pressure of 2.8 kg/cm² (40 psi). The pressure drop after 90 min indicated that the reduction was 92% complete. Fresh catalyst (0.25 g) was added and shaking under hydrogen was continued for 15 min with no additional hydrogen absorption. The mixture was filtered by suction under nitrogen and the pale yellow filtrate, which started to turn dark immediately, was acidified with dry HCl. The precipitated amine hydrochloride was filtered by suction under nitrogen and immediately dried under vacuum. The green-gray powdery product (9.4 g, 81%) was very hygroscopic and on exposure to air turned instantly to a black tar; it was used in the subsequent preparation without further purification.

Sodium nitrite (1.4 g, 0.02 mole) in 3 ml of water was added dropwise during 10 min to a stirred and cooled (0–5°) solution of 7.8 g (0.02 mole) of crude N,N-bis(2-methanesulfonoxyethyl)-*p*-phenylenediamine hydrochloride in 11 ml of fluoroboric acid (48–50%). The black tarry mixture was stirred an additional 10 min at 5° and the water layer was decanted from the sticky tar. The tar was dissolved in 60 ml of acetonitrile, stirred with decolorizing charcoal, and filtered, and ether was added to the filtrate (20 ml) until crystallization started. The mixture was cooled at −15° for 70 hr and then filtered by suction to give 3.5 g of brownish green crystals, mp 135° dec. The product was recrystallized twice from the same solvent mixture (with charcoal treatment) to give 1.7 g (20%) of dull yellow crystals: mp 135° dec; $\nu_{max}^{(halocarbon\ or\ Nujol)}$ 3030–2950, 2230, 2180, 1590, 1520, 1350, 1175 cm⁻¹.

Anal. Calcd for $C_{12}H_{18}BF_4N_3O_6S_2$: C, 31.94; H, 4.02; N, 9.31. Found: C, 31.43; H, 4.08; N, 9.56.

Acknowledgment.—We thank Dr. Wilson M. Whaley for his contributions and encouragement in the initial phases of our program, Mrs. Frances Potts Fernandes for technical assistance, and Dr. G. Richard Handrick for helpful discussions.

Tumor Localizing Agents. Radioactive Iodofluorenaminesulfonic Acids[1]

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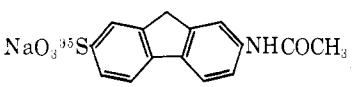
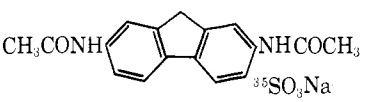
Received April 13, 1966

Two compounds, sodium N-2-(3-iodofluorenyl)acetamido-7-sulfonate-^{131}I (VI) and N,N'-2,7-(3-iodofluorenylene)bisacetamido-6-sulfonic-^{131}I acid (XI), were studied for distribution of radioactivity in tumor-bearing mice. The results clearly indicate that both compounds tend to localize in tumor tissue to a greater extent than in many organs or muscle. Compound VI gave the best ratios (concentration in tumor/concentration in tissue) at the end of 8 hr after injection. It showed a statistically significant difference at 0.01 level of probability with liver, kidney, stomach, muscle, and blood, and with spleen at 0.05 level of probability.

For some years, work in these laboratories has been aimed at finding a compound that would display preferential affinity for tumor tissue; if this compound were made radioactive, it could be used in the diagnosis and therapy of internal cancer. Our previous studies have shown that certain ^{35}S-labeled derivatives of fluorenesulfonic acids have an affinity for the tumor tissue.[2] But these compounds did not always give a favorable ratio of uptake of ^{35}S-labeled compound by tumor to other organs, especially liver and kidney. Recently we have elaborated[3] the fluorenesulfonic acid molecule by introducing an additional moiety, the basic amino group, to facilitate the protein binding of the compound.[4] These new fluorenaminesulfonic-^{35}S acids localized in tumor to a greater extent than in vital organs such as kidney, liver, and spleen. One of these compounds, sodium N-2-fluorenylacetamido-7-sulfonate (I), gave a favorable ratio (concentration in tumor/concentration in the tissue) with liver after 16 hr. The favorable ratios with kidney, spleen, and blood increased with increased time. This indicated that the compound is eliminated less readily from the tumor tissue than from the vital organs: it shows the affinity of the substance for the tumor tissue. The second compound, N,N'-2,7-fluorenylenebisacetamido-3-sulfonate-^{35}S (II), gave better ratios of about 4.0 or more at the end of 8 hr with the vital organs. These compounds have shown sufficient relative and absolute tumor tissue concentration to be most suitable for further investigation.



