The Uptake of As$^{74}$-labeled Arsonoazoproteins in Tissues of Tumor-bearing Mice*

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SUMMARY

The amounts of arsonoazoprotein-As$^{74}$ containing varied amounts of label were followed in tissues of tumor-bearing mice after intravenous injection. The uptake and clearance rates of this material were compared with those of uncoupled arsanilate-As$^{74}$. In terms of arsanilate, the uptake of a label into tumor from azoprotein approached that of the uncoupled material only with the use of extensively labeled protein (30 moles arsanilate/mole protein). Clearance of arsanilate-As$^{74}$ was virtually complete in about 24 hours, whereas 25–50 per cent of the initial concentrations remained in tumor 8 days following injection of arsonoazoprotein-As$^{74}$.

Large, persistent accumulations of arsonoazoprotein-As$^{74}$ were found within liver and kidney even with as little as 1–2 moles arsanilate/mole protein. These resemble the retention found after injection of other dye proteins and curb possible clinical applications. The possibility of overcoming this handicap with more precise, minimal labeling exists.

The uptake in kidney and the rate of disappearance of injected arsonoazoprotein-As$^{74}$ from blood varied directly with the amount of arsanilate bound to protein (within the range 2–30 moles of arsanilate/mole protein). The correlation between these factors was poor for liver and tumor and negative for muscle.

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arsanilate-As⁷⁴ was used as a tracer in the tissue uptake studies reported in this paper.

MATERIALS AND METHODS

A chemically induced, transplantable ependymoblastoma was used in these studies. This tumor was provided through the kindness of Dr. H. M. Zimmerman and Dr. R. G. Long. The solid tumor was macerated gently with a mortar and pestle in a solution of balanced mineral salts and glucose and strained through several layers of gauze to remove large particles. The suspension was injected subcutaneously into the inguinal region of young, male C57 mice. The tumors reached a palpable size in about 10 days and attained a weight of about 0.5—1 gm. 2—4 days later.

Crystalline bovine serum albumin was obtained from the Sigma Chemical Company. Arsanilic acid and other organic chemicals were obtained from Distillation Products, Eastman Organic Department. Arsenate-As⁷⁴ was obtained from Abbott Laboratories, Oak Ridge, Tennessee.

A tracerlab "1000" scaler and well counter were used for radioactivity measurements. Absorption measurements were made with a Model DU Beckman spectrophotometer with an ultraviolet attachment.

Preparation of mouse albumin.—After the chest cavity was opened, blood was drawn from the hearts of heparinized, anesthetized mice. The pooled blood from several animals was centrifuged and the plasma removed. Globulins were precipitated by half saturation with ammonium sulfate and centrifugation. The supernatant solution was dialyzed against distilled water for at least 2 days and then subjected to a further dialysis against 0.5 M carbonate buffer to complete the precipitation of the globulins. The solution was centrifuged to remove a small amount of precipitated protein and then the plasma removed. The solution was centrifuged to remove a small amount of precipitated protein and then the plasma removed. Globulins were precipitated by half saturation with ammonium sulfate and centrifugation. The supernatant solution was dialyzed against distilled water for at least 2 days at 4° C. with several changes to remove ammonium sulfate and to complete the precipitation of globulins. The solution was centrifuged to remove a small amount of precipitated protein and then subjected to a further dialysis against 0.5 M carbonate buffer, pH 10, for at least 8 hours. The crude albumin in 0.5 M carbonate buffer was stored frozen and used as required. Protein concentration was estimated according to the method of Gornall et al. (14).

Synthesis of arsanilic acid-As⁷⁴.—The synthesis was performed by the Bart reaction and was accomplished in three steps from sodium arsenate-As⁷⁴ (Na₂HAsO₄·7H₂O) and p-nitrobenzenediazonium borofluoride. The first step involved the reduction of sodium arsenate-As⁷⁴ with hydrazine sulfate and HBr. The arsenate-As⁷⁴ solution was pipetted into a separatory funnel, carrier sodium arsenate added, and the volume adjusted to 5 ml. for each 0.5 gm. arsenate. An equimolar amount of hydrazine sulfate was dissolved in the solution. Two volumes of concentrated HCl were added to this mixture followed by 1 volume of 48 per cent HBr. It was allowed to stand for about 5 minutes, and the arsenite was then extracted twice with 2½ volumes of benzene. The benzene was extracted twice with 10 ml. of N NaOH for each 0.5 gm. arsenate, and the alkaline extract was neutralized to about pH 10 with HCl. Arsenate was quantitatively reduced to arsinite by this procedure.

