Preparation of a Radioactive Iodotetrazolium Salt and Its Distribution in Mice*†

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Neoplastic tissues are said to reduce triphenyl tetrazolium chloride to a colored formazan more rapidly and completely than normal tissues (21). This difference was attributed to a higher aerobic glycolytic activity in tumors. If this is indeed the case, it is important to know whether or not tumors reduce tetrazolium compounds *in vivo* more rapidly and extensively than normal tissue. Tetrazolium salts are toxic in mammals (15). Mice will only tolerate the intravenous injection of 0.03 to 0.08 mg. This quantity of a colored pigment (formazan) would not be detectable in the tissues of a mouse. For this reason, a tetrazolium salt labelled in one benzene ring with radioactive iodine (I^{131}) was prepared, and the distribution of radioactivity in the tissues of normal and tumor-bearing mice was determined after intravenous injection.

Diphenyl *p*-iodophenyl tetrazolium chloride (IV) was prepared by a modification of the method for the synthesis of triphenyl tetrazolium chloride (12, 10). Aniline (1 mg.) was iodinated with radioactive iodine and diazotized, in a single reaction vessel (equations 1 to 3) (19). The diazonium compound (I) was coupled with benzal phenylhydrazone (II) (5) in pyridine, to yield a dark red formazan (III). The latter was oxidized with amyl nitrite and hydrochloric acid to the tetrazolium salt (IV).

EXPERIMENTAL

*Preparation of 2,5-diphenyl-3-*p*-radioiodophenyl tetrazolium chloride (IV).*—Iodination of aniline (13) and diazotization of the amine was accomplished with 0.02 millimole of iodine (excess) and 0.01 millimole of aniline according to equations 1 to 3. Reactions 1 and 3 must be conducted in strong acid, and reaction 2 in excess bicarbonate (19).

1) $6NaI + 2NaNO_2 + 8HCl \rightarrow 3I_2 + 8NaCl + 4H_2O + N_2$
2) $I_2 + C_6H_5NH_2 + NaHCO_3 \rightarrow IC_6H_4NH_2 + NaI + H_2O + CO_2$
3) $6IC_6H_4NH_2 + 6NaI + 8NaNO_2 + 20HCl \rightarrow 6IC_6H_4N_2Cl + 3I_2 + 14NaCl + 16H_2O + N_2$

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Carrier free I^{131} (3 to 4 mc.) in the form of the sodium salt was added to a solution of 6 mg. sodium iodide in a graduated conical centrifuge tube. The final volume was 2 cc. Free iodine was produced by adding 1.0 per cent sodium nitrite (0.1 cc.) and 10 per cent hydrochloric acid (0.1 cc.). The mixture was shaken and crystalline iodine separated (equation 1). After a few minutes 10 per cent sodium bicarbonate (0.1 cc.) followed by 1 per cent aniline (triply distilled from zinc dust) solution (0.1 cc.) was added. The color of iodine was discharged in the course of one hour as the aniline was iodinated (equation 2). The reaction mixture was cooled to 0° C. and diazotization was accomplished by adding 1 per cent sodium nitrite (0.3 cc.) and 10 per cent hydrochloric acid (0.5 cc.) (equation 3). A chilled solution of 10 mg. benzal phenylhydrazone (I) in 2 cc. pyridine was added to the solution of the diazonium salt (II). A dark red color was produced. The mixture was cooled in ice for 20 minutes and distilled water (10 cc.) was added slowly to precipitate the formazan (III). The mixture was centrifuged for 10 minutes at 2000 r.p.m., the supernatant was discarded, and the red precipitate was washed with three 10 cc. portions of water in order to remove pyridine and inorganic salts. The washed formazan was dissolved in absolute alcohol (3 cc.) and oxidized to the tetrazolium salt (IV) by the addition of amyl nitrite (3 drops) and of concentrated hydrochloric acid (3 drops). The alcohol solution was evaporated in a hot water bath to half its original volume, water (2 cc.) was then added, and the remainder of the alcohol was evaporated. The final volume was approximately 2 cc. The solution was cooled and shaken with chloroform (7 cc.). The aqueous layer was discarded (pipette), and the chloroform extract was concentrated to 3 cc. The tetrazolium chloride (IV) was precipitated by the addition of dry ether. It was washed 3 times with ether (centrifuge) and dried; yield 2.8 to 4.0 mgs. (sinters 160°, decomp. 170°, uncorr.).

