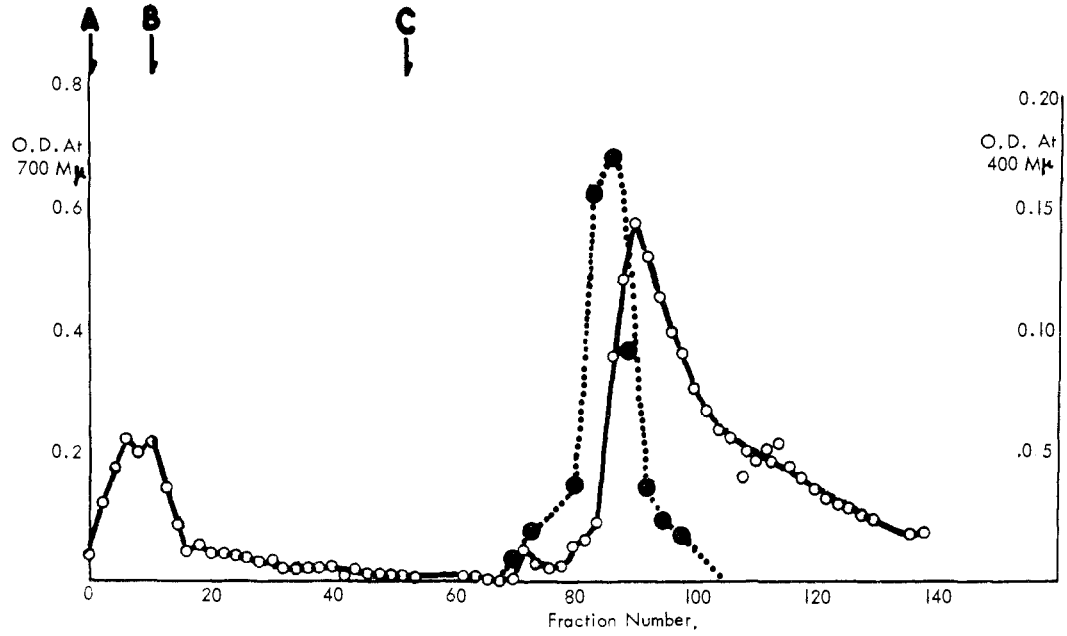

Fig. 1.—Chromatography of tyrosinase on DEAE-cellulose ion exchanger, using 3.0 g. of exchanger (capacity 0.59 meq./g.) in a column 26 × 1.2 cm. Collection after 1 hold-up volume (16 ml.), 2.1-ml. fractions. 0—0—0—0, Folin-Lowry[10] reaction with 0.1-ml. aliquots measured at 700 mμ, Coleman spectrophotometer; ●···●···●, 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-

lyzes the incorporation of serine-C^{14} into sphingosine and to identify some of the cofactors required for this conversion. The incorporation of radioactivity from acetate-1-C^{14} and L-serine-3-C^{14} into sphingosine has been observed *in vivo*.[1,2]

Brain tissue obtained from 10–14 day old rats was homogenized with 3 volumes of isotonic sucrose or 0.1 *M* potassium phosphate buffer (*p*H 7.8), and differential centrifugation was accomplished according to Brody and Bain.[3] The enzymes required for the conversion of L-serine to sphingosine were found in the supernatant solution obtained after sedimenting the particulate matter by centrifuging at 25,000 × *g* for 30 minutes. The enzyme preparation was dialyzed for 4 hours against 100 volumes of a solution containing 0.01 *M* potassium phosphate buffer (*p*H 7.0), 0.001 *M* cysteine hydrochloride and 0.001 *M* disodium ethylenediaminetetraacetic acid.

