Tumor Localizing Agents I.
Synthesis and Tissue Distribution of ^{14}C-Labeled and Radioiodinated 5,6-Diacetoxyindole

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Interest in the development of radiopharmaceuticals suitable for the diagnosis and treatment of malignant melanoma prompted the present study. One of the first approaches to this problem has involved the synthesis of suitably labeled precursors of melanin with the hope that these substances would be selectively taken up by the pigmented tumor and rapidly converted to a radiolabeled melanin within the tumor. Since 5,6-dihydroxyindole is a late precursor of melanin, the synthesis of this material labeled with ^{14}C was undertaken. This substance, however, proved to be too unstable and was therefore converted to the diacetate for animal studies. The radioiodine-labeled analog was prepared by iodine exchange of 5,6-diacetoxy-7-iodoindole with sodium iodide-^{125}I. Neither 5,6-diacetoxyindole-2-^{14}C nor the ^{125}I analog showed a predilection for melanomas in mice.

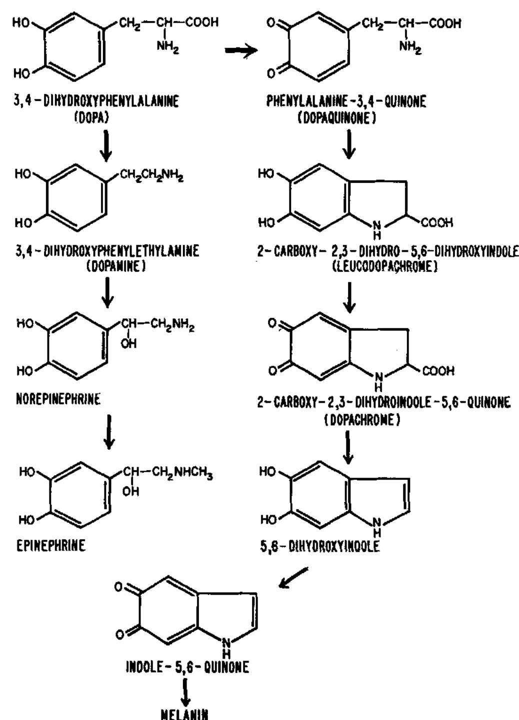
IN A RECENT REVIEW on the clinicopathological status of malignant melanoma, Raven (1) emphasized the need for new diagnostic and therapeutic agents for this disease. For several years now, the authors have been involved in a broad program aimed at the synthesis of substances which will localize in various tumors or organs. One objective of this program has been to find compounds that will selectively concentrate in melanotic tissues. Such compounds, when labeled with γ-emitting radionuclides, can be readily detected by external photoscanning techniques and thus afford a means for determining the location and metastatic nature of melanotic tumors.

One approach to the development of radiopharmaceuticals having a predilection for melanomas has involved an investigation of radio-labeled precursors of melanin. Since melanin appears to be an end product of tyrosine metabolism, it seemed possible that suitably labeled precursors of melanin could be selectively taken up by the pigmented tumor and rapidly converted to a labeled melanin which would remain within the tumor. Recent publications by several investigators have served to support the validity of this hypothesis. Blois and Kallman (2) studied the uptake of *dl*-dihydroxyphenylalanine-2-^{14}C (dopa-2-^{14}C) in mice with deeply pigmented melanomas and found the radioactivity of the different tissues to be in the order: melanoma $>$ adrenal $\gg$ kidney $>$ liver $>$ spleen. Similarly, Fimiam and Dowd (3) noted that when *dl*-tyrosine-2-^{14}C was administered to melanotic mice significantly greater radioactivity was associated with melanoma than with any other tissue studied.

On the basis of this information, the authors elected to synthesize radiolabeled compounds structurally similar to biological precursors of melanin. As indicated in the proposed pathway of melanin biosynthesis (Scheme I), tyrosine and dopa serve as precursors for not only melanin but also catecholamines (4). Since a high target–nontarget ratio in melanomas compared to surrounding tissues was desired, it seemed advisable to select precursors formed in the later stages of melanogenesis for the studies. The synthesis and preliminary tissue distribution data for two radiolabeled derivatives of 5,6-dihydroxyindole are reported here.

