Anal. Calcd. for $C_{15}H_{10}Cl_6N_2O_9$: C, 31.33; H, 1.75; N, 4.87; Cl, 37.00. Found: C, 31.92; H, 1.83; N, 4.68; Cl, 36.40.

The infrared spectrum showed maxima (5% in chloroform) at 1764 (C=O); 1560, 1351 (NO_2); 1274, 1161, 1130, 1082, 1020, 966; 815 cm.$^{-1}$ (CCl_3).

Decomposition of Chloretyl on Heating in Vacuum.—When either α- or β-chloretyl was sublimed at 135–140° (0.2 mm.), a small amount of unstable colorless compound, m.p. 102° dec., was obtained as the more volatile fraction. This substance was tentatively assigned the structure V, **2-methyl-5-trichloromethyl-1-oxacyclopentan-2-ol-3-one**, on the basis of analysis and infrared absorption spectrum: ν_{max}^{Nujol} 3355 (OH); 1751 (C=O); 1185, 1156, 1139, 1099, 995, 936, 911, 869; 799 cm.$^{-1}$ (CCl_3).

Anal. Calcd. for $C_6H_7Cl_3O_3$: C, 30.86; H, 3.02. Found: C, 30.89; H, 2.82.

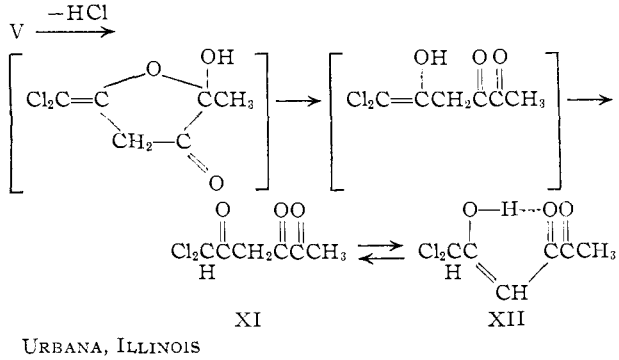
This material soon decomposed on standing at 25° to a yellow oil, with apparent loss of HCl. The infrared spectrum of the oil is consistent with the structure XI, **6,6-dichlorohexane-2,3,5-trione**, which probably exists largely as the enol XII.[27] The infrared spectrum showed maxima (5% in chloroform) at 3600–3000 (broad absorption with much fine structure, characteristic of bonded OH); 1720 (C=O); 1642 (conj. C=O); 1602 (C=C); 1423–1416, 1365, 1320, 1240–1213, 1115, 1098, 977, 840 cm.$^{-1}$.

Preparation of 2,2,2-Trichloroethyl 3,5-Dinitrobenzoate.[26]—To a cold solution of 1.42 g. (6.7 mmoles) of 3,5-dinitrobenzoic acid and 2.54 g. (13.6 mmoles) of *p*-toluenesulfonyl chloride in 20 ml. of cold dry pyridine was added 1.0 g. (6.7 mmoles) of 2,2,2-trichloroethanol. The mixture was allowed to stand at 0° for 1.5 hr. and was poured into 300 ml. of ice-water with vigorous stirring. The resultant peach-colored solid was collected by filtration and recrystallized from 95% ethanol to yield 1.55 g. (68%) of ivory platelets, m.p. 128–131°. Treatment with decolorizing carbon and repeated recrystallization from ethanol yielded an analytical sample of white platelets, m.p. 140–141°.

Anal. Calcd. for $C_9H_5Cl_3N_2O_8$: C, 31.45; H, 1.48; N, 8.15. Found: C, 31.70; H, 1.58; N, 8.33.

The infrared spectrum (5% in chloroform) showed maxima at 1755 (C=O); 1634, 1603 (aromatic); 1554, 1351 (NO_2); 1274, 1163 (C–O); 835 cm.$^{-1}$ (non-enhanced CCl_3). It will be noted that the position of the C=O maximum is at lower frequency than that exhibited by α-chloretyl mono-3,5-dinitrobenzoate.