A non-radioactive specimen was prepared in the same way on a larger scale. The formazan (III) after recrystallization from dilute alcohol melted at 195°–196° C. (uncorr.). The melting point of the tautomer prepared from *p*-iodophenyl hydrazine (4) is reported to be 185°–186° C. The tetrazolium salt (IV) was soluble in alcohol, chloroform, and water. In aqueous solution, it was reduced to the insoluble formazan with ammonium sulfide.

Analysis calculated for $C_{19}H_{14}N_4ClI$: C, 49.48; H, 3.03;

Found: C, 49.52; H, 3.16

Distribution of Radioactivity in Tissues of Mice.—The distribution of radioactivity in tissue at intervals after intravenous injection of radioactive iodotetrazolium chloride (IV) was determined by methods reported elsewhere (20, 18, 17, 16). One experiment (0.1 mg.) was performed with normal Swiss mice. Two experiments were done with Swiss mice 12 to 14 days after subcutaneous transplantation of sarcoma 37. A sublethal dose of the radioactive iodotetrazolium chloride (IV), which on a molar basis was one-fourth as toxic as triphenyl tetrazolium chloride (15), was given to each mouse. The doses used were 0.1 mg. and 0.2 mg. in each experiment respectively.

The results are shown in Tables 1 and 2 and are expressed as a ratio in per cent of the radioactivity per 0.2 cc. of blood and 200 mg. of wet tissue, to the radioactivity of 0.2 cc. of blood at zero time, assuming the circulating blood volume to be 10 per cent of the body weight (18, 17).

Following intravenous injection (tail vein) in both normal and tumor-bearing mice, the radioactivity disappeared rapidly from the circulating blood. In 30 minutes the level of radioactivity was 3 to 4 per cent and in 8 hours the level was 0.5 per cent of the activity at zero time. The highest concentration of radioactivity was found in kidney, liver and lung, in that order, after injection of 0.1 mg., while the lungs showed the highest concentration after injection of 0.2 mg. All other tissues, including sarcoma 37, were less radioactive.

The radioactivity of lung declined more slowly than that of other tissues in the first 12 hours (Table 1) and this was also the case with liver and spleen after the injection of the larger dose of radioactive iodotetrazolium salt (Table 2). The radioactivity of mesenteric fat showed a rise in the first few hours after injection and declined slowly thereafter. The accumulation and persistence of radioactivity in fat may have been related to the lipoid solubility of the formazan. A similar rise and fall in radioactivity of thyroid was noted and was produced presumably by the release of ionic iodine from the tetrazolium salt or the formazan.

Radioactivity of sarcoma 37 was less than most tissues and disappeared at about the same rate. There was no appreciable difference in the levels of radioactivity in the tissues of normal and tumor-bearing mice (Table 1).

The rate of disappearance of radioactive ionic iodine from blood and tissues of mice, reported previously (18), was much more rapid than was found with the radioactive iodotetrazolium salt described above.

DISCUSSION

Tumor cells have been reported to derive energy largely from aerobic glycolytic reactions which involve fermentation rather than oxidation (22). However, a comparison of the metabolism of tumors of liver and skin *in vitro* with that of the tissue of origin, revealed a higher degree of both aerobic and anaerobic glycolysis in tumors, although a marked decrease in specialized oxidative functions was observed in the tumors (2). Deficiencies in the various oxidative systems of tumors have been reported (1, 11, 9). The cytochrome content of tumors is deficient (3, 6). These deficiencies might lead to accumulation of reducing substances in neoplastic tissue. Triphenyl tetrazolium chloride was reported to be reduced more extensively by tumor than normal tissue *in*