A modification of the procedure of Ruddy and Starkey (21) was used for the next step—the synthesis of p-nitrophenyl-As⁷⁴ arsonic acid. About 25 mg. cuprous chloride catalyst was added to the arsenite solution and a mechanical stirrer attached. An equimolar amount of dry p-nitrobenzenediazonium borofluoride (prepared according to Ruddy and Starkey) was introduced in small amounts over a period of about 1 hour; pH was maintained at 7—10 with 10 per cent NaOH. Mixing was continued for an additional hour and then for 30 minutes at 65°—70° C. on an oil bath. The pH was adjusted to about 7 with N HCl and the mixture filtered with suction on a small, sintered glass funnel.

p-Nitrophenylarsenic acid was reduced to arsanilic acid with iron powder and HCl in the third step. Four drops of concentrated HCl (per 0.5 gm. arsenate used) and an equal volume of ethanol were added to the yellow filtrate. About 5—10 equivalents of iron powder were introduced, and the mixture was refluxed for at least 2 hours. At the end of this time the mixture was chilled and the arsanilic acid-iron complex filtered off with the aid of suction on a sintered glass funnel. The filtrate was discarded. Arsanilate-As⁷⁴ was dissolved from the iron by mixing a few ml. of 0.5 N NaOH with the material on the funnel and filtering. The elution was repeated at least once.

Arsanilic acid was assayed by absorption at 256 mμ. The molar extinction coefficient at this wave length was 1.36 X 10⁴. For assay, the material was diluted 1:1000 with distilled water to which was added 0.2 ml. N HCl/100 ml solution. Although the synthesized arsanilic acid was used for coupling to protein without further purification, the material could be purified by crystallization at pH 3 and treatment with activated charcoal. The unpurified preparations contained about 5—20 per cent nonarsanilate arsenic and were often of a slightly yellow coloration. The ultraviolet absorption spectrum of a typical preparation was identical with that of a recrystallized commercial material. The synthesis described above results in about 50—50 per cent of the theoretical yield.

In the preparation of arsanilic acid-As⁷⁴ for
coupling to mouse protein and injection, 50 mg.
carrier sodium arsenate (160 μmoles) were dis-
solved in sufficient arsenate-As⁷⁴ solution to yield a
specific activity of about 3 μc/μmole. The specific
activity of the solution was carefully determined
later use in the estimation of the quantity of
arsanilate coupled to protein.

Arsanilate-As⁷⁴ which was used for injection as
such was prepared from 200 mg. sodium arsenate
and sufficient arsenate-As⁷⁴ to yield a specific ac-
tivity of about 1 μc/mg. This material was crystal-
lized from water by adjusting to pH 3 with HCl,
dissolved in 3 N HCl, treated with activated char-
coal and heat, filtered, and adjusted to pH 7 with
NaOH.

**Diazotization of arsanic acid-As⁷⁴ and coupling
to protein.**—Arsanilic acid-As⁷⁴ was diazotized and
coupled to protein essentially as described by
Tabachnick and Sobotka (25). Arsanilic acid-As⁷⁴
was neutralized to about pH 3 with HCl, and
about 5 moles excess HCl was added for each mole
of arsanilic acid. The solution was chilled in an
ammonium sulfate-ice bath, an equimolar quanti-
ty of aqueous sodium nitrite was introduced over
a period of 10 minutes with stirring, and the mix-
ture then was allowed to set on ice for 1 hour. The
diazotate was added to protein in 0.5 M carbonate
buffer, pH 10, over a period of several minutes
with stirring. The concentration of carbonate buf-
fer in the reaction mixture was sufficient to prevent
pH change during the coupling process. At least 4
hours were allowed to complete the reaction. The
mixture was dialyzed at 4° C. for at least 3 days
against 2 liters of 0.1 M NaCl and 0.005 M phos-
phate buffer, pH 7, with daily changes of the
dialyzing solution. Essentially all reversibly bound
radioactivity was removed in this time. The azo-
protein solutions remained optically clear at all
times during dialysis, and there was no evidence of
precipitation up to the time of injection.