In the preliminary experiments, 5,6-dihydroxyindole proved to be too unstable for use in the tissue distribution experiments. The protected *O*-acetylated product, however, proved to be much more stable and suitable for the animal studies.

Among the various methods previously reported for the synthesis of 5,6-dihydroxyindole and its derivatives, the approach of Beer and co-workers



Biosynthesis of Melanin and Epinephrine

Scheme I

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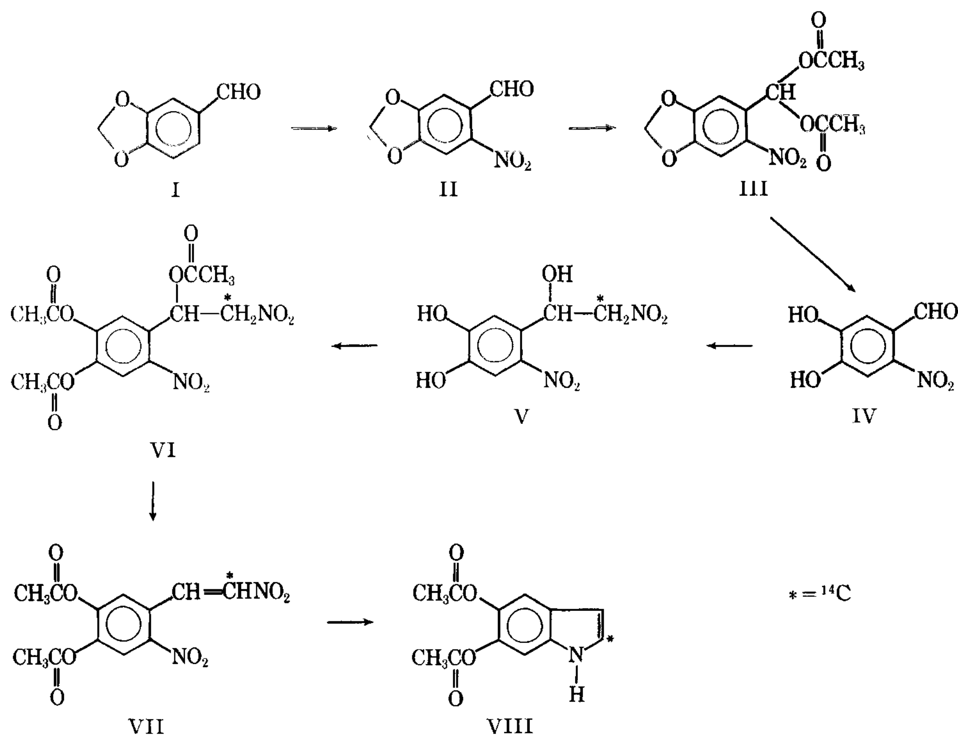
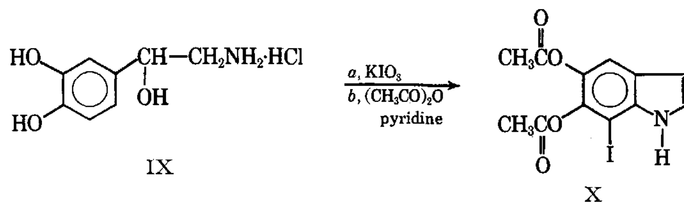


Synthesis of 5,6-Diacetoxyindole-2-^{14}*C*

Scheme II



Synthesis of 5,6-Diacetoxy-7-iodoindole

Scheme III

(5) seemed the most amenable for preparation of ^{14}C-labeled 5,6-diacetoxyindole. As outlined in Scheme II, piperonal (I) served as the starting material and was readily converted to 6-nitropiperonal (II). This product was then converted to acylal (III) and demethylenated according to the method of Parijs (6).

The radiolabel was introduced at this stage by condensation of IV with nitromethane-^{14}C. The subsequent steps were studied in detail in preliminary experiments so that the authors could proceed directly to the final product without isolating intermediates and thus minimize losses of radioactivity. Accordingly, the nitroalcohol V was immediately acetylated to give the triacetate (VI). Dehydroacetylation and reductive ring closure with iron in acetic acid afforded, after chromatography, 5,6-diacetoxyindole-2-^{14}C (VIII) in 43% over-all yield from IV.