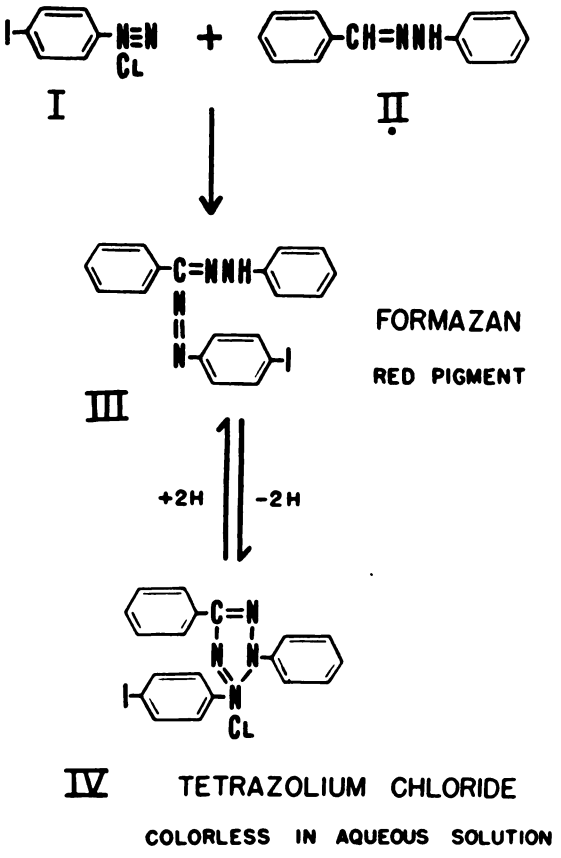

FIG. 1

vitro and by application of the material to the surface of tumor *in vivo* (21). On the other hand, no significant increase in the water-soluble substances capable of reducing methylene blue was found in tumor (14). Ability to decolorize methylene blue was reported to be decreased in neoplastic tissue (1, 7, 8).

In the *in vivo* experiments with radioactive tetrazolium salt, reduction to an insoluble formazan would be expected to result in prolonged localization of radioactivity in the tissues. However, the level of radioactivity was lower in sarcoma 37 than in most normal tissues after intravenous injection. Furthermore, radioactivity disappeared from the tumor at the same rate as with other tissues. The

tissues which normally show high dehydrogenase activity (liver and kidney) were the most radioactive. It may be concluded that sarcoma 37 does not reduce the tetrazolium salt *in vivo* more extensively than most of the normal tissues. The same results were obtained *in vitro* with a homogenate of sarcoma 37 at pH 7.0 to 7.6 (unpublished) (15). Another tetrazolium salt was reduced to its formazan (blue) much more slowly (or not at all) by this tumor than by liver and kidney mash. Although the degree of reduction was increased by anaerobiasis, it was also increased for the normal tissues. Similar results were noted with homogenates of Walker carcinoma 256, of Bagg lymphosarcoma and of several carcinomas from humans (unpublished) (15). No evidence is available from our experiments that neoplastic tissue is able to reduce tetrazolium salts *in vivo* or *in vitro* more readily or extensively than most normal tissues.

SUMMARY

The synthesis of 0.01 millimole of diphenyl *p*-iodophenyl tetrazolium chloride from radioactive iodine (I^{131}) is described. Following the intravenous injection of the radioactive tetrazolium salt into normal and tumor-bearing mice, radio-

TABLE 1

RADIOACTIVITY OF BLOOD (0.2 CC.) AND TISSUES (200 MGS.) OF EACH OF 8 NORMAL AND 8 TUMOR-BEARING MICE, EXPRESSED IN PER CENT OF THE RADIOACTIVITY OF BLOOD AT ZERO TIME MEASURED AT INTERVALS AFTER INTRAVENOUS INJECTION OF 0.1 MG. DIPHENYL *p*-RADIOIODOPHENYL TETRAZOLIUM CHLORIDE

