The Distribution of Stilbamidine in the Livers of Normal and Sarcoma-bearing Mice*

EDWARD L. BENNETT, DONALD E. PACK, BARBARA J. KRUECKEL, AND JOHN C. WEAVER

(Radiation Laboratory and Donner Laboratory of Medical Physics, University of California, Berkeley, Calif.)

In experiments preliminary to the possible use of carbon-14-labeled stilbamidine in the treatment of humans with multiple myeloma (2, 9, 26) it was found in normal mice that no abnormal concentration of stilbamidine persisted in any tissue and that over 90 per cent of the injected radioactivity was excreted within a month (20). A tracer dose of C14-labeled stilbamidine was then given to a human with advanced multiple myeloma, and at autopsy 3 months later it was found that 60 per cent of the activity of the administered C14 was present in the liver. Because of this finding, mice bearing several types of tumors were studied; and it was found that much larger amounts of C14 (up to 40 per cent of the injected dose 4 days after administration) were concentrated in the liver of strain A mice bearing transplantable sarcomas and of mice with certain other tumors than in that of normal controls (27). Inasmuch as this very likely reflects important differences in the livers of normal and tumor-bearing mice, this paper describes an investigation that has been made of the site of retention of the stilbamidine in the livers of such mice. These investigations have established that the stilbamidine is bound to the mitochondria or to a particle of very similar physical properties. The binding appears to be an association or adsorption rather than chemical in nature. It does not appear to be bound mainly to the nucleic acid, as has been suggested for the stilbamidine which is deposited in the cytoplasm of myeloma cells (26).

METHODS

Preparation of Stilbamidine-Amidine-C14

4,4'-trans-stilbenedicarboxamidine was prepared by a procedure modified from that reported earlier (20) and previously described methods (19) modified for tracer synthesis were employed. Potassium cyanide and cuprous cyanide were prepared as described (25).

* The work described in this paper was sponsored by the United States Atomic Energy Commission.

Received for publication September 9, 1952.

Cuprous cyanide-C14 (349 mg.; 1.94 μm; 19.6 μc.), trans-dibromostilbene (657 mg.; 1.94 μm) (3), and cupric sulfate (45 mg.) were sealed in a 15 X 180 mm. pyrex ignition tube together with 5 ml of pyridine (distilled from barium hydride). The contents were mixed and heated with shaking for 2 hours at 250°. The bomb and contents were allowed to cool to room temperature, and then the ignition tube was cooled in liquid nitrogen before opening. Subsequently, 10-15 volumes of cold 6 M hydrochloric acid were added, and the product was removed by filtration, washed with acid, water, a small amount of cold absolute ethanol, and air-dried to yield 492 mg. (101 per cent) of 4,4'-trans-dicyano-C14-stilbene.

This compound (440 mg.) was sealed with 1.5 gm. of dried ammonium thiocyanate in an ignition tube, mixed, and heated at 185° ± 5° for 14 hours. The tube was cooled in liquid nitrogen before being opened. The contents of the tube were extracted with 7-8 ml. of cold 0.5 M ammonium hydroxide and filtered. The precipitate was extracted with 10 ml. of 1 M hydrochloric acid at 80°, filtered, and extracted with 50-ml. portions of warm water, and then with acid and water again until the total volume of the extract was about 100 ml. Subsequently, the combined extracts were warmed, decolorized with 80 mg. of Norite, made 8 M in hydrochloric acid, and stored at 0° overnight. 4,4'-trans-stilbene-dicarboxamidine hydrochloride was dissolved in 10 ml. of water, filtered, precipitated with ammonia, and subsequently obtained as the disethionate. The yield was 815 mg., an over-all yield of 82 per cent from cuprous cyanide, and the specific activity was 19.5 μc/mg. From other preparations yields of 50-55 per cent have been obtained. The compound had the same infrared spectrum as an authentic sample. Paper chromatography of the product indicated that only one radioactive compound was present with the same Rf as an authentic sample (40 wt. per cent butanol-25 wt. per cent propionic acid-35 wt. per cent water used as the solvent system).