The radioiodine-labeled product was prepared by isotope exchange of 5,6-diacetoxy-7-iodoindole (X) with sodium iodide-^{125}I. The iodinated indole was readily obtained by potassium iodate oxidation of *dl*-norepinephrine hydrochloride (IX) according to the procedure of Heacock *et al.* (7) (Scheme III). Glacial acetic acid proved to be the most suitable solvent for the iodine exchange, and the rate of exchange could be readily followed by radioscanning of thin-layer chromatograms (TLC).

Tissue Distribution Studies[1]—Table I compares the distribution of radioactivity in various tissues after administration of *dl*-dopa-2-^{14}C and the radiolabeled indole derivatives to mice with deeply pigmented melanomas. In contrast with Blois and Kallman's results (2), the *dl*-dopa-2-^{14}C showed a greater concentration of radioactivity in adrenal than in melanoma. At 24 hr., for example, the concentration in adrenal was almost 15 times that in melanoma. Similar results were obtained by Hempel and Deimel (9) with tritiated *dl*-dopa in mice with Harding-Passey melanoma.

[1] A more detailed description of these findings and related material has been reported elsewhere (8).

TABLE I—TISSUE DISTRIBUTION OF RADIOACTIVITY AFTER INJECTION OF RADIOLABELED MELANIN PRECURSORS IN MELANOTIC MICE[a]

Compd.	Time Sacrificed After Injection, hr.	No. of Mice[b]	Melanoma	Liver	Adrenal	Kidney	Injection Site
dl-Dopa-2-^{14}C	0.5	9	1.2	2.6	2.3	6.0	...
	2	2	2.4	2.2	9.0	3.0	...
	4	10	3.2	2.2	13.6	...	...
	24	5	4.0	2.3	59.8	7.0	...
5,6-Diacetoxyindole-2-^{14}C	0.5	2	0.1	0.5	0.9	5.0	4.5
	2	4	0.4	0.4	0.5	9.2	3.5
	4	2	0.5	0.4	0.6	14.0	12.6
	24	2	0.7	0.8	0.6	6.1	10.7
7I odo-5,6-diacetoxy-indole-^{125}I	0.5	3	0.1	0.4	0.4	4.1	22.4
	2	3	0.3	0.4	0.3	2.1	...
	4	3	0.3	0.4	0.4	2.1	15.0
	24	5	0.4	0.3	0.4	0.9	16.8

[a] Tissue–serum ratios in c.p.m./mg. [b] Male mice with transplanted deeply pigmented B16 melanomas obtained from Jackson Laboratory, Bar Harbor, Me.

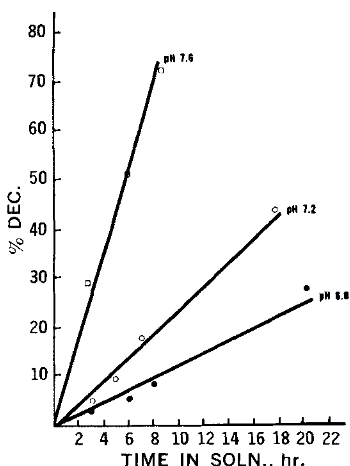


Fig. 1—Decomposition of 5,6-diacetoxyindole in aqueous solution at three pH values.

Neither 5,6-diacetoxyindole-2-^{14}C nor the ^{125}I analog showed selective uptake by the tumor. The site of injection, however, blackened rapidly after administration, suggesting that the diacetoxyindoles hydrolyzed to the dihydroxyindoles and rapidly polymerized to melanin-like materials. This undoubtedly accounted for the high levels of radioactivity at this site. The kidneys also became dark and the urine brown-black in color following administration of these compounds.[2] Presumably the concentration of radioactivity in the kidney represents renal excretion of these products or their metabolites. The rapid formation of dark melanin-like products at these sites indicates that a metabolic precursor of melanin preceding dihydroxyindole must enter the melanocyte prior to oxidative polymerization to melanin. Other potential precursors of melanin are currently under investigation.