I



II

^{35}S-labeled compounds, while useful for animal experimentation, have the disadvantages associated with low-energy β emission (0.168 mev) for clinical use. It was, therefore, felt that the labeling of these potentially interesting compounds with ^{131}I (a γ and β emitter) might combine ease of detection and estimation with even a possible therapeutic dose of radiation derived from the compound itself since ^{131}I-iodide is used in the therapy of thyroid carcinoma. The use of a γ-emitting isotope could make possible the visualization of tumor tissue by photoscanning. Our attention was focused on the iodination of fluorenaminesulfonic acids because the fluorenamine molecule has been found to retain iodine despite metabolic processes.[5] This should be a great advantage over such compounds as tetrasodium 2-methyl-3-halo-1:4-naphthohydroquinone diphosphate in which the substituted halogen atom at the 3 position was quickly removed from the hydroquinone ring.[6] In the present work, therefore, we

(1) This investigation was supported by U. S. Public Health Service Grant CA 08186 from the National Cancer Institute.

(2) (a) M. F. Argus, *Brit. J. Cancer*, **7**, 273 (1953); (b) M. F. Argus and K. Hewson, *ibid.*, **8**, 698 (1954).

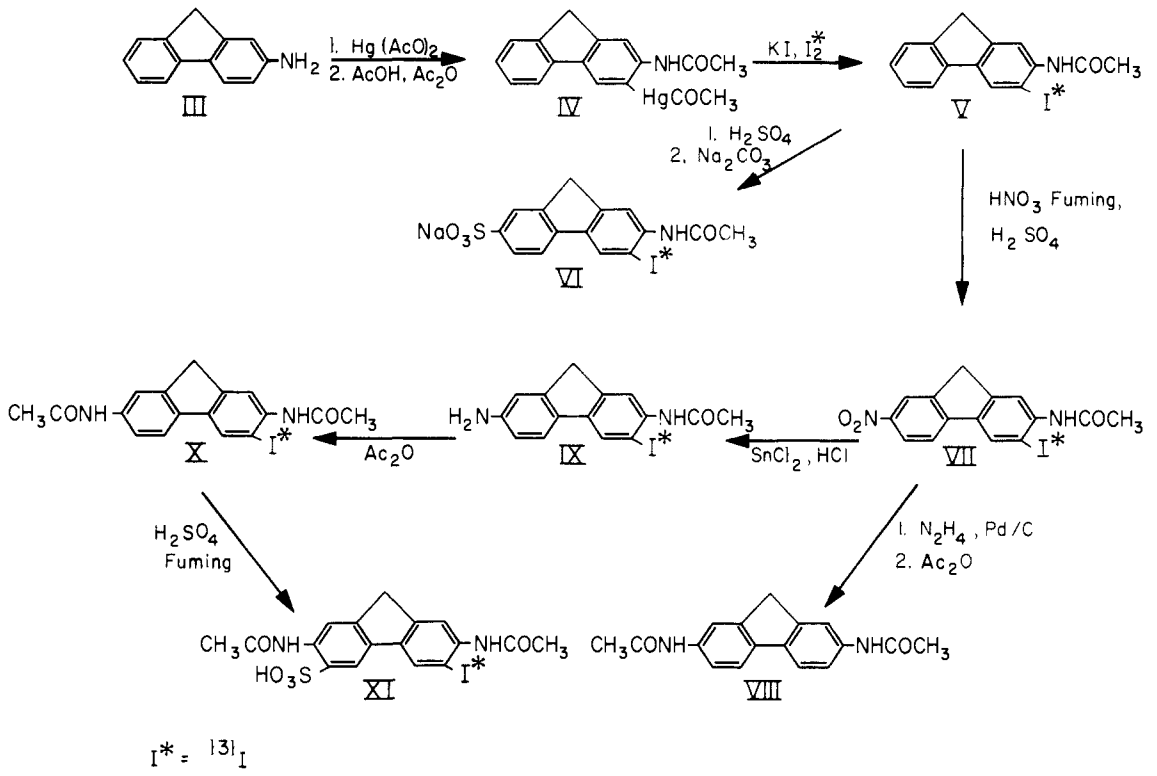
(3) F. E. Ray and K. C. Agrawal, *Cancer Res.*, in press.