(27) The formation of XI may be conceived as



URBANA, ILLINOIS

COMMUNICATIONS TO THE EDITOR

THE ENZYMATIC CONVERSION OF MEVALONIC ACID TO SQUALENE

Sir:

The recent isolation and identification of mevalonic acid (DL-β,δ-dihydroxy-β-methylvaleric acid, MVA) a new precursor of cholesterol[1–3] constitute an important advance in the understanding of terpene and steroid biogenesis. In this communication, we wish to report the transformation of mevalonic acid to squalene by soluble yeast enzymes and to describe experiments bearing on the mechanism of MVA utilization. Particle-free extracts of dried baker's yeast have been prepared which catalyze the conversion of DL-2-C^{14}-MVA to a radioactive lipid, shown to be squalene by alumina chromatography and by preparation of the thiourea adduct[4] and of the crystalline hexahydrochloride.[5] No sterols are synthesized from MVA under these conditions. Dialysis and treatment with charcoal to remove cofactors render the yeast extract completely inactive. Activity is restored by addition of Mn^{++}, ATP[6] and pyridine nucleotide (Table I). The reaction proceeds anaerobically as well as in air. Experiments bearing on the mechanism of the condensation process have been carried out with 2-C^{14},5-di-T-mevalonic acid. Tritium was introduced by partial reduction of β-hydroxy-β-methylglutaric acid with $LiAlT_4$. The purified product, when combined with authentic DL-2-C^{14}-MVA and recrystallized several times as the dibenzylethylenediamine salt or chromatographed on paper, showed no change in the T:C^{14} ratio. Squalene synthesized by yeast extracts from the doubly labeled MVA contained T and C^{14} in the same ratio as the precursor (Table II). From the work of Tavormina and Gibbs,[7] it is known that C_1 of mevalonic acid is lost during the conversion to cholesterol and hence the isoprenoid chain must be formed by condensations linking C_5 of one MVA residue (or a derivative thereof) to C_2 of another. The retention of T during squalene formation clearly rules out a preliminary oxidation of mevalonic acid to β-hydroxy-β-methylglutaric acid (or a derivative). Though the unchanged T:C^{14} ratio suggests that both hydrogen atoms at C_5 (δ-carbon) of MVA are

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(6) The following abbreviations are used: DPN and TPN, di- and tri-phosphopyridine nucleotide; ATP, adenosine triphosphate.

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TABLE I

CONVERSION OF DL-2-C^{14}-MVA TO SQUALENE IN YEAST EXTRACTS

Each experimental flask contained 1 ml. of yeast extract, 1 mg. of nucleotide as indicated, Mn^{++} or Mg^{++} 10^{-3} M, 8 μg. of DL-2-C^{14}-MVA (2500 c.p.m.). Incubation in air at 30° for 3 hr.

Additions							c.p.m. in squalene formed by extract	
DPN	DPNH	TPN	TPNH	ATP[a]	Mn^{++}	Mg^{++}	A[b]	B[c,d]
+				+	+		277	192
	+			+	+		407	178
		+		+	+		93	108
			+	+	+		364	308
+				+		+	169	24
+					+		59	18
+				+			4	12
				+	+		36	36

[a] Cryst. ATP-Na salt (Pabst & Co.); the same activity was obtained by preparations of 95% purity. [b] Dried baker's yeast was extracted by incubation with 3 vol. of 0.066 M $(NH_4)_2HPO_4$ for 3 hr. at 37°, the mixture kept at 4° for 16 hr. and centrifuged at 25,000 × g for 30 min. The supernatant was recentrifuged at 105,000 × g for 2 hr., dialyzed for 12 hr. against 4 changes of 0.005 M phosphate buffer pH 7.0, and finally treated with acid-washed Norite-A. [c] The material precipitating from extract A with $(NH_4)_2SO_4$ at 30% saturation was dialyzed for 12 hr. against 0.03 M phosphate buffer, pH 7.0 and centrifuged for 2 hr. at 105,000 × g. This supernatant (prep. B) contained 1.5 mg. of protein per ml. as compared to 12 mg. protein per ml. in prep. A. [d] Experiments carried out by Dr. H. Danielsson.