After dialysis the volume of the azoprotein solu-
tion was adjusted to 10 mg albumin/ml with dis-
tilled water. The quantity of arsanilate-As⁷⁴
coupled on a molar basis was estimated from the
specific activity of the arsanilate-As⁷⁴ with 67,000
used as the molecular weight of albumin. It was
assumed that the molecular weight of mouse al-
bumin does not differ significantly from that of
other mammalian species (80). Loss of protein
from the dialysis bags during dialysis was assumed
to be negligible (25).

The amount of arsanilate-As⁷⁴ coupling to pro-
tein could be approximately predicted by the molar
eration of diazotized arsanilate to protein in
the reaction mixture under the conditions de-
scribed above. Chart 1 shows that diazotized ar-
sanilate coupled stoichiometrically up to a molar
ratio of about 100 to 1. Although not shown in this
chart, the linearity of the plot was maintained up
to a ratio of 200 to 1. At higher ratios the per cent
of material irreversibly coupling to protein de-
creased, and considerably longer periods of dialysis
were required to remove reversibly bound materi-
All azoproteins, including those prepared with
8,000 times molar excess arsanilate, showed no
evidence of insolubility at the concentrations em-
ployed.

**Injection of mice and assay of uptake.**—0.1 Ml. of
solution containing 1 mg. azoalbumin-As⁷⁴ or 0.5—
1 mg. arsanilate-As⁷⁴ was injected into the tail vein
with a 30-gauge needle. About 10 minutes before
sacrifice, the mice were given injections of 0.05 ml.

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**Table 1** illustrates the correlation between the
number of arsanilate groups coupled to albumin
and the rate of clearance from the blood stream
after injection. The rate was approximately pro-
portional to the number of arsanilate groups on the
protein molecule. 0.2 Per cent of the injected dose

---

The animals were anesthetized with ether,
the chest cavities were opened, and 0.2 ml. blood
was withdrawn from the hearts. The mice were
then perfused with 0.9 per cent NaCl until the
liver and kidneys were blanched. The kidney,
liver, tumor, and a portion of leg muscle were re-
moved, weighed, and counted for 10 minutes in a
well counter. All counts were corrected for back-
ground and decay. Results were expressed as per-
cent of injected dose per gm. tissue and were nor-
malized to a 25-gm. mouse (multiplying by wt. of
mouse/25).

**RESULTS**

Table 1 illustrates the correlation between the
number of arsanilate groups coupled to albumin
and the rate of clearance from the blood stream
after injection. The rate was approximately pro-
portional to the number of arsanilate groups on the
protein molecule. 0.2 Per cent of the injected dose
(per ml. blood) of the lightly coupled protein remained in the circulation 8 days after injection; this same concentration was found with the heavily coupled protein after 24 hours. Table 1 also shows the rate of clearance of uncoupled arsanilate-As³⁴ from the blood stream. As expected, this material was removed from the circulation considerably more quickly than even the most heavily coupled azoprotein. After 24 hours the arsanilate was barely detectable. Table 2 shows the concentration of azoproteins and free arsanilate in the liver up to 8 days after injection. The uptake of azoprotein did not vary appreciably with extent of coupling. There appeared to be a slightly more uptake in this organ with greater than 1–2 moles of arsanilate per mole protein, but this disparity disappeared after about 16 hours. The liver concentrations rose above those in blood after the first hour.

The uptake of azoprotein into muscle also did not appear to be related to the coupling values (Table 3). This tissue also did not take up uncoupled arsanilate in appreciable amounts, the levels being less than those in the blood stream at all times after injection. The rate of clearance of azoprotein from muscle tissue was very slow, especially in the case of the lightly labeled material. The concentration after 8 days was not appreciably different from that observed during the first few hours.