(4) H. M. Dyer and H. P. Morris, *J. Natl. Cancer Inst.*, **17**, 677 (1956).

(5) H. M. Dyer, *ibid.*, **16**, 11 (1955).

(6) D. H. Marrian and D. R. Maxwell, *Brit. J. Cancer*, **10**, 739 (1956).

SCHEME I



have synthesized the iodinated fluorenaminesulfonic acids using ^{131}I. It could be that the addition of iodine atom (at. wt 131) to the molecule might alter the properties of the parent compound. Therefore, biological studies of these compounds were undertaken.

Chemistry.—Sodium N-2-(3-iodofluorenyl)acetamido-7-sulfonate (VI) was prepared by sulfonation of N-2-(3-iodofluorenyl)acetamide (V, see Scheme I). Direct iodination of N-2-fluorenylacetamido-7-sulfonic acid (I) was unsuccessful. The synthesis of the intermediate V was achieved by the mercuration of 2-fluorenamine (III) to give a 2-amino-3-acetoxymercurifluorene complex. Acetylation of this product with acetic anhydride at room temperature yielded 2-acetylamino-3-acetoxymercurifluorene (IV). Replacement of the acetoxymercuri group by iodine (^{131}I) gave the intermediate V. Direct iodination of 2-fluorenamine produces a 7-iodo derivative, but mercuration has been shown to occur at the 3 position.[7]

N,N′-2,7-(3-Iodofluorenylene)bisacetamido-6-sulfonic acid (XI) was obtained by sulfonation of N,N′-2,7-(3-iodofluorenylene)bisacetamide (X). Attempts to obtain compound XI by iodination of II were unsuccessful. Compound X also could not be made by the iodination of VIII. Compound V was, therefore, nitrated with fuming nitric acid to give N-2-(3-iodo-7-nitrofluorenyl)-acetamide (VII). Hydrogenation in the presence of platinum and by hydrazine hydrate in presence of catalytic Raney nickel or palladized charcoal dehalogenated the molecule to give N-2-(7-aminofluorenyl)-acetamide. The latter compound on acetylation gave N,N′-2,7-fluorenylenebisacetamide (VIII). This reaction gave proof that nitration in IV took place at the 7 position. Reduction with stannous chloride and hydrochloric acid gave the desired compound N-2-(3-iodo-7-aminofluorenyl)acetamide (IX). During this reaction the acetyl group at position 2 remained intact, possibly due to the steric hindrance of the bulky *o*-iodo group. Compound IX on acetylation gave N,N′-2,7-(3-iodofluorenylene)bisacetamide (X) which on sulfonation yielded the desired XI, N,N′-2,7-(3-iodofluorenylene)bisacetamido-6-sulfonic acid.

(7) (a) E. H. Huntress, K. Pfister, and K. H. T. Pfister, *J. Am. Chem. Soc.*, **64**, 2845 (1942); (b) E. K. Weisburger, J. H. Weisburger, and F. E. Ray, *J. Org. Chem.*, **16**, 1697 (1951).

