INTRODUCTION

Materials which on administration to tumor hosts will localize in tumor tissues have been sought for many years (1, 2, 6, 9—14, 20). If such a localizing material could be found, radioactive atoms might be incorporated into it to provide a diagnostic tool or possibly a therapeutic agent. Search for such a material was begun here some years ago (1), and the present report is a continuation of that work. A radioactive dye was prepared by the reaction of diazotized trypan blue with sodium radioiodide 131 (NaI*). This was administered intravenously to mice bearing transplanted tumors, and the distribution of the radioactivity in their tissues was measured.

Preparation and analysis of the radioactive dye.—The dye used for injection was a mixture of compounds resulting from the replacement of the amino groups of trypan blue with iodine1 or hydroxyl groups. It was prepared by synthesizing trypan blue from H-acid and ortho-tolidine (8), then replacing the amino groups by use of the Sandmeyer reaction. The method of synthesis employed was that recommended by Bloch and Ray (1), using 1 millimole of trypan blue, approximately 3.5 milli-curies of radioactive iodine (I131), and 92 millimoles of sodium iodide which was added after the radioactive iodide. The crude dye thus prepared was reprecipitated four times, each time by dissolving the material by warming in approximately 5 ml. of water and then adding 150 ml. of a 1:2 ethanol-ether mixture. The dye was then mixed with 50 ml. of ethanol saturated with NH3. After an hour it was again precipitated by addition of 100 ml. of ether and was washed with a 1:2 ethanol-ether mixture. The recoveries of dye and of radioactivity at various stages of the preparation are given in Table 1. The product had an activity of 0.4 micro-curie per milligram at the time that it was injected into mice.

TABLE 1

Recoveries of Dye and of Radioactivity in the Preparation of Dye Used in the Animal Experiments

<table>
<thead>
<tr>
<th>Stage in preparation of dye</th>
<th>Yield of dye (mg.)</th>
<th>Specific radioactivity (counts/min./mg. dye)*</th>
<th>Initial radioactivity recovered in dye (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original precipitate</td>
<td>1423</td>
<td>21,300</td>
<td>36.7</td>
</tr>
<tr>
<td>After 8d and 9d precipitations</td>
<td>1050</td>
<td>25,300†</td>
<td>32.2†</td>
</tr>
<tr>
<td>After 4th precipitation</td>
<td>1000</td>
<td>29,400†</td>
<td>33.6</td>
</tr>
<tr>
<td>After 5th precipitation</td>
<td>950</td>
<td>30,000</td>
<td>32.7</td>
</tr>
<tr>
<td>After NH-EtOH treatment</td>
<td>900</td>
<td>31,200</td>
<td>32.1</td>
</tr>
</tbody>
</table>

* Corrected for radioactive decay.
† An error in measurement of radioactivity is presumed here.

The composition of the dye mixture was determined from a series of analyses, data from which are presented in Table 2. The dye, after an initial heating at 80° C. and storage over Drierite, lost 5 to 6 per cent of its weight on further heating at 80° to 90° C. This dried dye had approximately the following composition: 52 per cent dihydroxy trypan blue, 40 per cent monoiodo monohydroxy trypan blue, 3 per cent di-iodo trypan blue, and possibly some unchanged trypan blue.

Nitrogen was determined by the Dumas method (15), sulfur by the Carius method (5), sodium by ashing with sulfuric acid (15), and iodine by wet...
combustion and distillation (18), using the recovery of added radioactive iodine as the criterion for recovery of the inert iodine.

**ANIMAL EXPERIMENTS**

Swiss A mice, each bearing 2 subcutaneous implants of mammary spindle-cell carcinoma 15091a (3, 4, 16), were obtained from the Jackson Memorial Laboratory, together with normal mice of the same strain. They were kept individually in beakers. When the tumors were 192 days old, the mice were given, by tail vein, a solution of the radioactive dye in 0.9 per cent NaCl, having a concentration of 8.63 mg. of dye per milliliter. The dose administered, determined by weighing the syringe before and after each injection, averaged 8.81 mg. of this dye solution per gram of mouse; 21 of the 24 tumor mice received within ± 15 per cent of this dose. No symptoms were observed. Excreta were collected daily for 3 days. The mice were sacrificed by a blow on the head, 1, 92, 3, 4, or 5 days after injection.