The kidney was the only organ studied in which uptake appeared to be directly related to the extent of coupling. Table 4 shows the proportionately greater uptake of the more heavily labeled

### Table 1

**Radioactivity Remaining in the Blood Stream after Injection of Azoalbumin-As³⁴ in Tumor-bearing Mice**

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Aarsanilate-As³⁴</th>
<th>Azoalbumin-As³⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1-2*</td>
<td>4-6*</td>
</tr>
<tr>
<td>1</td>
<td>1.2 ± 0.1</td>
<td>22 ± 1.8</td>
</tr>
<tr>
<td>2</td>
<td>0.5 ± 0.1</td>
<td>20 ± 1.0</td>
</tr>
<tr>
<td>4</td>
<td>0.2 ± 0.02</td>
<td>12 ± 1.3</td>
</tr>
<tr>
<td>8</td>
<td>9 ± 0.6</td>
<td>5 ± 0.1</td>
</tr>
<tr>
<td>16</td>
<td>0.06 (1)</td>
<td>0 ± 0.0</td>
</tr>
<tr>
<td>24</td>
<td>0.06 (1)</td>
<td>0 ± 0.0</td>
</tr>
<tr>
<td>48</td>
<td>0.06 (1)</td>
<td>0 ± 0.0</td>
</tr>
<tr>
<td>96</td>
<td>0.06 (1)</td>
<td>0 ± 0.0</td>
</tr>
<tr>
<td>192</td>
<td>0.06 (1)</td>
<td>0 ± 0.0</td>
</tr>
</tbody>
</table>

* Moles arsanilate-As³⁴/mole protein.

Results are expressed as per cent of injected dose per ml. of blood normalized to a 25-gm. mouse (multiplying by wt. of mouse/25). One mg. As³⁴-labeled azoprotein and 0.5 mg. arsanilate-As³⁴ were injected. In this and the following tables, each figure represents the average of at least five mice. Figures without standard error represent the means of one to three mice; the number of mice studied is indicated in parentheses.

### Table 2

**Uptake of Arsanilate-As³⁴ and Azoalbumin-As³⁴ in Liver of Tumor-bearing Mice**

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Aarsanilate-As³⁴</th>
<th>Azoalbumin-As³⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1-2*</td>
<td>4-6*</td>
</tr>
<tr>
<td>1</td>
<td>1.3 ± 0.08</td>
<td>17 ± 0.5</td>
</tr>
<tr>
<td>2</td>
<td>1.2 ± 0.11</td>
<td>21 ± 1.7</td>
</tr>
<tr>
<td>4</td>
<td>1.0 ± 0.08</td>
<td>32 ± 1.8</td>
</tr>
<tr>
<td>8</td>
<td>1.0 (1)</td>
<td>17 ± 1.1</td>
</tr>
<tr>
<td>16</td>
<td>0.6 (1)</td>
<td>15 ± 0.5</td>
</tr>
<tr>
<td>24</td>
<td>0.4 (1)</td>
<td>20 ± 0.8</td>
</tr>
<tr>
<td>48</td>
<td>15 ± 0.1</td>
<td>15 ± 0.1</td>
</tr>
<tr>
<td>96</td>
<td>9 ± 1.0</td>
<td>9 ± 1.0</td>
</tr>
<tr>
<td>192</td>
<td>9 ± 1.0</td>
<td>9 ± 1.0</td>
</tr>
</tbody>
</table>

* Moles arsanilate-As³⁴/mole protein.

Results are expressed as per cent of injected dose per gm. tissue normalized to a 25-gm. mouse; 1 mg. As³⁴-labeled azoprotein and 0.5 mg. arsanilate-As³⁴ were injected.
material by this tissue and suggests a capacity of this organ to detect abnormal or modified proteins in a quantitative manner. At the present time, no explanation for the mechanism of this capacity is possible. The kidney also showed a relatively high uptake of uncoupled arsanilate; however, this was confined to the first 24-hour period and appeared to be compatible with the excretory pattern.

There were no clear differences in the uptake of variously labeled proteins into tumor. The data in Table 5 show that the highest concentrations were reached between 8 and 24 hours, with 1–2 moles arsanilate per mole protein. With more arsanilate coupled, the concentrations were high up to 4 hours after injection and then fell progressively. This result may be a reflection of the more rapid clearance of highly coupled azoproteins from the blood stream noted in Table 1. Although the uptake of azoproteins appeared to be highest with the more lightly labeled proteins (up to 4.6 per cent of the injected dose per gm. of tissue), the differences in uptake of the various azoproteins was not nearly as striking as in the case of kidney. In fact, no differences in uptake were observed among the proteins with more than 1–2 moles arsanilate per mole protein. The elimination of azoprotein from tumor appeared to be slower with the more lightly coupled material. After 8 days there was 3 times more lightly coupled protein in the tumor than in the material containing 8–10 moles arsanilate per mole protein. The uncoupled arsanilate deposits were very low in tumor; these were virtually eliminated in 24 hours.