Experimental Methods

To establish the site and nature of retention of stilbamidine in the livers of the tumor-bearing mice, the following technics were used: (a) Fractionation with ultracentrifugation, (b) disintegration of mitochondria by sonic disintegration or by release of gas pressure, (c) enzymatic treatment of centrifuged fractions.

Liver cell fractionation procedure (Chart 1).—For the studies reported here, 6-8-month-old, normal or tumor-bearing (Sarcoma-1) A strain mice, maintained on a diet of Purina Laboratory Chow, were used. In each mouse of the tumor series, two sarcomas were implanted subcutaneously 7-10 days prior to
administration of the stilbamidine. At the time of sacrifice, the total tumor weight per mouse ranged from 0.8 to 6.3 gm. The mice were given intraperitoneal injections of 0.45 mg. of a freshly prepared solution of C\(^{14}\)-labeled stilbamidine diisethionate in 0.10 ml of 0.9 per cent saline. Unless otherwise indicated, the mice were sacrificed by decapitation 96 hours after injection, and the livers were removed and fractionated by the methods described below. The mice were kept in cages designed to prevent contamination of food and water by excreta.

The livers were weighed, and an aliquot was removed to determine the radioactivity present by combustion and subsequent determination in the form of barium carbonate. The remainder of the liver was forced through a masher to remove connective tissue. The pulp was homogenized in approximately 10-12 volumes of 0.85 mosm sucrose. The homogenate (F-1) was centrifuged 10 minutes at top speed (about 2,000 r.p.m.) in an International clinical centrifuge. The sediment (F-1) of nuclei, unbroken liver cells, and red blood cells was generally rehomogenized and recentrifuged to yield the final sediment (F-2-A) and a supernatant (F-2-B), which was added to the first supernatant (F-3) and centrifuged at 9,000 r.p.m.\(^{1}\) (5,440-7,350 g.) for 15 minutes, unless otherwise noted, to yield a first supernatant (F-3) and centrifuged at 9,000 r.p.m.\(^{1}\) (3,440-4,800 g.) for 15 minutes at top speed (about 2,000 r.p.m.) in an International clinical centrifuge. The sediment (F-4) of nuclei, unbroken liver cells, and red blood cells was generally rehomogenized and recentrifuged to yield the final sediment (F-4-P), consisting mainly of mitochondria and a supernatant (F-4-S) containing microsomes and "soluble" constituents of the liver. The precipitate was rehomogenized in sucrose solution and recentrifuged at 18,000 r.p.m. (13,800-20,400 g.) for 15 minutes. This precipitate (F-4-P) was suspended in 0.25 M sucrose or phosphate buffer, pH 7.7, depending upon the nature of further experiments with this fraction. All supernatants (F-3) were combined.

**Disintegration of Mitochondria**

**By sonic disintegration (11).—**The precipitate containing mitochondria (F-4-P) (Chart 1) was homogenized briefly in water, sucrose solution, or phosphate buffer. (0.002 M \(\text{KH}_2\text{PO}_4\) or 0.016 M \(\text{K}_2\text{HPO}_4\), pH 7.7) and subjected to sonic disintegration for 60 minutes at 2°-4°. The disintegrated mitochondria (F-D) were centrifuged at 9,000 r.p.m. for 15 minutes to yield a gray precipitate (F-D-1) and a supernatant, which was centrifuged at 31,000-120,000 g.) for 90 minutes to yield a reddish pellet (F-D-2) and the final supernatant (F-D-3).

**By release of gas pressure (7).—**The mitochondrial precipitate (F-4-P), suspended in 0.25 M sucrose or phosphate buffer, pH 7.7, was subjected to one disintegration cycle with nitrous oxide and subsequently centrifuged to yield precipitates at 9,000 r.p.m. (F-B-1) and at 38,000 r.p.m. (F-B-2) and a supernatant (F-B-3).

**Enzymatic treatment of fractions.—**The mitochondrial precipitate (F-4-P) and the disintegrated mitochondrial precipitate (F-D-P) were suspended in 0.25 M sucrose and 0.02 M phosphate buffer, pH 7.3, and were subjected to the action of crystalline ribonuclease (Armour) or trypsin (Armour crystalline) at \(37^{\circ}\). Subsequently, the reaction mixture was fractionated by centrifugation.