The various organs and tissues removed for analysis were immediately weighed (Table 3) and suspended in 1 per cent NaOH. Whole organs or pairs of organs were used to minimize sampling errors. An aliquot of 0.20 ml. of each sample, representing 20 mg. of fresh tissue, was dried on a 22-mm. diameter microscope cover glass. Thyroids with accompanying tracheal tissue were dried directly on cover glasses, and the pinnae of the ears were glued on cover glasses. Measurements of ra-
dioactivity were made with Victoreen mica-window counters connected to scalers using circuits of Higinbotham's design with scales of 1 to 64. All aliquots were counted at least 200 impulses above background count, while the liver, spleen, kidney, and tumor samples were counted at least 770 impulses above the background count. To check on the operating condition of these instruments, a sample of radium D + E was counted twice daily. Aliquots of the original I* solution from which the dye was prepared were mixed with silver nitrate solution and dried on cover glasses; these were counted two or three times daily. All sample counts reported here are in terms of the ratio of such counts to the average count of these standard I* cover glasses multiplied by the factor 4.43, to convert to the basis of micrograms of iodine. This calculation assumes that the ratio of inert to radioactive iodine was the same in all iodine compounds present in the dye mixture. The standard I* cover glasses decayed, with an 8-day half-life over a period of more than a month.

RESULTS

The concentrations of I* in both the larger and the smaller tumors were several times greater than those in the leg muscle or skin (pinna of the ear) and were nearly as great as in the heart or stomach. The concentration of I* in the tumors was directly proportional to the amount of dye injected. The concentration of I* in the larger of the two tumors on each mouse was directly proportional to that in the smaller tumor, though the relation was not striking. A higher degree of correlation was found between the average I* concentrations in the tumors and those in the liver, kidneys, and stomach, in part because of the effect of varying doses. The heavier the tumors, the lower I* concentrations they contained. With lengthening time between injection and sacrifice, the I* concentrations in the tumors were lower, partly because of the rapid growth of the tumors. But the total I* contents of the tumors were directly proportional to their weights.

Concentrations of I* in the other tissues examined were probably highest in the thyroids; but, since these glands were not weighed, only the total I* recovered in them is reported in Table 3. These recoveries averaged 0.3 per cent of the administered dose, suggesting that most of the I* remained in organic combination in the mice. Concentrations of I* in the livers of the tumor mice were twice those found in their kidneys (Fig. 1). The concentrations of I* were lower in the livers of control mice than in those of most of the tumor mice. This difference may have occurred because larger livers showed lower concentrations and the control mice had livers which constituted a larger proportion of their body-weight than was the case with tumor mice. Even among the tumor mice themselves, these relations among body-weight (corrected for tumor-weight), liver-weight, and liver concentration of I* were found to obtain.

Concentrations of I* in the spleens of the control mice were higher, for a given dose, than were those in the spleens of the tumor mice. Enlarged spleens,
ly after injection and might decrease with time. The present experiment was therefore extended over a period of 5 days. This was insufficient time, since the I* concentrations found in the livers was as great 4 or 5 days after injection it was as 1 day after.

The dose of dye administered was determined in part by the specific radioactivity of the dye. Sufficient activity had to be administered to allow measurements of radioactivity in the tissues to be made by the technic employed. Although 2 to 5 mg. of radioactive di-brom trypan blue per mouse were employed in similar work (13), it was there stated that Duran-Reynals (6) had found maximal selective staining with doses around 0.1 to 0.5 mg. in mice. In the work presented here the average dose was about 1 mg. of dye per mouse, the minimal amount which appeared suited to the conditions noted above. Selective staining of the tumors was obtained, in the sense that the tumors were much bluer than the surrounding skin and muscle.