Stability Studies—The high concentration of radioactivity at these sites prompted the authors to determine the effect of pH on the stability of 5,6-diacetoxyindole. The rate of decomposition at pH 6.8, 7.2, and 7.6 was ascertained by measurement of the absorbance of buffered aqueous solutions at 218 mμ. As shown in Fig. 1, decomposition of 5,6-diacetoxyindole increased with increasing pH. The high concentration of activity at the site of injection could therefore be related to the slightly basic pH at this site (10) or to the presence of an esterase. With regard to the latter possibility, Riley (11) recently demonstrated that 5,6-diacetoxyindole is rapidly converted to melanin in sections of human skin and presented evidence to support the presence of an indole-5,6-acetyl esterase.

EXPERIMENTAL[3]

5,6-Diacetoxyindole-2-^{14}C (VIII)—A solution of 6-nitro-protocatechuic aldehyde (5, 6) (IV, 2.5 Gm., 13.6 mmole) and nitromethane-^{14}C (0.84 Gm., 13.8 mmoles, 1.13 mc.)[4] in ethanol (25 ml.) was cooled to 0°. To this solution was added dropwise with stirring a solution of potassium hydroxide (1.8 Gm.) in 95% ethanol (25 ml.) while maintaining the reaction temperature at 0°. Following the addition period of about 1 hr., the reaction mixture was stored at 0° for 24 hr. and diluted with water (30 ml.). The mixture was acidified with 10% HCl

[2] Radiochromatogram analysis of the solutions used in the animal experiments indicated that no decomposition of the compounds had taken place prior to the tissue localization studies

[3] Melting points were taken on a Fisher-Johns melting point apparatus and are corrected. Ultraviolet spectra were recorded on a Beckman DK2A spectrophotometer in phosphate buffer system (pKa 6.8). Thin-layer chromatograms of radiolabeled materials were run with 1-in. wide Eastman chromatograms, type K301R with fluorescence indicator, and the compounds were detected with ultraviolet light. The distribution of radioactivity on the developed chromatograms was determined by scanning each strip with an Atomic Associates RCS-363 radiochromatogram scanner. The specific activities were determined with an Atomic Associates well scintillation counter model 810C and scintillation spectrometer model 530. The silicic acid used in the column chromatography was Baker and Adamson reagent grade.

[4] Purchased from New England Nuclear Corp., Boston, Mass.

and extracted with ether (6 × 25 ml.). The extract was washed with 10% sodium bisulfite solution (100 ml.) and dried over a mixture of anhydrous sodium sulfate and a decolorizing agent.[5] Removal of the solvent gave a solid (crude V) which was acetylated directly with acetic anhydride (10 ml.) containing a few drops of 70% perchloric acid. The acetylation mixture was allowed to stand at room temperature for 30 min. Water (25 ml.) was then added and the product was extracted with chloroform (5 × 25 ml.). Evaporation of the dried chloroform solution (anhydrous sodium sulfate) furnished the solid triacetate (VI).

This triacetate (VI), without further purification, was treated with acetic anhydride (12 ml.) and anhydrous sodium acetate (6.0 Gm.) at 140° for 2 min. The cooled reaction mixture was diluted with water (25 ml.) and extracted with chloroform (5 × 40 ml.). The extract was washed with 5% sodium bicarbonate solution (100 ml.), dried (anhydrous sodium sulfate), and evaporated to afford the solid, dinitrostyrene derivative (VII).

Crude VII was suspended in glacial acetic acid (65 ml.) and added dropwise over a 3-hr. period to a stirred mixture of glacial acetic acid (50 ml.) and iron powder (18.6 Gm.). No external heat was applied. The reaction mixture was stirred for an additional 3 hr. at room temperature. The solid residue was removed by filtration and washed well with hot ethanol. The resulting filtrate was diluted with water (50 ml.), neutralized with solid $KHCO_3$, and extracted with ether (7 × 50 ml.). The extract was treated with anhydrous sodium sulfate and the decolorizing agent and the solvent were removed *in vacuo*. The resulting solid brown residue was dissolved in a minimum of benzene, and adsorbed onto silica gel (25 Gm.), and the column was eluted with benzene. The solvent was removed from the combined indole-containing fractions[6] under reduced pressure to give pure VIII (1.37 Gm., 43%) m.p. 131–133° [lit. (5) m.p. 134–136°] with a specific activity of 90 μc./mmole.