**Analytical Methods**

The measurements of radioactivity were made by direct plating of aliquots of the fractions (precipitates were suspended by brief homogenization in water or sucrose solution, depending upon the requirements of subsequent experimental procedure), and the determinations were made with a proportional counter.\(^{4}\) Self-absorption corrections were made where necessary. All determinations were made in duplicate. Checks were made by combustion and determination of the radioactivity in the form of barium carbonate by conventional technique.

Nitrogen was determined colorimetrically after Kjeldahl digestion (18). Readings were made at 440 mč in a Beckman Model DU spectrophotometer.

Nucleic acids were precipitated in the cold by the addition of 50 per cent triethanolamine acid (TCA) to a final concentration of 12 per cent.\(^{4}\) The precipitate was washed twice with cold 6 per cent TCA, then 8 times with 0.5 per cent ethanol, and finally extracted with 6 per cent TCA or perchloric acid for 15 minutes at 90°. Ribonucleic acid was determined by the orcinol reaction (16, 17), and deoxyribonucleic acid by the diphenylamine reaction (6). Readings were made in a Beckman Model DU spectrophotometer at 660 mč and 200 μ, respectively. Commercial yeast nucleic acid (Schwarz Laboratory) and sperm desoxyribonucleic acid (National Biochemical Corporation) were used as standards.

Sucinoxidase activity was determined manometrically by the method of Schneider and Potter (84).

In all cases, results were corrected for aliquots removed for analytical purposes.

**RESULTS**

**Site of stilbamidine-C\(^{14}\) concentration in liver cells.—**The experiments indicate that a large fraction, if not all the stilbamidine\(^{3}\) in the livers of sarcoma-bearing and normal A strain mice 4 days after the intraperitoneal injection of 0.45 mg. stilbamidine diisethionate is in the mitochondrial fraction.

Table 1 summarizes the results of numerous ultracentrifugal fractionations of these mouse livers. As can be seen from the table, the stilbamidine is found almost entirely in the mitochondrial fraction (F-4-P), and the distribution is the same whether the livers are from normal or sarcoma mice. About 14 per cent of the stilbamidine comes down with the slowly centrifuged nuclear fraction (F-2-A), and about the same amount remains in the final supernatant (F-5). This will be considered further.

Table 1 also shows the deoxyribonucleic acid, further.

\(^{3}\) Nucleometer, Radiation Counter Laboratories, Chicago.

\(^{4}\) Unaccountably low and erratic ribonucleic acid analyses were obtained when the nucleic acid was precipitated with 12 per cent perchloric acid (85). Investigation indicated that, although essentially identical results were obtained by the use of either acid immediately after sacrifice of the mice, in the 6-8 hours required for a complete fractionation, the apparent PNA content obtained when \(\text{HClO}_4\) was used as the precipitant decreased by 50 per cent or more. The deoxyribonucleic acid content obtained by either method remained essentially the same.

The radioactive compound present in the liver is referred to in this paper as stilbamidine. We have only preliminary evidence that the compound present in the cell may still be stilbamidine; further work is in progress to determine definitely the chemical nature of the radioactive compound.
LIVER MASH
Homogenized in 0.25 M Sucrose
Centrifuged 10 minutes in clinical centrifuge

PRECIPITATE F-2
Nuclei, unbroken liver cells, red blood cells

SUPERNATANT F-3
Mitochondria, microsomes, soluble constituents

Rehomogenized, recentrifuged

PRECIPITATE F-2-A
Mainly nuclei, red blood cells

SUPERNATANT F-2-B
Mitochondria, etc.