The activity of As$^{74}$-labeled azoproteins declined, in all tissues, at a very slow rate. After 8 days, most tissues retained 25–50 per cent of their initial activity. This fact, plus the negligible loss of radioactivity after 3 days' dialysis, suggests that the arsanilate is firmly bound to protein. In general, uncoupled arsanilate remained in the tissues

<table>
<thead>
<tr>
<th>TABLE 3</th>
<th>UPTAKE OF AZOALBUMIN-AS$^{74}$ AND ARSANILATE-AS$^{74}$ IN MUSCLE OF TUMOR-BEARING MICE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TIME (HOURS)</strong></td>
<td><strong>ABSANILATE-AS$^{74}$</strong></td>
</tr>
<tr>
<td>****</td>
<td><strong>Azoalbumin-As$^{74}$</strong></td>
</tr>
<tr>
<td></td>
<td>1–2$^a$</td>
</tr>
<tr>
<td>1</td>
<td>0.2 ± 0.02</td>
</tr>
<tr>
<td>2</td>
<td>0.1 ± 0.03</td>
</tr>
<tr>
<td>4</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td>8</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>16</td>
<td>0.8 ± 0.18</td>
</tr>
<tr>
<td>24</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>48</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>96</td>
<td>0.5 ± 0.3</td>
</tr>
</tbody>
</table>

* Moles arsanilate-As$^{74}$/mole protein.

Results are expressed as per cent of injected dose per gm. tissue normalized to a 25-gm. mouse; 1 mg. azoprotein-As$^{74}$ and 0.5 mg. arsanilate-As$^{74}$ were injected.

<table>
<thead>
<tr>
<th>TABLE 4</th>
<th>UPTAKE OF AZOALBUMIN-AS$^{74}$ AND ARSANILATE-AS$^{74}$ IN KIDNEY OF TUMOR-BEARING MICE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TIME (HOURS)</strong></td>
<td><strong>ABSANILATE-AS$^{74}$</strong></td>
</tr>
<tr>
<td>****</td>
<td><strong>Azoalbumin-As$^{74}$</strong></td>
</tr>
<tr>
<td></td>
<td>1–2$^a$</td>
</tr>
<tr>
<td>1</td>
<td>7.6 ± 0.9</td>
</tr>
<tr>
<td>2</td>
<td>7.6 ± 0.9</td>
</tr>
<tr>
<td>4</td>
<td>6.0 ± 0.1</td>
</tr>
<tr>
<td>8</td>
<td>1.0 (1)</td>
</tr>
<tr>
<td>16</td>
<td>0.4 (1)</td>
</tr>
<tr>
<td>24</td>
<td>2.3 (1)</td>
</tr>
<tr>
<td>48</td>
<td>12 ± 1.4</td>
</tr>
<tr>
<td>96</td>
<td>12 ± 1.4</td>
</tr>
<tr>
<td>192</td>
<td>12 ± 1.4</td>
</tr>
</tbody>
</table>

* Moles arsanilate-As$^{74}$/mole protein.

Results are expressed as per cent of injected dose per gm. tissue normalized to a 25-gm. mouse; 1 mg. As$^{74}$-labeled azoprotein and 0.5 mg. arsanilate-As$^{74}$ were injected.
for relatively brief periods at comparatively high levels. The tumor contained about 0.8 per cent of the injected dose per gm. tissue 1 hour after injection. Since 0.5 mg. was injected, this corresponds to about 0.004 mg. delivered to 1 gm. tumor. When 1 mg. or 1/69 mmole protein (to which is attached about 2/69 mmole arsanilate) was injected, about 1.7 per cent of the injected dose or 0.017 X 2/69 mmole of arsanilate was delivered to the tumor at the end of 1 hour. One mmole is equal to 0.217 mg.; hence, this corresponds to about 0.0001 mg. With 30 moles arsanilate per mole protein and 3.3 per cent of the injected dose taken up at the end of 1 hour, this corresponds to about 0.003 mg. arsanilate, or nearly the same concentration as with the uncoupled material, in spite of the fact that about one-fifth the amount of arsanilate was injected. At the end of 24 hours, the tumor contained only 0.00015 mg. of uncoupled arsanilate per gm. tissue, whereas the lightly labeled protein level rose to 0.0008 mg., and the arsanilate-As⁷⁴ from the heavily coupled protein fell to 0.002 mg.