Biological Results and Discussion

The distribution in tumor-bearing CAF_1/Jax mice at different time intervals following intraperitoneal injection of VI and XI are given in Tables I and II, respectively. The ratios of concentration of the compound in tumor to the concentration in tissue are also given. Time intervals between the injection of ^{131}I-labeled compounds and killing of the animals were chosen to be the same as in the case of noniodinated compounds so that maximal comparison could be made to determine the optimum molecular size for tumor localization. Compound VI localized in tumor almost four times as much at 8 hr as after 6 hr, while the reverse was true for liver, liver having four times as much concentration at 6 hr as at 8 hr. The ratio of the concentration of radioactivity between tumor and liver then remained relatively constant up to 16 hr. The concentration of radioactivity decreased in all the tissues except the tumor while going from 6 to 8 hr. Thus, the ratios which were not favorable at 6 hr improved to a great extent at 8 hr. This increase in ratios indicates the selective localization of VI in the neoplastic tissue. These observations were similar to those in the case of noniodinated compound[3] but the ratios of the iodinated compound were better. By 16 hr, elimination of the radioactivity appeared to be more general than selective. The ratios with kidney and spleen were increased

TABLE I

COMPARISON OF THE CONCENTRATION OF RADIOACTIVITY IN TUMOR TISSUE TO THE CONCENTRATION IN OTHER TISSUES FOLLOWING INTRAPERITONEAL INJECTION OF SODIUM N-2-(3-IODOFLUORENYL)ACETAMIDO-7-SULFONATE-^{131}I (VI)

	6 hr			8 hr			16 hr		
Tissue	C^a	R^b	t^c	C^a	R^b	t^c	C^a	R^b	t^c
Tumor	25.9			94.7			27.1		
Liver	65.2	0.40	<1	16.7	5.7	37.8[d]	4.8	5.6	33.8[d]
Kidney	50.7	0.51	<1	24.9	3.8	34.7[d]	4.3	6.3	26.6[d]
Stomach	38.5	0.67	<1	35.0	2.7	24.3[d]	44.9	0.60	<1
Intestine	955.0	0.03	<1	419.0	0.22	<1	283.0	0.10	<1
Spleen	43.6	0.59	<1	80.1	1.2	5.6[e]	12.0	2.2	13.6[d]
Muscle	15.5	1.7	19.3[d]	9.7	9.7	57.2[d]	7.5	3.6	19.3[d]
Blood	35.7	0.73		9.5	9.9	31.6[d]	6.3	4.3	54.9[d]
Urine	21.5[f]			28.7[f]			41.5[f]		

[a] Concentration in micrograms of compound/gram of tissue or milliliter of blood (wet weight or volume). Average value from three animals. [b] Ratio of concentration in tumor/concentration in tissue. [c] t is the value of "Paired Difference t-Test" on the log concentrations. [d] t is significant at the 0.01 level of probability. [e] t is significant at the 0.05 level of probability. [f] Per cent of administered dose based on recovered radioactivity.

TABLE II

COMPARISON OF THE CONCENTRATION OF RADIOACTIVITY IN TUMOR TISSUE TO THE CONCENTRATION IN OTHER TISSUES FOLLOWING INTRAPERITONEAL INJECTION OF N,N'-2,7-(3-IODOFLUORENYLENE)BISACETAMIDO-6-SULFONIC-^{131}I ACID (XI)

	8 hr			16 hr		
Tissue	C^a	R^b	t^c	C^a	R^b	t^c
Tumor	30.4			17.0		
Liver	55.5	0.54	<1	7.6	2.2	83.4[d]
Kidney	469.2	0.06	<1	41.8	0.41	<1
Stomach	273.8	0.11	<1	1133	0.01	<1
Intestine	1580	0.02	<1	966.0	0.02	<1
Spleen	25.1	1.2	7.0[d]	13.5	1.2	4.5[e]
Muscle	26.1	1.2	3.2[e]	13.5	1.2	4.4[e]
Blood	35.0	0.87	<1	12.5	1.4	5.5[e]
Urine	4.4[f]			14.5[f]		