Centrifuge 9000 RPM, 15 minutes
3440-7350 g

PRECIPITATE F-4
Mitochondria

Rehomogenized, recentrifuged
18000 RPM, 15 minutes
13800 - 29400 g

PRECIPITATE F-4-P
Mitochondria

SUPERNATANT F-5
Microsomes, soluble constituents

Chart 1.—Fractionation procedure for mouse liver

Table 1
Distribution of Radioactivity in the Livers of Normal and Sarcoma-bearing A Strain Mice Four Days After Injection of Stilbamidine-Amidin-C34 Disethionate

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear ppt. (F-2-A)</td>
<td>14 (11-19)</td>
<td>15 (8-20)</td>
<td>78 (71-83)</td>
<td>76 (67-85)</td>
<td>10 (7-15)</td>
<td>15 (7-15)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitochondrial ppt. (F-4-P)</td>
<td>75 (67-70)</td>
<td>75 (67-70)</td>
<td>10 (6-15)</td>
<td>10 (7-15)</td>
<td>25 (18-32)</td>
<td>25 (18-32)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant (F-5)</td>
<td>12 (7-18)</td>
<td>12 (7-18)</td>
<td>11 (10-15)</td>
<td>11 (10-15)</td>
<td>64 (52-75)</td>
<td>64 (52-75)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Per cent injected dose in liver</td>
<td>8.2  (5.0-5.5)</td>
<td>25 (11-15)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Nine normal A strain male mice, 6-8 months, average weight, 24 gm., average liver weight 1.1 gm., are represented. Twelve sarcoma-bearing A strain male mice, 6-8 months; average weight, 23 gm.; average liver weight, 1.2 gm.; and average tumor weight (two tumors), 3.7 gm. (0.8-6.3 gm.) are represented. Total N averaged 31 mg. (28-33 mg.); total DNA, 3.3 mg. (2.9-4.1) in normal mice (4) and 5.5 mg. (2.0-3.5) in sarcoma-bearing mice (6). Total PNA averaged 13.5 mg. (11.1-14.8 mg.).
† Range.

* Nine normal A strain male mice, 6-8 months, average weight, 24 gm., average liver weight 1.1 gm., are represented. Twelve sarcoma-bearing A strain male mice, 6-8 months; average weight, 23 gm.; average liver weight, 1.2 gm.; and average tumor weight (two tumors), 3.7 gm. (0.8-6.3 gm.) are represented. Total N averaged 31 mg. (28-33 mg.); total DNA, 3.3 mg. (2.9-4.1) in normal mice (4) and 5.5 mg. (2.0-3.5) in sarcoma-bearing mice (6). Total PNA averaged 13.5 mg. (11.1-14.8 mg.).
ribonucleic acid, and nitrogen percentages in the different fractions. These values are in general agreement with those previously reported for liver fractionations (15, 21, 23, 24).

It seemed likely that the stilbamidine found in other than the mitochondrial fraction (Table 1) represented incomplete separation of mitochondria from the nuclear fraction and the final supernatant during centrifugation. In order to confirm this, a comparison of succinoxidase activity and C14 activity was made. It has been shown that succinoxidase activity resides solely in the mitochondria (12), so the finding of some succinoxidase activity in the nuclear fraction would indicate "contamination" with mitochondria. Table 2 shows that this is actually the case.

The distribution of succinoxidase activity closely corresponds to the distribution of radioactivity. The fractions used for this correlation were the nuclear (F-2-A) and the two mitochondria-containing fractions (F-2-B and F-3) (Chart 1). Since succinoxidase activity resides only in the mitochondria, this indicates that the succinoxidase activity present in the nuclear fraction was due to incomplete separation of mitochondria. Furthermore, the close correlation with radioactivity percentages in these fractions is strong presumptive evidence that the radioactivity is associated with the mitochondria.

To test further the postulate that stilbamidine is associated with the mitochondria, an experiment was performed using only a non-nuclear mitochondrial fraction (F-3). Portions of this fraction were centrifuged at speeds from 1,800 to 9,000 r.p.m.