Attempts were made to determine the distribution of radioactivity among cell fractions after injection of As⁷⁴-labeled azoproteins into mice. In initial experiments, tissues which were removed and counted for the data described in Tables 2–5 (30 moles arsanilate per mole protein) were stored frozen and homogenized in 0.9 per cent NaCl with a mortar and pestle. The volume of homogenate was measured and a sample counted. The total homogenate was then centrifuged at 20,000–30,000 X g for 15 minutes in a refrigerated centrifuge to remove most of the particulate material, and a sample of the supernatant fluid was counted. Fifty per cent of the radioactivity was removed from the homogenate by this procedure; tumor, liver, kidney, and muscle behaved similarly in this respect. In later experiments, normal mice (without tumors) were given injections of 1 mg. As⁷⁴-labeled azoprotein containing 8 moles arsanilate-

### TABLE 5

**UPTAKE OF ARSANILATE-AS⁷⁴ AND AZOALBUMIN-AS⁷⁴ IN TUMORS OF EPENDYMOBLASTOMA-BEARING MICE**

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Arsanilate-As⁷⁴</th>
<th>Azoalbumin-As⁷⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1-4h</td>
<td>4-6h</td>
</tr>
<tr>
<td>1</td>
<td>0.8±0.1</td>
<td>1.7±0.2</td>
</tr>
<tr>
<td>2</td>
<td>0.2±0.05</td>
<td>0.9±0.1</td>
</tr>
<tr>
<td>16</td>
<td>0.04±0.05</td>
<td>0.03±0.02</td>
</tr>
<tr>
<td>48</td>
<td>0.3±0.2</td>
<td>0.3±0.2</td>
</tr>
<tr>
<td>96</td>
<td>0.5±0.3</td>
<td>0.5±0.3</td>
</tr>
<tr>
<td>192</td>
<td>0.9±0.5</td>
<td>0.9±0.5</td>
</tr>
</tbody>
</table>

* Moles arsanilate-As⁷⁴/mole protein.
Results are expressed as per cent of injected dose per gm. tissue normalized to a 25-gm. mouse; 1 mg. As⁷⁴-labeled azoprotein and 0.5 mg. arsanilate-As⁷⁴ were injected.

### TABLE 6

**DISTRIBUTION OF RADIOACTIVITY AMONG CELL FRACTIONS IN LIVERS OF MICE GIVEN INJECTIONS OF AS⁷⁴-LABELED AZOPROTEIN**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Time after injection (hr.)</th>
<th>Per cent of total liver radioactivity in particulate fractions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>900 X g 10 min.</td>
<td>8,000 X g 10 min.</td>
</tr>
<tr>
<td>Injected</td>
<td>1</td>
<td>32</td>
</tr>
<tr>
<td>Injected</td>
<td>24</td>
<td>46</td>
</tr>
<tr>
<td>Added to homog.: incubated 2 hr.</td>
<td>2.6</td>
<td>2.0</td>
</tr>
</tbody>
</table>
protein was added to a liver homogenate prepared in the same manner from uninjected mice, incubated for 2 hours at room temperature, and subjected to the same centrifugation. The data obtained from these experiments are shown in Table 6. These observations suggest that a significant proportion of the injected azoproteins were taken into the cell and not merely trapped in the interstitial fluid or bound to the cell surface.

**DISCUSSION**

Our studies of the experimental tumor concentrations of arsanaoazalbumin indicate one advantage which is of interest in considering a possible application for protein-coupled cytotoxic groups. This is the prolonged retention of conjugate within tumor. The levels of uncoupled arsanilate, although initially high, fell very rapidly in the first few hours; the protein accumulations did not follow this desorptive course but rose quickly to high levels which were maintained for several days. Unfortunately, as indicated above, the absolute amounts of conjugated arsanilate in tumor were relatively low save with the use of heavily labeled material (20–30 moles/mole protein).