TABLE II[a]

CONVERSION OF DL-2-C^{14},5-DI-T-MVA TO SQUALENE

	C^{14}, d.p.m.	T, d.p.m.	T/C^{14}
(1) C^{14}, T analyses of MVA[b]			
Preparation IA	2440	7300	3.00
IB	2220	6600	2.97
(2) Incubation experiments[c]			
Squalene formed from IA	450	1400	3.10
IA	560	1520	2.71[d]
IB	360	1040	2.90
IB	1140	3380	2.97

[a] The T and C^{14} analyses were performed with a Packard Liquid Scintillation Counter by the New England Nuclear Corporation, Boston, Massachusetts. [b] The product obtained on treatment of β-hydroxy-β-methylglutaric acid with $LiAlT_4$ in dioxane was adsorbed on Dowex-1-formate and eluted with 0.1 N formic acid. Authentic 2-C^{14}-MVA was added and the mixture crystallized several times as the dibenzylethylenediamine salt.[1] This, after removal of the amine, was chromatographed on paper in an ethanol-ammonia system. Strips were cut separately from the slower moving section (IA) and the middle portion (IB) of the radioactive peak (R_f = 0.57), the MVA eluted with water and aliquots assayed for T and C^{14}. [c] For each experiment aliquots of the 2 fractions (IA and IB) containing 120 μg. of MVA were incubated with 15 ml. of a crude yeast extract (supernatant from 25,000 × g centrifugation, Table I, footnote b) in the presence of 9 mg. DPN, Mn^{++} 10^{-3} M, and 30 mg. of hexose diphosphate, for 3 hr. at 30° in air. In all expts. approximately 30% of the radioactivity was recovered in the hydrocarbon fraction. [d] Isolated and analyzed as the crystalline squalene hexahydrochloride.

retained, the possibility that the R·CH_2O–group is oxidized to R·CHO cannot be ruled out because large rate differences are known to exist for the breaking of C–H and C–T bonds.[8] In any event, these results eliminate a Claisen-type condensation mechanism and raise the distinct possibility that the condensing unit is MVA itself or a derivative of the same oxidation level. The data of Table I indicate that the reduced forms of the pyridine nucleotides are the more efficient cofactors, as would be expected from the reductive nature of the over-all transformation: 6MVA + 2H → squalene + $6H_2O$ + $6CO_2$. The concurrent participation of the oxidized nucleotides nevertheless cannot be excluded at this time. The participation of Mn^{++} is of interest since Mn^{++} is known to accelerate both enzymatic[9] and non-enzymatic[10] decarboxylations. A stimulation of cholesterol synthesis from acetate by Mn^{++} previously has been reported.[11] The dependence of the process on ATP suggests that a phosphorylation step is involved in the formation of squalene from mevalonic acid. This phosphorylation probably is not associated with the activation of carboxyl groups, since no requirement for Coenzyme A could be demonstrated.

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(12) Holder of a Union Carbide and Carbon Fellowship in Biochemistry.

CONVERSE MEMORIAL LABORATORY
HARVARD UNIVERSITY B. H. AMDUR
DEPARTMENT OF CHEMISTRY H. RILLING[12]
CAMBRIDGE 38, MASSACHUSETTS K. BLOCH

RECEIVED APRIL 11, 1957

PREPARATION OF A SOLUBLE MAMMALIAN TYROSINASE[1]

Sir:

Mammalian tyrosinase, unlike the soluble, highly purified,[2] well characterized[3] plant product, has been reported to be firmly bound to melanized cellular particles.[4] In view of the emphasis which has been placed on the difficulty of removing this enzyme from particulate matter,[4,5] we would like to report a relatively simple technique for the preparation of a soluble, melanin-free mammalian tyrosinase.

(1) This work was supported by the National Cancer Institute, grant C-2620.

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