized for 1 minute in CO_2. To sterilize the external surface, they were passed successively through 5 percent ethyl alcohol, 4 percent formaldehyde (immersion of the head was avoided), and two changes of 70 percent ethyl alcohol. The excess alcohol was removed on blotting paper, and the dissection was performed in a sterile petri dish with iridectomy scissors. The thoracic segments of five to ten larvae were collected in phosphate buffered saline. They were washed in five changes of the saline to eliminate possible microbial contaminants.

The saline of Dulbecco and Vogt (*7*) was used, with the following modifications: (i) NaCl 8.0 g, KCl 0.2 g, Na_2HPO_4 1.15 g, KH_2PO_4 0.2 g, phenylthiourea (twice recrystallized from hot ethyl alcohol) 2.08 g, water (distilled and deionized) 800 ml; (ii) $CaCl_2$ 0.2 g, $MgCl \cdot 6H_2O$ 0.1 g, phenylthiourea 0.52 g, water 200 ml. The phenylthiourea was included to inhibit the tyrosinase activity in the tissue (*1*). The two solutions were autoclaved separately and combined when cooled. Prior to use, penicillin (200 units/ml) and streptomycin (100 μg/ml) were added.

Disaggregation of the tissue was attempted, initially, with trypsin and Versene, under conditions used routinely in the preparation of vertebrate cell cultures. Although many cells were liberated, they were injured in the process. Cytoplasmic extrusions indicated damage to the cellular membranes which could not be arrested by washing with saline or serum.

Liberation of undamaged cells was accomplished with an extract prepared from the hepatopancreas and crop of the snail *Helix aspersa* Müller. Such an extract is a rich source of hydrolytic enzymes. The snails were starved for 4 to 7 days to empty their digestive tracts of solid materials. The hepatopancreas and crop were removed, their wet weight was determined (0.55 to 1.20 g) and they were immediately ground with an equal weight of distilled water in a Tenbroek tissue grinder, cooled to approximately 4°C. The resultant homogenate was centrifuged at 24,000 *g* for 15 minutes in a refrigerated centrifuge, and the supernatant was stored in a frozen state at −20°C. Prior to use, the extract was thawed, recentrifuged, diluted with two volumes of saline, and sterilized by passage through a Millipore filter.

Disaggregation was performed at room temperature in a 50-ml erlenmeyer flask containing the thoracic segments of five larvae and 5 ml of hepatopancreas-crop extract. The contents were stirred gently with a magnetic stirrer. Liberation of the cells was completed in 5 to 7 minutes, as evidenced by the transparency of the larval cuticle and the cloudiness of the suspension. The fluid was pipetted into a centrifuge tube and allowed to stand for 5 minutes to permit the tissue fragments to settle. The cell suspension was decanted into a second tube and centrifuged for 1 minute at 1700 rev/min (clinical centrifuge). The supernatant was discarded, the cells were washed in the saline, and an aliquot was counted in a hemocytometer chamber. The thoracic segments of each larva yielded approximately 160,000 cells. They appeared round and clear, with well-defined nuclear and cytoplasmic membranes.

The cells were recentrifuged, the saline wash was decanted, and they were suspended in the culture medium. This consisted of medium 199 [modified by increasing the amino acids to the level recommended by Wyatt (*6*)], 20 percent human serum, and 5 percent chick embryonic extract. Culture tubes were seeded with approximately 400,000 cells in 1 ml of medium and held at room temperature. The cells attached themselves readily to the glass, either singly or in packets of five to ten. They were maintained for 4 to 5 days in a healthy state, flattened, optically clear, and with

normal-appearing nuclei. No effort was made at this time to study the growth requirements of the cells.

This method for preparing cell suspensions from insect tissue is reproducible and makes possible quantitative studies involving large numbers of cells. Conceivably it could provide monolayer cultures for viral research. We are hopeful that the liberated cells may dedifferentiate rapidly and be capable of growth in a less complex medium than is required, apparently, for explanted insect tissues.

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Protection of Fungi against Polyene Antibiotics by Sterols

While we were studying the structural chemistry of the polyene antifungal agent, filipin, the question arose of the possible mechanisms of its antifungal action (*1*). The presence of a conjugated polyene structure in filipin suggested that perhaps filipin interfered with the synthesis or the function of carotenoids in the fungi. In order to investigate this hypothesis, a mixture of carotenoids was obtained from carrots. The hexane extract, prepared according to the method of Loomis and Shull (*2*), was tested by the assay disc method on *Penicillium oxalicum* (*3*). The inhibitory activity of filipin was, indeed, completely prevented, and the organism grew normally in the presence of filipin plus the crude hexane extract.