As$^{74}$-labeled azoproteins appear to be unsuitable for any therapeutic or diagnostic applications because of the excessively high and persistent renal and liver accumulations. These, interestingly, were approximately 5–15 times greater than the tumor deposits and also greatly exceeded renal and liver levels found with other trace-labeled protein (6–8, 16). Long et al. (19) obtained approximately the same per cent uptake in tumors as those noted in Table 5 after injection of radioiodinated human serum albumin in mice bearing the same experimental tumor as the one used in this study. Blood levels, after injection of iodinated albumin, also did not differ greatly from those obtained with 1–2 moles arsanilate per mole protein. The major differences were in uptake by liver and kidney. In the study by Long et al., the tumor concentrations were approximately 5 times higher than kidney and 5 times higher than those attained in the livers 9 hours after injection. Our azoproteins—even those containing one conjugated arsanilate group—appear to be retained in liver much in the same way as the dye proteins of Sabin (22) and Kruse and McMaster (18) and the highly iodinated globulin of Francis and Hawkins (18).

Within the range 1–50 moles/mole protein, the extent of coupling greatly influenced the uptake in kidney. The absence of such an effect in other tissues (such as muscle) and the evenness of the 1-hour blood samples of varied labels suggest that this was not entirely related to a retention in blood. This renal detention is of particular interest in view of the demonstration by Smetana and Johnson (23) of a prolonged (1 year) deposition of dye-proteins within the cells of renal tubules. Tumor uptake, like muscle, did not appear to vary significantly with the coupling. This finding agrees with the observations of Starbuck and Busch for tumor (24). Kidney and, to a lesser extent, liver, therefore, seem able to recognize grossly altered proteins; muscle and perhaps tumor fail in this discrimination.

Heavily labeled arsanaoazalbumin again resembles the dye-protein of Sabin in that both azo compounds are cleared from blood much more rapidly than are native or trace-labeled proteins. Dixon et al. (10) and Gitlin et al. (18) have noted this acceleration with dye-proteins; Haurowitz and Crampton (15) and Francis and Hawkins (12) have shown a similar rapid loss with extensively iodinated globulin. Homogeneity of labeling was not insured in our protein preparations; however, the direct correlation between coupling and clearance rates (Table 1) indicates that the allotment in each of the four groups was grossly accurate. This correlation parallels a similar effect noted with lightly and heavily brominated protein by Fine and Seligman (11). Alterations induced by the addition of the first arsanilate moiety seemed to influence the clearance rate proportionately more than later increments. However, our As$^{74}$ arsanilate was of relatively low specific activity, and it is possible that improved labeling technics (with more active material) might allow precise, minimal protein labeling and reveal more subtle changes.

Changes in physical characteristics such as alteration in tertiary structure, isoelectric point, etc., would be expected to occur upon the addition of charged groups to protein molecules. These changes in physical and chemical properties should be evidenced by altered solubility, electrophoretic mobility, etc. None of the azoproteins prepared in the present study showed evidence of decreased solubility, and all preparations, although highly colored, remained optically clear at the pH's and concentrations used. The introduction of charged groups could probably affect interaction with other proteins in the blood stream. A negatively charged group such as arsenate would be expected to lower the isoelectric point, and it is conceivable that some interaction with normal proteins to form aggregates could occur. The possibility for aggregation would be enhanced with increasing numbers of introduced negative charges and, hence, could explain the correlation between the numbers of added arsanilate groups and the uptake by the
kidney which were observed in this study. However, there was no visible evidence of aggregation or precipitation when azoproteins were added to normal serum in vitro and incubated at 37°C. A more plausible explanation for this correlation is that the kidney is sensitive to small changes in isoelectric point of proteins. The effect of alteration of isoelectric point on the uptake of proteins into tissues is currently under study in this laboratory.

The high tumor pickup of azoprotein suggests a useful application for radioencephalographic positron scanning. Tumor to brain ratios were 2-8 times greater than those found with either arsanilate or inorganic arsenic. However, we feel that the significant liver and renal deposits bar its clinical use as a scanning source and emphasize the factor of retentivity as a major obstacle to the practice of safe, clinical scanning. In this instance, the dangers from prolonged retention appear greater than those emphasized by Blau and Bender for iodinated albumin (5) and those noted by Brinkman et al. for a labeled micromolecular diuretic (40-56 rad local renal radiation) (4).

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The Uptake of As$^{74}$-labeled Arsonoazoproteins in Tissues of Tumor-bearing Mice

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