[a–f] See corresponding footnotes in Table I.

but at the same time the ratios with stomach, intestine, muscle, and blood decreased. The increase of radioactivity in stomach tissue indicated that the stomach was not excreting the compound competitively. This was consistent with the results obtained from noniodinated compounds. It is likely that the high level of radioactivity in the gastrointestinal tract was a result of accumulation in the contents rather than actual tissue localization, because the mice were not fed during the time of experiment. Radioactivity was slowly excreted in the urine, about 41% of a 5-mg dose appearing during 16 hr.

Statistically, at the end of 8 hr the concentration of radioactivity in tumor was significantly different with liver, kidney, stomach, muscle, and blood at the 0.01 level of probability. Spleen showed significant difference at the 0.05 level of probability. The ratios of about 2–3 over the surrounding tissue have been found to give sufficient concentration of the radioactive compound for successful tumor diagnosis.[8] The measurement of the radioactivity of ^{131}I-labeled compounds VI and XI in tissues appears to be an accurate reflection of the actual concentration of the unaltered drug, since the halogen is not metabolized to any degree.[5] Therefore, the results obtained with VI are encouraging in this approach.

Compound XI with two acetylamino groups (Table II) did not reflect any improvements on the results obtained with VI; on the contrary the ratios were mostly unfavorable. At the end of 8 hr liver, kidney, stomach, and blood gave ratios of less than 1. Spleen and muscle were the only tissues giving favorable ratios. With increased time, the compound seemed to be eliminated somewhat faster from the liver and blood than from tumor. The ratios with spleen and muscle showed very little variation. A high level of radioactivity was still present in kidney after 16 hr. Stomach showed increase in concentration of radioactivity to a great extent. Statistically, spleen, muscle, and blood were significantly different at 0.05 level of probability, while liver differed at the 0.01 level of probability. Excretion through urine was very slow, only 14.5% of a 5-mg dose appearing during 16 hr. From these data, we may conclude that because XI was relatively larger in size (mol wt 486), the kidney was not able to excrete substantial amounts. It has been shown previously that similar compounds are taken up by the phagocytic reticulum cells which are most effective on the larger molecules.[9]

Compound II gave the ratios of about 4.0 or more with all the tissues[3] but the addition of iodine atom in the molecule changed the properties of the parent compound to a considerable extent, giving unfavorable or lower ratios. But this was not so in the case of VI, where the ratios have become more favorable after introducing iodine in I. We may, therefore, conclude that VI gave us an optimum molecular size for selective tumor localization in mice which might be extended to successful tumor diagnosis and possible therapy.

Experimental Section[10]

2-Acetylamino-3-acetoxymercurifluorene (IV).—To a magnetically stirred solution of 2-fluorenamine (III, 18.12 g, 0.1 mole) in 400 ml of ethanol, cooled at 25°, was slowly added during 15 min 32 g (0.1 mole) of mercuric acetate dissolved in 200 ml of water containing 4 ml of acetic acid to prevent hydrolysis. A greenish precipitate was formed and was allowed to stand overnight whereupon the color changed to brown. The precipitate was filtered, washed, and after drying at room temperature weighed 42 g; it could not be recrystallized without decomposition. This crude product was stirred mechanically for 4 hr in a mixture of 350 ml of glacial acetic acid and 50 ml of acetic anhydride. The solution was then heated over steam bath for 15 min and poured into 2 l. of water. After standing for a few hours the light brown precipitate was filtered off, washed, and

(8) W. DiGiulio and W. Beierwaltes, *J. Nucl. Med.*, **5**, 417 (1964).

(9) M. F. Argus, K. Hewson, and F. E. Ray, *Brit. J. Cancer*, **10**, 321 (1956).