Purity of the final product was checked by mixed melting point with an authentic sample, and by ascending paper chromatography using benzene as the solvent. Chemical and radiochemical purity were confirmed when the radiochromatogram scan of the developed chromatogram showed only one peak, corresponding to the same position as the indole compound.

5,6-Diacetoxy-7-iodoindole-^{125}I (X)—An aqueous solution of $Na^{125}I$ (1 ml., 350 μc.)[7] was evaporated under nitrogen (45°), and a solution of 5,6-diacetoxy-7-iodoindole (7) (101.2 mg.) in acetic acid (5 ml.) was added. The solution was refluxed for 30 min. and diluted with water (20 ml.), and solid sodium bicarbonate was added in small portions to neutralize the acetic acid. The resulting mixture was extracted with benzene (5 × 10 ml.), and the extract was washed with 5% sodium bicarbonate solution and dried (anhydrous sodium sulfate). Removal of the solvent *in vacuo* gave a residue which was purified by column chromatography over silica gel (5 Gm.) and elution with chloroform–benzene (1:1). The indole-containing fractions were combined and concentrated under nitrogen. Petroleum ether (b.p. 30–40°) was added periodically until a white crystalline material appeared. Removal of the supernatant afforded pure X (93 mg.), m.p. 125.7–127.1° [lit. (7) m.p. 125–126.5°], with a specific activity of 484 μc./mmole. Exchange occurred to the extent of 35.6%. Purity of the final product was established by TLC using ethyl ether as the eluant. The developed chromatogram was subsequently subjected to a radiochromatogram scan in which only one area of radioactivity was found which was superimposable with the developed indole spot.

Spectrophotometric Analysis of 5,6-Diacetoxyindole Solutions—Standard phosphate buffer solutions (pKa 6.8) were prepared for pH values of 6.8, 7.2, and 7.6, and their lack of absorbance in the ultraviolet region (210–360 mμ) was established. The buffered stock solutions of 5,6-diacetoxyindole were prepared by dissolving the indole in a minimum volume of methanol and diluting to volume with the respective standard phosphate buffer solutions. Subsequent dilutions of the indole stock solutions for U.V. determinations were made with the corresponding phosphate buffer. The pH of each solution was checked experimentally before and after spectrophotometric determination. Data from these experiments provided standard linear curves of concentration of indole *versus* absorbance for each pH value. The rates of decomposition for these dilute solutions of 5,6-diacetoxyindole were determined from the standard linear curves. Results are shown in Fig. 1.

[5] Norit, American Norit Co., Jacksonville, Fla.
[6] Indole-containing fractions were identified with Erlich's reagent.
[7] Purchased from Nuclear Science and Engineering Corp., Pittsburgh, Pa.

REFERENCES

(1) Raven, R. W., *Ann. N. Y. Acad. Sci.*, **100,** 142(1963).
(2) Blois, M. S., and Kallman, R. F., *Cancer Res.*, **24,** 863(1964).
(3) Fimiam, W. J., and Dowd, G. A., *Proc. Soc. Exptl. Biol. Med.*, **102,** 95(1959).
(4) Meister, A., "Biochemistry of the Amino Acids," vol. II, 2nd ed., Academic Press Inc., New York, N. Y., 1965, pp. 909, 919.
(5) Beer, R. J. S., Clarke, K., Khorana, H. G., and Robertson, A., *J. Chem. Soc.*, **1948,** 2223.
(6) Parijs, A. H., *Rec. Trav. Chim.*, **49,** 33(1930).
(7) Heacock, R. A., Mahon, M. E., and Scott, B. D., *Can. J. Chem.*, **39,** 231(1961); Heacock, R. A., Hutzinger, O., Scott, B. D., Daly, J. W., and Witkop, B., *J. Am. Chem. Soc.*, **85,** 1825(1963).
(8) Meier, D. A., Counsell, R. E., and Beierwaltes, W. H., *Cancer Res.*, to be published.
(9) Hempel, K., and Deimel, M., *Strahlentherapie*, **121,** 22(1963).
(10) Best, C. H., and Taylor, N. B., "The Physiological Basis of Medical Practice," 7th ed., Williams and Wilkins Co., Baltimore, Md., 1961, p. 131.
(11) Riley, P. A., *Nature*, **213,** 190(1967).