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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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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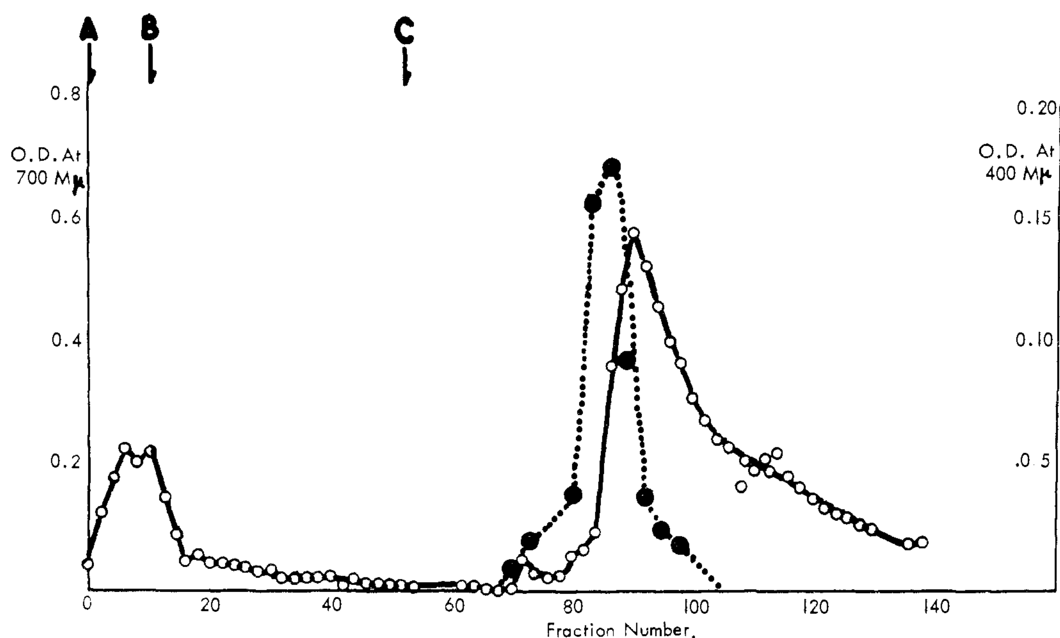

Fig. 1.—Chromatography of tyrosinase on DEAE-cellulose ion exchanger, using 3.0 g. of exchanger (capacity 0.59 meq./g.) in a column 26 × 1.2 cm. Collection after 1 hold-up volume (16 ml.), 2.1-ml. fractions. 0—0—0—0, Folin-Lowry[10] reaction with 0.1-ml. aliquots measured at 700 mμ, Coleman spectrophotometer; ●··●··●, tyrosinase activity of 0.1-ml. aliquots in terms of melanin produced after incubation with tyrosine in 2.7 ml., *p*H 6.8 phosphate buffer for 3 hours. O.D. measured at 400 mμ. A, 0.005 *M* phosphate buffer, *p*H 6.0, plus 0.05 *M* NaCl. B, 0.005 *M* phosphate buffer, *p*H 7.0, plus 0.05 *M* NaCl with gradient change to 0.05 *M* phosphate buffer, *p*H 8.0, through 50 ml. mixing chamber. C, Gradient change to 0.15 *M* phosphate buffer, *p*H 8.0.

Harding-Passey melanoma (415 g.) was homogenized with 2100 ml. of cold distilled H_2O in a Waring Blendor and centrifuged for one hour at 600 × *g*, and the extraction repeated. The supernatant fractions (*p*H 6.9) were combined and made 0.1 satd. with $(NH_4)_2SO_4$. An equal volume of cold acetone was added and the black precipitate was removed on a buchner funnel. These steps are critical to tyrosinase solubility which appears to depend on the ammonium sulfate salting the enzyme into the 50% acetone–water solution. Another volume of acetone was added to the clear filtrate and the precipitate was removed on a buchner, washed with acetone, and dried over P_2O_5 to yield 4.8 g. of a white powder.

The product (3.65 g.) was dissolved in 200 ml. of H_2O, the *p*H was kept at 6–7 with a few drops of 1 *N* NaOH, and was dialyzed for 24 hours against 2 liters of cold distilled H_2O. Lyophilization gave 0.96 g. of active material or 1.2% of the original dry weight of tumor. To establish the absence of cellular particles, a 1% solution of this material was subjected to centrifugation in the ultracentrifuge (Spinco model E). After 1 hour at 260,000 × *g*, the activity remained in the upper part of the cell and the fastest boundary indicated had a sedimentation constant of 3.4, although the material was not homogeneous.[6]

Tyrosinase activity was assayed manometrically against L-tyrosine plus small amounts of dopa.[4a] For the calculation of activity, the unit of Hogeboom and Adams[7] has been used but referred to the protein nitrogen content to obtain specific activity as units per mg. The original homogenate had a specific activity of 2.6 units/mg. N. The product had a specific activity of 25 units/mg. N and contained 18% of the starting activity.

Further purification was attempted by chromatography on a DEAE cellulose[8] column using differential elution.[9] The results are shown in Fig. 1.

The most highly purified fraction (82) from the chromatogram gave a positive biuret test. The remainder of the activity peak was pooled and was shown to catalyze the oxidation of both L-dopa and L-tyrosine through some pink intermediate, presumably dopachrome, to black melanin. Heating at 100° for 30 minutes completely destroyed the activity.

Although preparations having 500 units/mg. N have been obtained, Figure 1 indicates that optimum conditions for complete chromatographic separation were not established. Experiments to improve these conditions are being carried out as rapidly as starting material becomes available.[10]

(6) We are indebted to John A. Cooper for the ultracentrifuge study.

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BIOSYNTHESIS OF SPHINGOSINE IN VITRO

Sir:

We wish to report the preparation of an enzyme system obtained from rat brain tissue which cata-