(10) Melting points were determined on a Thomas–Hoover capillary apparatus and are recorded as obtained. Analyses were performed by Schwarzkopf Microanalytical Laboratory, Woodside 77, N. Y.

dried at room temperature. The crude product was crystallized from chloroform to give 10 g (22% in various runs) of IV, mp 230–232°. Two recrystallizations from chloroform gave white microcrystals, mp 238° (lit.[7b] mp 237°).

N-2-(3-Iodofluorenyl)acetamide (V).—A mixture of 10 g of IV, 20 g of KI, 7 g of iodine, 200 ml of chloroform, and 300 ml of water was refluxed for 30 min with stirring. The chloroform layer was removed and washed with dilute bisulfite solution and then with water. The solvent was evaporated and the residue was crystallized twice from ethanol using charcoal to yield 6 g (80%) of fluffy white needles, mp 217° (lit.[7b] mp 204–206°).

The synthesis of N-2-(3-iodofluorenyl)acetamide-^{131}I was carried out essentially in the manner described above except with the following experimental modifications. A mixture of 2.5 g of IV, 75 ml of $CHCl_3$, and 25 ml of water was heated over a steam bath. Potassium iodide (50 mg) dissolved in 5 ml of water was mixed with the 0.2 ml of ^{131}I solution (2.5 mcuries).[11] It was then added dropwise to the chloroform solution of IV. Iodine (1.5 g) and KI (4 g) were dissolved in 50 ml of water and added slowly to the solution. The mixture was refluxed for 30 min and the chloroform layer was treated as mentioned above. This compound had a specific activity of 0.2 μcurie/mg.

Sodium N-2-(3-Iodofluorenyl)acetamido-7-sulfonate (VI).—Compound V (7.5 g) was mixed with 50 g of concentrated H_2SO_4 in small portions and heated over a steam bath for 10 min. The mixture was allowed to cool and was then decomposed in 100 g of crushed ice. The acid was neutralized by adding Na_2CO_3 in small portions. The hot solution was filtered and allowed to crystallize. The sodium salt was filtered, washed with cold water, and dried to give 8.0 g (82%) of product. The analytical sample was its 2-benzylthiuronium derivative, mp 243°.

Anal. Calcd for $C_{23}H_{22}IN_3O_4S_2$: C, 46.38; H, 3.70; I, 21.34; N, 7.06; S, 10.75. Found: C, 46.65; H, 3.43; I, 21.41; N, 7.21; S, 10.80.

Compound VI (^{131}I) was made essentially in the manner described above by sulfonating 0.3 g of V (^{131}I) with 2 g of concentrated H_2SO_4. This compound was recrystallized to a constant specific activity of 0.15 μcurie/mg.

N-2-(3-Iodo-7-nitrofluorenyl)acetamide (VII).—Compound V (4 g) was dissolved in 200 ml of glacial acetic acid by heating and the solution cooled to 35°. Fuming HNO_3 (20 ml, d 1.50) was added dropwise while stirring. The temperature of the mixture rose to 40° and it was further heated to about 45°. The stirring was continued for 15 min at this temperature, while a light yellow substance precipitated. The reaction mixture was then allowed to cool to room temperature, filtered, and washed with a small amount of cold glacial acetic acid and then with water, to give 3.6 g (80%), mp 258–260°. An analytical sample was prepared by two recrystallizations from toluene to give light yellow fluffy needles, mp 268°.

Anal. Calcd for $C_{15}H_{11}IN_2O_3$: N, 7.28. Found: N, 7.08.

The radioactive synthesis was carried out in the same manner as described above with 0.3 g of V (^{131}I).