**TABLE 4**

**AVERAGE RECOVERIES OF INJECTED IODINE FROM THE FECES AND URINE OF MICE AFTER INJECTION OF IODINATED TRYPAN BLUE DYE MIXTURE**

<table>
<thead>
<tr>
<th>Micrograms of iodine found</th>
<th>In feces</th>
<th>In urine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st day</td>
<td>2nd day</td>
</tr>
<tr>
<td>Tumor mice</td>
<td>15</td>
<td>4</td>
</tr>
<tr>
<td>Control mice</td>
<td>14</td>
<td>9</td>
</tr>
</tbody>
</table>

A transplanted tumor was used in this work because it provided a single, uniform kind of tumor tissue, several tumors could be obtained on one animal, the tumors would be of similar histological character and of definite age in animals of the same age; and the tumor-bearing animals could be obtained on a schedule that could be related to the decay of the radioactivity in the dye. Mice were selected rather than larger animals, to avoid undue exposure of personnel to radiation hazards. Tumor 15091a was recommended as being especially suitable for easy removal. It is described as a mammary spindle-cell carcinoma (16), although Ewing first classified it as an adenocarcinoma of the thyroid (19).

The assumption made in presenting the data on iodine in Table 3 was that the inert iodine and the I* in the administered dye mixture remained in the same proportion to each other (except for radioactive decay) throughout the experiment. Since the inert and radioactive iodides were presumably thoroughly mixed in the dye synthesis, it is probable that, whatever compounds were present in the administered mixture, the ratio of inert iodine to I* in each of them was the same. It is possible that the amount of radioactivity and the amounts of either iodinated or noniodinated dye did not remain in a fixed ratio in the animals, although two lines of evidence suggest that this was true of the iodinated dye, for the most part: (1) differences in the distribution of radioactivity in the tissues and excreta of the mice and the distribution of radioactivity found after administration of NaI*; (2) similarity of the tissue distribution of radioactivity in these mice and in the mice given radioactive di-brom trypan blue or radioactive di-brom Evans blue (13). On the other hand, the fact that the bile and feces showed no blue color and that no blue color could be leached from the feces with 1 per cent NaOH suggests that the dye was broken at the azo linkages in the liver or intestine or both.

In comparing the tissue distribution of radioactivity found in tumor-bearing mice after administration of the radio-brominated dyes and of the radio-iodinated dyes, data on five tissues are available: tumor, liver, kidney, spleen, and muscle. With each of the three dye preparations, the tumors contained an average of 3 per cent of the injected radioactivity per gram of tissue and the muscle averaged 1 to 2 per cent per gram. The spleens averaged 4 to 8 per cent, the kidneys 7 to 11 percent, and the livers 8 to 25 per cent per gram. Data from the iodinated trypan blue more nearly resembled that from the brominated trypan blue than that from the brominated Evans blue. These similarities in tissue distribution of radioactivity were found despite the fact that (1) different radioactive elements were employed, (2) the radioactive bromine was attached to the tolidine group and the I* was attached to the naphthol—sulfonic acid groups of the dye molecule, and (3) the basic amino groups present in the brominated compounds were replaced in the iodinated compound by iodine or by acidic hydroxyl groups. These differences were not of major importance in determining the tissue distribution, which must, therefore, be principally the result of the similarities in the dyes.

In comparing the tissue distribution of radioactivity found in tumor-bearing mice injected with the radioactive trypan blue dye mixture and those injected with NaI*, unpublished data are available on 8 ABC mice bearing tumor 15091a, which received, on the average, one-third as much iodine (given as NaI*) as did the mice injected with the dye. They were killed after 25 hours. The ratios of radioactivity per gram of fresh tissue after administration of NaI* to that after administration of the radio-iodinated dye mixture were as follows: tumor, 1.7; muscle, 3.0; liver, 0.16; kidneys, 0.5; lungs, 1.8; heart, 2.3; spleen, 2.2; and stomach, 7.5. There is wide variation in these ratios, indi-
cating noticeably dissimilar behavior of radioactive iodine administered as NaI\(^*\) and that administered as the radio-iodinated trypan blue.

**SUMMARY**

The distribution of radioactivity in the tissues of 24 Swiss A mice, each bearing two subcutaneous implants of tumor 15091a, and 5 control mice was measured after intravenous injection of a radioactive dye mixture prepared by iodination of trypan blue in the presence of radioactive iodide. The tumor tissue showed several times as high a concentration of radioactivity as did the skeletal muscle or skin, but less than the liver, spleen, or kidneys. The data are compared with those obtained by others using radio-brominated trypan blue and Evans blue to show the similar behavior of these different radioactive dye preparations.

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