### Table 2

**Comparison of Succinoxidase Activity and Radioactivity in Fractions**

(Results expressed as per cent of total)

<table>
<thead>
<tr>
<th>Fraction</th>
<th>M-11$^*$</th>
<th>M-18*</th>
<th>M-19*</th>
<th>M-20*</th>
<th>M-65†</th>
<th>M-66†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear F-2-A</td>
<td>S.O.$^+$</td>
<td>C&lt;sup&gt;14&lt;/sup&gt;</td>
<td>S.O.</td>
<td>C&lt;sup&gt;14&lt;/sup&gt;</td>
<td>S.O.</td>
<td>C&lt;sup&gt;14&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mitochondrial F-2-B</td>
<td>15 15 15 15</td>
<td>15 15 15 15</td>
<td>15 15 15 15</td>
<td>15 15 15 15</td>
<td>15 15 15 15</td>
<td>15 15 15 15</td>
</tr>
<tr>
<td>Mitochondrial F-3</td>
<td>73 73 73 73</td>
<td>73 73 73 73</td>
<td>73 73 73 73</td>
<td>73 73 73 73</td>
<td>73 73 73 73</td>
<td>73 73 73 73</td>
</tr>
</tbody>
</table>

* A strain, sarcoma-bearing mice.
† A strain normal mice.
‡ S.O. = succinoxidase.

Total mm<sup>3</sup> O<sub>2</sub>/hr consumed varied from 15,000 calculated for the total liver.

### Table 3

**Distribution of C<sup>14</sup> Activity, Nitrogen, PNA, and Succinoxidase Activity upon Centrifugation at Several Speeds**

<table>
<thead>
<tr>
<th>Speed (r.p.m.)</th>
<th>Relative g.</th>
<th>Per cent of total</th>
<th>Per cent of total ptted. at 9,000 r.p.m.</th>
<th>Per cent of total ptted. at 9,000 r.p.m.</th>
<th>Per cent of total ptted. at 9,000 r.p.m.</th>
<th>Per cent of total ptted. at 9,000 r.p.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,800</td>
<td>157–290</td>
<td>17</td>
<td>19</td>
<td>29</td>
<td>29</td>
<td>29</td>
</tr>
<tr>
<td>3,750</td>
<td>525–1,375</td>
<td>55</td>
<td>60</td>
<td>69</td>
<td>69</td>
<td>69</td>
</tr>
<tr>
<td>5,300</td>
<td>1,150–2,545</td>
<td>78</td>
<td>89</td>
<td>80</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>7,000</td>
<td>2,070–4,450</td>
<td>85</td>
<td>97</td>
<td>95</td>
<td>95</td>
<td>95</td>
</tr>
<tr>
<td>9,000</td>
<td>3,440–7,350</td>
<td>88 (100)</td>
<td>90 (100)</td>
<td>90 (100)</td>
<td>90 (100)</td>
<td>90 (100)</td>
</tr>
</tbody>
</table>

* Sarcoma-bearing mice were used for the above experiments.
† Minimum and maximum refer to the g at the top and the bottom of the tubes.

The sediment and supernatant of each centrifugation were measured for succinoxidase activity and radioactivity. Table 3 shows the comparison of radioactivity and succinoxidase activity in the sediment of each centrifugation expressed as "per cent of total precipitated at 9,000 r.p.m." and indicates for each centrifuged speed the percentage of radioactivity and succinoxidase sedimented, as compared to the total precipitated at 9,000 r.p.m. It may be seen that there is a good correlation between the concentration of succinoxidase and that of stilbamidine. The largest deviation occurs in the mitochondrial sample sedimented at 3,500 r.p.m. There the sediment showed a C<sup>14</sup> activity of 60 per
The mitochondrial sediment was suspended and disintegrated. This was then centrifuged at 9,000 r.p.m. for 15 minutes producing sediment F-D-1 (Table 4). The supernatant was then centrifuged at 38,000 r.p.m. for 30 minutes producing sediment F-D-2. The supernatant of this sediment is labeled F-D-3 in Table 4.

