

# The Fate of Rat Tumor Cells Labeled with Sulfanilic-S<sup>35</sup> Acid in the Host\*

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## SUMMARY

A method of labeling rat ascites tumor cells with diazotized sulfanilic-S<sup>35</sup> acid is described. The distribution of radioactivity in host rats with labeled subcutaneous or ascites tumor implants was studied; radioactivity was found to be present mainly in the tumor, kidneys, spleen, liver, and blood, decreasing in specific activity in that order. In general, the tissue localization of the label derived from the labeled tumor cells in the host rat resembled the patterns observed by Haurowitz *et al.* (5) following injection of labeled sulfanil-azotyrosine and sulfanil-azoalbumin and differed from the distribution of free sulfanilic-S<sup>35</sup> acid administered intraperitoneally.

Studies of the metabolic activity of tumor cells with a labeled amino acid used as a tracer have been undertaken by various investigators (1-3). Investigations of this type often yield valuable information regarding the kinetics and mode of metabolic activity of the tumor cells. However, the data obtained are sometimes difficult to interpret, since the labeled compound may be re-utilized following its introduction into the metabolic cycle. In order to circumvent the re-entry of the label into the metabolic pool and to obtain more definitive information concerning the activity of the catabolic phase of the tumor cell metabolism, a labeled compound which is foreign to the metabolic process may profitably be used. In these studies S<sup>35</sup>-labeled sulfanilic acid was used to label tumor cells in order to investigate its value as a tracer. The fate of tumor cells labeled in this manner was also studied.

## MATERIALS AND METHODS

*Labeling of tumor cells.*—The tumor cells used for tagging were obtained from the ascitic fluid of rats bearing a fibrosarcoma which arose in this strain of Slonaker rats and which is able to grow in ascites form or as a solid tumor (11). The red cells in the ascitic fluid, when present in excessive amount, were removed by hemolysis after adding

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an equal volume of distilled water according to the method of Morgan *et al.* (8). The ascitic fluid after harvesting was immediately chilled to 0-3° C. and centrifuged. The packed tumor cells ranging from 1.5 to 2.5 ml. in volume were washed twice with chilled Hanks balanced salt solution (4) and then suspended in an equal volume of the salt solution for coupling with the diazotized sulfanilic-S<sup>35</sup> acid.

The diazotized sulfanilic acid solution was prepared from sulfanilic-S<sup>35</sup> acid, which was synthesized in this laboratory with a specific activity of approximately 1 mc/mg (6). The procedure for diazotization and coupling was that of Ingraham (7), modified as follows: 0.64 mg. of radioactive sulfanilic acid in 0.5 ml. solution was acidified with 0.03 ml. of 2.0 N hydrochloric acid and diazotized with 0.09 ml. of 0.1 N sodium nitrite. After 10 minutes at room temperature, 0.1 ml. of 0.2 M urea was added to decompose the unreacted sodium nitrite. The solution was allowed to stand at room temperature for 10 minutes before being chilled to 0-3° C. Just prior to mixing with the washed tumor cells, 0.1 ml. of 0.5 M sodium phosphate buffer, pH 7.65 (glass electrode), and 0.18 ml. of 0.2 M sodium carbonate were added, and the solution was then transferred dropwise with gentle stirring to a 1:1 suspension of tumor cells in Hanks' balanced salt solution. The coupling was allowed to continue at 0-3° C. for 1 hour with frequent and gentle shaking. Approximately 50-60 per cent of the label from the diazotized acid was attached to the cells. The treated cells were centrifuged and

washed 3 times with 4 times their volume of chilled balanced salt solution. Finally, the washed labeled tumor cells were suspended in the salt solution at 1:1 dilution for injection into the peritoneum of a host rat. The ascitic fluid formed thus provided a source of viable labeled tumor cell for metabolic studies.

**Sampling preparation and counting.**—The tissue sample from distribution studies was digested with concentrated hydrochloric acid and oxidized with Benedict's solution to convert the organic sulfur to inorganic sulfate according to a procedure previously reported (9). The radioactivity of the resulting barium sulfate was measured by a thin, large end-window Geiger-Müller counter, and the self-absorption of the sample was corrected accordingly (9). For liquid samples, such as urine, etc., the method of liquid scintillation counting was employed. The liquid phosphor was a 10-ml. aliquot of a dioxane solution (1 l.) containing 2,5-diphenyloxazole (7 gm.), 2,2'-*p*-phenylenebis(5-phenyloxazole) (50 mg.), and naphthalene (50 gm.). An appropriate amount of the radioactive solution to be assayed was added to the liquid scintillation solution, and the radioactivity was read on a liquid scintillation spectrometer. Quenching of the sample was corrected by a method reported earlier (10).