Following incubation, the lipids were quantitatively extracted with *n*-butanol and 18 μmoles of sphingosine sulfate were added to each sample as carrier. The mixtures were refluxed for 6 hours in 85% methanol made 1.2 *M* with respect to H_2SO_4, and subsequently extracted with petroleum ether which was back-washed two times with dilute acid. The methanolic solution containing the sphingosine sulfate was concentrated under vacuum, and the sphingosine base was recovered from salt-saturated alkaline aqueous phase by extracting with 2% isoamyl alcohol in heptane.[4] The solvents were evaporated under reduced pressure and the sphingosine was recrystallized two times from petroleum ether. Approximately 80% of the carrier sphingosine was recovered with the use of this procedure. The twice recrystallized sphingosine was found to have constant specific radioactivity.

As shown in the table, the conversion of serine-3-C^{14} to sphingosine requires the presence of pyridoxal phosphate, magnesium chloride, triphosphopyridine nucleotide, diphosphopyridine nucleotide, and an as yet unidentified substance present in a kochsaft obtained from rat liver tissue. The recovered sphingosine was degraded with sodium periodate,[4,5] and the radioactivity of the residual aliphatic aldehyde was determined. It is apparent from these data that the radioactivity from the serine-C^{14} is preferentially localized in positions 1 and 2 of sphingosine and that the reaction does not represent random incorporation of radioactivity via an active one or two carbon fragment. These findings have been confirmed with the use of L-serine-U-C^{14} as substrate in this system.

TABLE I

ENZYMATIC CONVERSION OF SERINE-3-C^{14} TO SPHINGOSINE

Except as noted, the incubation mixtures contained 1.5 ml. of dialyzed supernatant solution obtained by centrifuging a 1:3 (w./v.) cell-free preparation of rat brain tissue at 25,000 × *g* for 30 minutes, 150 μmoles of potassium phosphate buffer (*p*H 7.8), 4 μmoles of DL-serine-3-C^{14} (4 μC.), 1 μmole of pyridoxal phosphate, 5 μmoles of $MgCl_2$, 0.3 μmole of triphosphopyridine nucleotide (TPN), 0.3 μmole of diphosphopyridine nucleotide (DPN), 20 μmoles of nicotinamide, 2μmoles of uridine triphosphate (UTP), 0.4 μmole of cytidine triphosphate (CTP), 5 μmoles of adenosine triphosphate (ATP), and 0.1 ml. of liver kochsaft in a total volume of 2.0 ml. Incubation time, 3 hours at 38° in air.

Reactant omitted	Distribution of radioactivity in recovered sphingosine: Sphingosine, c./m./μmole	Residue after periodate degradation, c./m.μmole
None	155	26
None, heated at 100° for 10 min.	3	..
Kochsaft	11	..
Pyridoxal phosphate	35	23
$MgCl_2$	71	20
TPN	90	20
DPN	93	22
Nicotinamide	110	25
UTP	140	26
CTP	165	25
ATP	170	28

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A NOVEL ORGANO-CHROMIUM COMPOUND

Sir:

The known organo-chromium compounds, apart from the anionic cyanide and ethynyl complexes[1] of chromium(III) are compounds of chromium(0), chromium(I) or chromium(II)[2] which generally do not contain simple carbon–chromium bonds. We wish to report the first preparation (in solution) of a simple organo-chromium compound of the type $Cr(H_2O)_5R^{++}$ where R is benzyl. The compound has not yet been obtained crystalline, but solutions of the pure perchlorate in dilute perchloric acid solution have been obtained and are fairly stable (half-life in absence of oxygen at room temperature of about 1.5 days). The structure of the compound, benzylpentaaquochromium(III) perchlorate, can be regarded as analogous to that of the chloride complex, $Cr(H_2O)_5Cl^{++}$, of chromium(III); *i.e.*, the compound is a complex of Cr(III) with the *benzyl anion*. The remarkable stability of this organo-metallic compound in acid solution is undoubtedly related to the inertness of Cr(III) complexes to substitution.[3]

The benzylchromium compound was prepared by reducing benzyl chloride (or bromide or iodide) with aqueous 1 *M* chromous perchlorate in 1 *M* perchloric acid, either heterogeneously or in presence of alcohol or other suitable organic solvents. Countercurrent distribution of the product at 5° with the solvent system 0.01 *M* perchloric acid-butanol gave, after 70 transfers, a separation of the organo-chromium compound (partition coefficient

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