Several interesting results were obtained in this experiment. Following sonic disintegration, as seen in Table 4, an average of 58 per cent of the C"C (stilbamidine) was sedimented at 38,000 r.p.m., and only 27 per cent was spun down at the slower speed of 9,000 r.p.m. About 15 per cent of the stilbamidine remained in the supernatant after the high speed centrifugation, suggesting either that this portion of the stilbamidine was detached from its binding to the mitochondria, or that this

<table>
<thead>
<tr>
<th>TABLE 4</th>
<th>FRACTIONATION OF MITOCHONDRIA DISINTEGRATED BY SEVERAL METHODS</th>
</tr>
</thead>
<tbody>
<tr>
<td>EXPERIMENT</td>
<td>SONIC DISINTEGRATION IN 0.05 M PHOSPHATE BUFFER, pH 7.7</td>
</tr>
<tr>
<td>Fraction</td>
<td>Per cent</td>
</tr>
<tr>
<td>Sedimented at 9,000 r.p.m.</td>
<td>27</td>
</tr>
<tr>
<td>F-D-1</td>
<td>(26-28)</td>
</tr>
<tr>
<td>Sedimented at 38,000 r.p.m.</td>
<td>58</td>
</tr>
<tr>
<td>F-D-2</td>
<td>(52-61)</td>
</tr>
<tr>
<td>Supernatant F-D-3</td>
<td>15</td>
</tr>
</tbody>
</table>

* The figures represent the average of three experiments; sarcoma-bearing mice were used in two experiments, normal mice in the third.
† F-D-1 sedimented by centrifuging disintegrated mitochondria at 9,000 r.p.m. for 15 minutes; F-D-2 subsequently sedimented by centrifugation at 38,000 r.p.m. for 30 minutes; and F-D-3 is the remaining supernatant material.
‡ Range.

Manner in which stilbamidine is attached to mitochondria.—Preliminary experiments showed that stilbamidine is related to the liver cell mitochondria either by association or as a loose complex, because the stilbamidine can be readily extracted by dilute hydrochloric acid or ammonium hydroxide in the cold. Since stilbamidine is not soluble in cold alcohol, warm alcohol, or ether, it is not surprising that efforts to extract radioactivity with these solvents were unsuccessful. Dialysis in the cold against sucrose and against 0.9 per cent sodium chloride removed little of the radioactivity. The lability of stilbamidine to acid excluded the use of trichloroacetic acid or perchloric acid to separate the nucleic acid in order to determine whether stilbamidine is combined with the ribonucleic acid.

The problem was next attacked by breaking up the mitochondria in two ways: (a) sonic disintegration (11) and (b) disintegration by release of gas pressure (7). In both techniques of disintegration, the experiments were performed on the mitochondrial sediment of differentially centrifuged liver.

amount of stilbamidine was attached to small protein or other molecules. The fact that the great bulk of stilbamidine was sedimented indicates that there is binding with larger particulate constituents of the mitochondria. Furthermore, only 15 per cent of the ribonucleic acid content of the mitochondria remained in the final supernatant (Table 4). The inference might be, then, that the stilbamidine is combined with the ribonucleic acid in mitochondria, but this is later shown not to be the case. This also indicates that mitochondria contain particles rich in ribonucleic acid, or are surrounded by a membrane rich in ribonucleic acid, or both. About 50 per cent of the nitrogen content of the mitochondria was freed by sonic disintegration. This indicates that half of the protein of these mitochondria was released in soluble form, while the other half belonged to the ribonucleic acid-rich particles and membrane.

Further evidence that these mitochondria consist of a membrane, particles, and an interior soluble portion rich in protein is obtained by mitochondrial disintegration by release of gas.

cent versus a succinoxidase concentration of 80 per cent. Furthermore, in this experiment there is a close relationship between the total nitrogen and the radioactivity (stilbamidine) in the centrifuged sediment. Two correlations indicating association of stilbamidine with the mitochondria are thus established by this experiment: (a) The percentage of succinoxidase activity (which is found only in mitochondria) correlates very closely with the percentage of radioactivity (stilbamidine) in the different centrifuged fractions. (b) There is a close relationship between total nitrogen content of the sediments and the C"C activity. Since it almost certainly can be assumed that the nitrogen content of the sediment is chiefly from the mitochondria, this is good evidence that the stilbamidine is associated with the mitochondria.