## RESULTS

**In vivo, regeneration of the treated tumor cells.**—The labeling of tumor cells by coupling with diazotized sulfanilic-S<sup>35</sup> acid was not a normal physiological process and would undoubtedly cause various degrees of injury to the cells. However, subcutaneous implantation of three different cell preparations in rats with an inoculum of a minimum of 10<sup>7</sup> cells gave 80, 60, and 0 per cent tumor "takes," indicating that some treated cells still retained viability in spite of the unphysiological condition of the labeling procedure. Results of these and other related studies also reflected a possible effect upon the number of surviving cells, owing to the age of the tumor cells used for labeling; however, this effect was not further investigated.

The chemical nature of the linkage between the label and the tumor cells was not specifically determined in this study. By analogy to *in vitro* diazotization reactions, the label would most likely be bound via an azo linkage to the aromatic nucleus of the tyrosine residue in the cell protein. The fact that a firm attachment was established between the cell and the label was inferred from the results of the following experiments: (a) Paper chromatography of the treated cells revealed that

approximately 79 per cent of the radioactivity applied to the paper remained at the origin. In the same solvent system (*n*-butanol-acetic acid-water; 4:1:5; v/v), sulfanilic-S<sup>35</sup> acid gave a R<sub>f</sub> value of 0.2. (b) Treatment with trichloroacetic acid precipitated approximately 80 per cent of the radioactivity of the treated cells. (c) Storage at 4° C. for various lengths of time did not change either the fraction of the activity precipitable by trichloroacetic acid or the fraction found remaining at the origin on paper chromatography.

The treated tumor cells were inoculated into the peritoneum of a host rat in order for the normal cell multiplication process to occur. In this manner, the dead and the irreparably damaged cells were discarded by the host through catabolic process, and the viable ones were allowed to regenerate. The daily excretion of the label from the host following intraperitoneal injection of the treated cells amounted to approximately 12.5 per cent at the end of the 1st day, decreasing exponentially to 1.7, 0.8, 0.6, 0.4, 0.4, and 0.18 per cent of administered dose, respectively, for each succeeding day. These data represent average values from ten rats. On the 6th or the 7th day after inoculation the ascitic fluid was removed from the rat to yield viable labeled cells which, after washing, were used in subsequent implant studies.

**Distribution of the label and fate of labeled tumor implants.**—The localization of the label in various tissues was studied in rats bearing either labeled ascites or subcutaneous tumors arisen from labeled tumor implants. Distribution of radioactive sulfanilic acid in normal and tumor-bearing animals was also investigated to compare the results with those obtained from the labeled tumor cells.

Table 1 shows the data from these studies. The distribution values reported here are the average values of five rats in the ascites tumor group, six rats in the subcutaneous tumor group, and two normal and two tumor-bearing rats that had received sulfanilic-S<sup>35</sup> acid directly as a single intraperitoneal injection. The values in Table 1 are expressed as percentage of the total sulfur-S<sup>35</sup> recovered in the rat per gram organ; the fraction of radioactivity excreted in the feces and urine was not taken into consideration in the computation.

The urinary excretion of sulfur-35 label in rats bearing subcutaneous labeled tumor implants was studied in groups of six animals. The average excretion at the end of the 1st day after implantation was less than 5 per cent of the administered dose, and in the following 2 days this value decreased to half and became approximately constant at this concentration for another 6 days before it gradually diminished. The fecal excretion of sulfur-35

was about half of the corresponding concentration in the urine, and it was believed that this value was higher than it might appear owing to urinary contamination of the feces. For this reason, determination of the fecal excretion of the label was not continued.

In rats given intraperitoneal inoculations of labeled tumor cells, the urinary excretion of sulfur-35 from seven animals varied from 3 to 8 per cent, averaging 6.2 per cent of the administered dose at the end of the 1st day. In subsequent days, the urinary and fecal excretions closely resembled the patterns observed in rats with subcutaneous tumors; the rats with ascites tumors excreted approximately 1 per cent more radioactivity in the urine than rats with subcutaneous tumors.

The activity of sulfur-35 found in subcutaneous tumors amounted to more than 80 per cent of the total activity recovered from the animal. In rats bearing ascites tumors, solid tumors were found in the peritoneum in addition to the ascitic fluid. The

solid peritoneal tumors retained approximately 40-50 per cent of the label recovered; this on a per gram weight basis had a higher specific activity than that of the ascitic fluid. Among the organs, the kidneys were observed to have a high concentration of sulfur-35; this was more than a hundred-fold that found in muscle. Other organs such as gastrointestinal tract, liver, spleen, and skin exhibited a greater concentration of sulfur-35 in rats with ascites than in rats with subcutaneous tumors.