Saponification of the hexane extract followed by chromatography on MgO : Hyflo Super Cel gave an active fraction which followed α- and β-carotene from the column. From this fraction a white material was obtained which was easily crytalized from methanol-water. This material was obviously not the usual type of carotenoid. Moreover, when crystalline preparations of α-carotene, β-carotene, or vitamin A were tested, they showed no activity at levels of 10 times that of filipin. The isolated material melted at 134.5° to 137°C and gave analytical data consistent with a $C_{20}H_{30}O$ structure. C-Methyl analysis completely ruled out the possibility that this material was a perhydrocarotenoid, for the material contained only 1.5 C-methyl groups per 20 carbons. An alternative possibility that the protecting agent might be a sterol was confirmed by positive Lieberman-Burchard and Salkowski tests. The analytical data are also in good agreement for sterols of the C_{29} series—that is, sitosterol and stigmasterol (4)—which have been isolated from carrots, if they are calculated for ½ H_2O of crystallization (*5*). $C_{29}H_{50}O \cdot ½ H_2O$: Calculated: C, 82.18 percent; H, 12.11 percent. Found: C, 82.01 percent; H, 12.46 percent.

The observation that the active agent is a sterol led to the testing of a number of such compounds for similar activity. Thus far, three fungi, *Penicillium oxalicum, Aspergillus niger,* and *Hansenula subpelliculosa,* have been used to study this phenomenon. The prevention of filipin inhibition of the *Penicillium* was observed in both the assay plate test and in liquid media. On agar plates, both carrot sterols and soybean sterols allowed growth of *P. oxalicum* to occur in the presence of filipin at a weight ratio of sterol to filipin of 0.5 : 1.0; *Hansenula* grew normally in shaken liquid culture at a ratio of 0.25 : 1.0. The effect of sterols on nine polyene antibiotics showed various degrees of protection, but no definite relationship between the number of unsaturated groups in the antibiotics and their vitiation could be established (*6*). Filipin and fungichromin inhibitions were most readily prevented, followed by amphotericin B, trichomycin, and rimocidin, while candicidin A, candicidin B, ascosin, and nystatin were only slightly affected. Unfortunately, only the purities of filipin and fungichromin were known so that the relative order of protection can only be tentatively suggested.

Of all the sterols examined thus far, cholesterol, ergosterol, sitosterol, stigmasterol, and to a slight degree, lanosterol, have been active in offsetting filipin inhibition of *H. subpelliculosa.* Ergosterone gives effective reversal of filipin activity; cholesterone is ineffective. None of the short-chain steroids that were tested had similar effects even at a ratio of steroid to filipin of 4 to 1. Apparent reversal occurs after long incubation periods, but preliminary data indicate the possible inactivation of filipin under these conditions.

The prevention of the antifungal activity of filipin by sterols has some interesting implications. Evidently, sterols play a much more important role in the growth processes of fungi than has hitherto been suspected. While a few microorganisms have been shown to require sterols for growth (*7–9*), our current studies indicate that such substances are probably essential metabolites for many fungi. This has not been recognized until now because these microbes are for the most part autotrophic for their sterol requirement. Except for *Labrynthula vitallina* var. *pacifica* (*9*) and *Saccharomyces cerevisiae* S C-1 when grown anaerobically (*7*), the need for this compound has not been demonstrated among the fungi.

The mechanism by which filipin and other polyenes inhibit fungi is intriguing. They might either prevent the synthesis of sterols which are necessary for growth or competitively replace the sterol as a cofactor of a reaction vital to the metabolism of the organism. If the synthesis of cholesterol is prevented, then the inhibition probably occurs at a stage beyond lanosterol formation (*10*). Squalene does not reverse the action of filipin on *Hansenula subpelliculosa* even at a squalene to filipin ratio of 20 : 1. Lanosterol reverses the antibiotic only very slightly at a lanosterol to filipin ratio of 4 : 1. This is a sharp contrast to cholesterol, which is active at a ratio of under 4 : 1 and probably at ¼ : 1.

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Pyrrolidine Metabolism: Soluble γ-Aminobutyric Transaminase and Semialdehyde Dehydrogenase

A strain of *Pseudomonas* isolated by the enrichment culture technique with pyrrolidine as the sole carbon source has been found to catalyze the following reactions designated as γ-aminobutyric-