The mitochondrial sediment was suspended and disintegrated. This was then centrifuged at 9,000 r.p.m. for 15 minutes producing sediment F-D-1 (Table 4). The supernatant was then centrifuged at 38,000 r.p.m. for 30 minutes producing sediment F-D-2. The supernatant of this sediment is labeled F-D-3 in Table 4.

Several interesting results were obtained in this experiment. Following sonic disintegration, as seen in Table 4, an average of 58 per cent of the C"C (stilbamidine) was sedimented at 38,000 r.p.m., and only 27 per cent was spun down at the slower speed of 9,000 r.p.m. About 15 per cent of the stilbamidine remained in the supernatant after the high speed centrifugation, suggesting either that this portion of the stilbamidine was detached from its binding to the mitochondria, or that this...
pressure. In Table 4 it is seen that essentially the same quantity of nitrogen was released into solution as with sonic disintegration. However, it is interesting that considerably more stilbamidine and nitrogen were centrifuged down at the slow speed (9,000 r.p.m.) than following sonic disintegration. This suggests that sonic disintegration is more efficient in breaking up particles, or the mitochondrial membrane, than is release of gas pressure.

Nature of the binding of stilbamidine in mitochondria.—In an effort to determine in what manner stilbamidine is fixed to the membrane and/or particles of the mitochondria, the effect of several enzymes was studied. If stilbamidine were chemically bound to proteins, the action of proteolytic enzymes would solubilize a large part of it. Similarly, the action of ribonuclease should be effective if the stilbamidine were selectively bound to the ribonucleic acid. These studies were made on both undisintegrated mitochondria (F-4-P) and on disintegrated (sonic) mitochondria (F-D-P). The results of these studies are given in Table 5. It is seen that, for the undisintegrated mitochondria, neither ribonuclease nor trypsin and chymotrypsin significantly changed the distribution of radioactivity from that of a similarly treated sample to which no enzymes were added. The sediment of the disintegrated mitochondria treated with proteolytic enzymes contains less stilbamidine than either the undisintegrated mitochondria, the ribonuclease action present so that, even in the undisintegrated mitochondria, ribonuclease had no effect on the stilbamidine content solution. However, it is interesting to note that even though only 15 per cent of the nitrogen was found in the sediment of the disintegrated mitochondria treated with ribonuclease or the sample to which no enzyme was added. This suggests that the sonic disintegration disrupted some large aggregates containing stilbamidine to the extent that the proteolytic enzymes were able to release some stilbamidine into solution. However, it is important to note that even though only 15 per cent of the nitrogen was found in the sediment of the disintegrated mitochondria treated with proteolytic enzymes, 40 per cent of the stilbamidine was present in this sediment. It appears unlikely, then, that there is a real chemical binding of the stilbamidine to protein. As with the undisintegrated mitochondria, the ribonuclease had no effect on the stilbamidine content of the sediment from the disintegrated mitochondria. In all experiments there was sufficient ribonuclease action present so that, even in the undisintegrated mitochondria, only a small percentage (maximum, 22 per cent) of the ribonuclease action remained, as determined by the conventional trichloroacetic acid method. These results almost certainly indicate that the stilbamidine is not bound selectively to the ribonucleic acid of the mitochondria.

Further evidence was obtained that would appear to exclude the possibility that the stilbamidine may be bound to the nucleic acid of liver. Ribonucleic acid and deoxyribonucleic acid were isolated from livers of sarcoma-bearing mice, to which stilbamidine had been previously administered, by a method in which cold saturated sodium chloride was used to extract the nucleic acid and the protein was subsequently denatured with ether (8). Following precipitation with ethanol, the nucleic acid was dialyzed and reprecipitated with ethanol. The nucleic acid isolated represented about 25 per cent of the initial ribonucleic acid present but contained less than 0.1 per cent of the initial radioactivity of the liver.