### DISCUSSION

From the data presented, the feasibility of the described method for labeling tumor cells with radioactive diazo sulfanilic acid is indicated. The label combines firmly with the cell. Return of the labeled tumor cells into the peritoneum of a rat affords a means of eliminating the badly damaged cells through the catabolic activity of the host and concurrently allowing the less impaired cells to recover. Although passage of the labeled cells

TABLE 1  
DISTRIBUTION OF SULFUR-35 IN RATS OF DIFFERENT STUDY GROUPS

ORGAN	RANGE OF SAMPLE SIZE PER ORGAN (GM.)	PER CENT OF RECOVERED RADIOACTIVITY PER GRAM OF WET TISSUE			
		Rats bearing labeled ascites tumor*	Rats bearing labeled subcutaneous tumor†	Rats dosed with sulfanilic-S <sup>35</sup> acid‡	
				Normal	Tumor-bearing
Blood	3 - 5	.27 ± .01§	.34 ± .02	.05	.14
Bone#	1.0- 1.8	.36 ± .02	.15 ± .01	.78	.20
Brain	1.0- 1.7	.11 ± .01	.09 ± .01	.40	.37
Heart	0.4- 0.6	.19 ± .02	.11 ± .02	1.03	1.47
Kidneys	0.9- 1.6	12.75 ± 1.10	5.50 ± .59	1.96	3.58
G. I. tract	8.8- 12.6	.52 ± .12	.037 ± .005	1.26	1.63
Liver	4.9- 8.6	.97 ± .14	.19 ± .04	.25	.36
Lungs	0.8- 1.4	.38 ± .06	.14 ± .01	.95	1.40
Muscle	4.3- 5.0	.038 ± .007	.038 ± .006	.15	.05
Skin**	10.5- 37.3	1.17 ± .41	.30 ± .04	.08	.16
Spleen	0.3- 0.6	1.59 ± .35	.64 ± .05	1.17	1.20
Tumor					
Subcutaneous	10.5- 28.2		3.27 ± .23		.23
Intraperitoneal	2.1- 4.5	18.70 ± 1.10			
Ascitic fluid	13 - 16	1.94 ± .42			
Balance of rat**	55 - 132	.12 ± .01	.11 ± .01	.51	.53
Urine††		(13.7 ± 5.4)	(12.3 ± 4.6)	(82.6)	(85.4)
Feces‡‡	2.5- 6.8	§§	§§	.80	.71

\* Data from five rats, sacrificed from 7 to 9 after days inoculation with labeled tumor cells.

† Data from six rats, sacrificed from 16 to 21 days after implantation.

‡ Average values from two rats, sacrificed 24 hours after intraperitoneal administration of the acid.

§ Standard error =  $\sqrt{\Sigma \text{dev.}^2/n(n-1)}$ .

# Sample included femurs and bone marrow of both hind legs of the animal.

|| Gastrocnemius muscle from both legs of the animal.

\*\* Only aliquots used for radioassay.

†† Expressed as per cent of administered dose.

‡‡ Expressed as per cent of administered dose per gram wet sample.

§§ See text.

through the host resulted in a net decrease of radioactivity associated with the cells, this method avoids the complications involved in the interpretation of the distribution data obtained from a direct use of the treated cells, containing both viable and dead labeled tumor cells.

Comparison of the distribution of radioactivity in rats bearing either subcutaneous or ascites labeled tumors with normal and tumor-bearing rats dosed with labeled sulfanilic acid revealed that the tissue organs of the animal in the former groups retained more of the radiosulfur than those of the rats in the latter group. The general elevation of the level of radioactivity in the tissues of rats with labeled tumors strongly suggests the circulation and selective cumulation in these organs of some breakdown cell fraction containing sulfur-35 whose metabolic fate differs from that of sulfanilic-S<sup>35</sup> acid. The high concentration of label observed in the kidneys of these tumor rats also favors such an assumption. Haurowitz *et al.* (5) in their study of the metabolic fate of sulfanil-azotyrosine and sulfanil-azoalbumin reported the localization of high levels of radioactivity in the kidneys of the animal; our results are in line with their findings.

From the low tissue retention and high urinary excretion of the label and its persistence in tumors, it becomes apparent that the radioactive sulfanilic acid is a suitable tracer for studying the fate of tumor implant. It is conceivable that, after the lapse of the period when the sulfur-35 from the damaged tumor cells is completely eliminated and when accumulation of the radioactivity in other

organs has attained equilibrium, the daily urinary excretion of the label may be interpreted as indicative of the metabolic activity of the tumor implant.

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