N-2-(3-Iodo-7-aminofluorenyl)acetamide (IX).—A mixture of 4 g of VII, 16 g of $SnCl_2 \cdot 2H_2O$, 20 ml of concentrated HCl, and 100 ml of ethanol was refluxed for 1 hr with stirring, then allowed to cool. The white precipitate was filtered, washed with cold water, and neutralized with aqueous NH_4OH. The cream-colored amine was filtered and washed with water to yield 3.2 g (86.5%), mp 232–234°. Recrystallization from 100 ml of ethanol raised the melting point to 235–236°.

Anal. Calcd for $C_{15}H_{11}IN_2$: N, 7.66. Found: N, 7.62.

The radioactive synthesis was carried out in the same manner as described above with VII (^{131}I).

N,N'-2,7-(3-Iodofluorenylene)bisacetamide (X).—Compound IX (2 g) was refluxed in a mixture of 10 ml of glacial acetic acid and 5 ml of acetic anhydride for 15 min. The solution was cooled and poured into 100 ml of water. The white precipitate was filtered and washed with water to give 2.0 g (90%), mp 270–272°. It was recrystallized from ethanol in fine needles, mp 278°.

Anal. Calcd for $C_{17}H_{15}IN_2O_2$: C, 50.24; H, 3.70; N, 6.90. Found: C, 49.98; H, 3.78; N, 6.93.

The radioactive synthesis was carried out in the same manner as described above with IX (^{131}I).

N,N'-2,7-(3-Iodofluorenylene)bisacetamido-6-sulfonic Acid (XI).—To a mixture of 7 ml of fuming H_2SO_4 (30%) and 7 ml of concentrated H_2SO_4, 2 g of powdered X was added in small portions at 10° with stirring. After mixing thoroughly the mixture was allowed to stand at room temperature for 10 min, then it was decomposed in 100 g of crushed ice. The precipitate of XI was filtered, washed with water and dissolved in hot 10% Na_2CO_3 solution. From the clear filtered alkaline solution XI was precipitated by acidifying with dilute HCl. The precipitate was filtered, washed with water, and dried to give 2.2 g (90%). The analytical sample was made from its S-benzylthiuronium derivative, mp 238°.

Anal. Calcd for $C_{31}H_{29}IN_4O_8S_2$: C, 46.01; H, 3.83; I, 19.47; N, 8.59. Found: C, 46.01; H, 3.86; I, 19.56; N, 8.69.

The synthesis of XI (^{131}I) was carried out essentially in the manner described above except that the precipitate of free acid was dissolved in dilute NaOH solution to avoid any spurting with the evolution of CO_2 as was the case with Na_2CO_3. This compound was recrystallized to a constant specific activity of 0.06 μcurie/mg.

Animal Experiments.—Male CAF_1/Jax hybrid mice were employed in this study. Three mice were used for each time-interval study and an average was calculated. Four weeks prior to administration of the compounds the mice received a subaxillary transplant of a keratinizing squamous cell carcinoma[12] now in its 178th transplant generation in our laboratory. ^{131}I-labeled compounds were administered intraperitoneally in a dose of 5 mg to each mouse. One per cent solution for injection of VI was made in water and that of XI in pH 7 buffered solution. Concentration and per cent recovery of radioactive materials in tissues were determined by methods previously described,[13] except for the modification that the tissues were homogenized for 5 min at 7000 rpm after refrigeration. Radioactivity measurements were made with a Tricarb liquid scintillation spectrometer, Model 314 EX, from the Packard Instrument Co. The efficiency of the instrument was 57%. Total blood volume in mice was calculated on the basis of 6.32 ml/100 g of body weight.[14]

(11) Radioactive iodine was obtained from New England Nuclear Corp., Boston, Mass.

(12) Line a, stomach carcinoma originally obtained from the animal supply and research units of the British Empire Cancer Campaign.

(13) M. F. Argus, T. L. Seepe, N. Gutierrez, K. Hewson, and F. E. Ray, *Brit. J. Cancer*, **12**, 636 (1958).

(14) C. L. Oakley and G. H. Warrack, *J. Pathol. Bacteriol.*, **50**, 372 (1940).