Perdeuterio Synthetic-Natural Rubber

The recently reported stereospecific polymerization (*1*) of isoprene and the availability of D_2O has made possible the preparation of synthetic-natural (SN) rubber in which most of the hydrogen atoms are D atoms.

Acetone-d_6 was prepared (*2*) from 99.5 percent D_2O and acetone-h_6 in the presence of K_2CO_3. Nine stages were applied. Each required fractionation of acetone from water, and it is estimated that each replaced approximately half of the light hydrogen remaining from the previous stage. Acetylene-d_2 was prepared from D_2O and calcium carbide. The procedure of Bergmann (*3*) for the Favorskiĭ (*4*) reaction of acetylene and acetone to form 2-methyl-3-butyn-2-ol was followed except that in our work perdeuterio reactants were used.

The triple bond of the butynol selectively absorbed D_2 over the Lindlar (*5*) catalyst, and the resulting 2-methyl-3-buten-2-ol dehydrated readily over Al_2O_3 to give isoprene-d_8. Standard fractionation techniques were used for the isoprene and for all the intermediates. The isoprene was finally distilled over sodium to remove acetylenes, acetone, and alcohols. The same methods were applied to the corresponding perprotio derivatives. Table 1 gives the properties of most of the compounds prepared and used in this study.

The isoprene-d_8 polymerization was initiated by $TiCl_4$ and triisobutyl-h_{27}-aluminum in both aliphatic and aromatic solvents. Hydrogen from these solvents did not appear to interchange with deuterium from the monomer of polymer. Isoprene-d_8 polymerizes faster and to a higher molecular weight than isoprene-h_8 under what are believed to be comparable conditions. Our best sample of D-SN rubber was prepared by hexane extraction of a raw polymer containing 25 percent gel. The soluble polymer ($M_n = 333{,}000$) milled (broke down) like natural rubber and had a limiting intrinsic viscosity in benezene at 25° of $[\eta]_0 = 5.8$.

The benzene solutions were found to degrade unless they were stabilized with a small amount of tetramethylthiuram disulfide (TMTD). D rubber evolved (*6*) a trace of D_2 when it was vulcanized with TMTD and ZnO. The vulcanizate, after removal of zinc compounds and other substances by short-path distillation techniques (*7*), was nearly transparent and displayed an x-ray diffraction pattern that was superposable over those of natural and SN rubbers. The density at 23°C of the vulcanizate was 1.010 g/ml (equivalent to 1.009 at 25°C); and the density of the polymer before cure was 1,005 g/ml (equivalent to 1.003 at 25°C). Accepting 0.906 g/ml at 25°C as the density of purified natural rubber (*8*) and multiplying this by the ratio (1.118) of the density of isoprene-d_8 to the density of isoprene-h_8 (see Table 1), we obtain a calculated value of 1.013 g/ml for the density of D rubber. Now the ratio of the formula weight (76.145) of isoprene-d_8 to that (68.119) of isoprene-h_8 is 1.118. It is probable that 0.906 is somewhat high as the density of perprotio all-*cis*, 1-4, head-to-tail polyisoprene, and values observed for some of our best samples of SN rubber are around 0.901. This, when multiplied by 1.118, gives 1.007 as the expected density of D-SN rubber. The good agreement between the found and expected values of the density shows that our specimen contains at most not more than a few hydrogen atoms.

Table 1. Properties of perprotio and perdeuterio derivatives of compounds and azeotropic mixtures.

Compound or azeotrope	Boiling point (740 mm)	Freezing point	Water (wt. %)	Nd^{20}	d_4^{20}
Perprotio derivatives of compounds and mixtures					
Acetone	55.3			1.3592	0.7895
Water	99.2	0.0	100	1.3333	0.99823
2-Methyl-3-butyn-2-ol	103	3.0		1.4215	0.8609
2-Methyl-3-butyn-2-ol water azeotrope	89.0	−10.5	27	1.4050	
2-Methyl-3-buten-2-ol	96.5	−28.0		1.4172	0.8231
2-Methyl-3-buten-2-ol water azeotrope	85.3	− 9.0	23.2	1.4078	
Isoprene	33.3			1.4219	0.6802
Perdeuterio derivatives of compounds and mixtures					
Acetone	54.3			1.3565	0.8719
Water	100.6	3.8	100	1.3286	1.1075
2-Methyl-3-butyn-2-ol	102	1.9		1.4188	0.9423
2-Methyl-3-butyn-2-ol water azeotrope	89.5		27	1.4034	
2-Methyl-3-buten-2-ol	96.0	−28.8		1.4134	0.9185
2-Methyl-3-buten-2-ol water azeotrope	86.0		20.0	1.4053	
Isoprene	31.8			1.4189	0.7604

Study of the boiling points given in Table 1 reveals, except for water where hydrogen-bonding is important, that the perdeuterio derivatives boil at lower temperatures than the corresponding perprotio compounds. Assuming boiling point to be a measure of molecular interaction in small molecules and to be of value for predicting segment interaction in a related polymer, it is expected that there would be less polymer-polymer interaction in the case of D rubber than in the case of H rubber. This expectation is confirmed by the interaction coefficient, μ_1, found by study of toluene solutions which revealed a value of 0.415 compared with 0.398 for H-SN rubber. It is tempting to predict, therefore, that the dynamic properties of D rubber will be found to be better than those of H rubber.

The infrared spectrum of D-SN rubber is of special interest. For example, it contains a band at 15.20μ corresponding to the 11.95-μ band in H rubber. This finding confirms the previously somewhat doubtful assignment of the 11.95-μ wavelength to the H atom on the double bond.

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Succinic Semialdehyde Oxidation by a Soluble Dehydrogenase from Brain

The identification of succinic semialdehyde as a product of transamination of γ-aminobutyrate by brain tissue (*1*) in vitro has led to an investigation of the further metabolism of succinic semialdehyde. Whole homogenates of rat, cat, and monkey brains have been shown to catalyze a diphosphopyridine nucleo-

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