	Hours							
Tissue	0.5	2	4	8	12	24	48	96
				Normal Mice				
Blood	3.4	2.6	1.4	0.5	0.4	0.07	0.08	0.09
Kidney	58.6	34.9	16.8	6.6	4.8	1.2	0.4	0.1
Liver	20.3	18.2	11.4	4.1	3.8	0.7	0.3	0.2
Lungs	12.7	15.2	11.0	5.8	6.4	1.2	0.8	2.1
Spleen	4.2	3.4	2.4	0.8	0.6	0.1	0.1	0.06
Intestine	8.5	6.1	3.5	1.2	1.1	0.1	0.08	0.2
Muscle	3.1	3.7	2.1	1.0	1.4	0.8	0.5	0.5
Nodes	0.4	0.4	0.4	0.1	0.5	0.3	0.1	0.2
Brain	0.1	0.4	0.5	0.4	0.5	0.3	0.1	
Mesenteric fat	0.9	4.8	1.8	2.0	5.1	3.1	2.6	1.9
Thyroid	1.6	3.2	1.4	0.8	12.5	3.1	1.2	3.4
				Tumor-bearing Mice				
Blood	3.7	3.3	1.7	0.6	0.4	0.1	0.1	0.1
Kidney	92.2	38.9	22.2	23.2	4.8	1.0	0.6	0.3
Liver	24.5	20.3	15.1	4.1	3.4	1.0	0.5	0.4
Lungs	15.3	14.0	12.0	6.5	7.0	2.4	1.6	0.5
Spleen	4.5	3.1	2.8	1.0	0.08	0.3	0.3	0.2
Intestine	7.8	6.0	5.1	1.4	1.4	0.4	0.4	0.1
Muscle	2.8	3.0	2.4	1.0	1.0	0.5	0.5	0.8
Nodes	0.8	0.4	0.3	0.1	0.3	0.3	0.1	0.3
Brain	0.4	0.4	0.5	0.5	0.5	0.3	0.1	0.1
Mesenteric fat	1.0	7.4	2.1	4.4	3.0	2.8	4.4	0.9
Thyroid	2.0	2.3	2.1	3.1	3.6	2.9	3.2	2.3
Tumor	2.4	1.5	1.9	1.8	1.5	1.6	0.4	0.3

TABLE 2

RADIOACTIVITY OF BLOOD (0.2 CC.) AND TISSUES (200 MGS.) OF EACH OF 8 TUMOR-BEARING MICE EXPRESSED IN PER CENT OF THE RADIOACTIVITY OF BLOOD AT ZERO TIME, MEASURED AT INTERVALS AFTER INJECTION OF 0.2 MG. DIPHENYL *p*-RADIOIODOPHENYL TETRAZOLIUM CHLORIDE

	Hours							
Tissue	0.5	2	4	8	16.5	24	49	120
Blood	4.5	2.9	2.3	0.6*	0	0	0	0
Kidney	45.3	34.4	26.9	13.2	6.4	3.9	2.7	0.8*
Liver	34.6	26.6	24.8	15.0	10.3	9.0	9.4	7.6
Lungs	111.5	84.4	55.6	48.2	46.4	20.9	30.2	22.2
Spleen	8.3	8.1	6.1	2.8	4.4	4.8	3.9	2.5
Intestine	6.5	3.2	9.7	2.0	0.5*	0.5*	0	0
Muscle	2.5	1.4*	1.1*	1.3*	0.4*	0.5*	0	0
Mesenteric fat	1.2*	1.4*	3.4	5.2	0.9*	2.0	1.7	1.3*
Thyroid	3.1	12.3	6.5	5.7	4.3	9.5	4.0	5.0
Tumor	2.4	1.6	2.3	1.8*	0.9*	0	0	0

* Less than 5× background.

activity disappeared rapidly from the circulating blood, and appeared in greatest amount in kidney, liver, and lung. Sarcoma 37 contained less radioactivity than most other tissues.

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