Comparison of site of localization of stilbamidine with that of colloidal chromic phosphate.—It is known that colloidal chromic phosphate is quickly removed from the blood by the Von Kupfer cells of the liver (6). It seemed of interest to compare the distribution of colloidal chromic phosphate with that of stilbamidine in the centrifuged liver fractions. Five days before autopsy two sarcoma-bearing mice were given intravenous injections of colloidal chromic phosphate (400–600 μc. P32). The following day the usual intraperitoneal injection of stilbamidine-C14 was made. The results of the fractionation are given in Table 6. It may be seen that the distribution of radioactivity from the two isotopes is quite different. The stilbamidine, as noted above, is largely in the mitochondrial fraction, while the colloidal chromic phosphate is about equally divided between the nuclear fraction and the mitochondrial fraction. Intact Von Kupfer cells probably would be sedimented entirely in the nuclear fraction. It is likely that

TABLE 5

<table>
<thead>
<tr>
<th>ENZYME TREATMENT</th>
<th>UNDISINTEGRATED MITOCHONDRIA (F-4-P)*</th>
<th>DISINTEGRATED MITOCHONDRIA (F-D-P)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>No added enzyme</td>
<td>71% 66% 62% 68%</td>
<td>71% 66% 62% 68%</td>
</tr>
<tr>
<td>Ribonuclease (2.7 mg.)</td>
<td>74% 68% 65% 56%</td>
<td>74% 68% 65% 56%</td>
</tr>
<tr>
<td>Trypsin (4.3 mg.) and chymotrypsin (1.7 mg.)</td>
<td>71% 41% 42% 15%</td>
<td>71% 41% 42% 15%</td>
</tr>
</tbody>
</table>

*Six A strain sarcoma-bearing mice were used in this experiment; therefore, in each incubation the mitochondria from the liver of approximately one mouse was used. Incubations were carried out at 37°C, for 2 hours with shaking in Warburg vessels in 0.15 M sucrose and 0.01 M phosphate buffer, pH 7.4. F-4-P represents the precipitate obtained upon subjecting an aliquot of F-4-P to sonic disintegration for 1 hour at 2°–4° and subsequently centrifuging at 38,000 r.p.m. for 30 minutes. In each experiment 34 per cent of the C14 activity, 6 for per cent of the nitrogen, and 86 per cent of the PNA were obtained in F-D-P. The percentages in the table above are those found in the precipitate upon centrifuging the incubated fractions at 38,000 r.p.m. for 30 minutes. The remainder of the activity and of the nitrogen was found in the supernatant.
many of the Von Kupfer cells were broken in the macerating process. These would release their content of colloidal chromic phosphate, which it might be surmised would then be sedimented with the mitochondrial fraction. Further consideration of this is beyond the scope of this paper.

This experiment appears to indicate that stilbamidine is not handled by the liver as a colloid, although the possibility exists that the stilbamidine is collected by Von Kupfer cells and passed across the liver cell boundary into its cytoplasm and then adsorbed to the membrane of the mitochondria.

**DISCUSSION**

The above experiments appear to show conclusively that stilbamidine present in the liver is nearly all localized in or on the mitochondria. This is just as true of the livers of mice with sarcomas as of normal mice. Therefore, since much larger amounts of intraperitoneally or intravenously injected stilbamidine are concentrated in the livers of sarcoma-bearing mice than in the livers of normal mice (27), it seems likely that there is a significant difference in the liver cell mitochondria when sarcoma is present elsewhere in the body.

A qualitative difference between the soluble proteins of the liver cell mitochondria of C3H mice and the mitochondria of Hepatoma 98/15 has been shown (10). Since the tumor used in our experiment is a sarcoma and since we have observed no metastases in sections studied, it would be interesting to determine whether the soluble proteins of liver mitochondria in the mice with sarcomas are also altered. Such studies are in progress. The possibility also of qualitative differences in the non-soluble portions of the mitochondria is suggested by our findings. Until the nature of the binding of stilbamidine is more clearly known, it cannot be decided whether the primary difference here is a physical or a chemical one.

Other evidence linking mitochondrial alterations in mice with malignancy (sarcoma and carcinoma) has recently been presented by Annau et al. (1). These workers have demonstrated marked histologic changes in the liver cell mitochondria of these tumor mice. These changes are characterized by a change in the size and shape of the liver cell mitochondria as compared to normal and are more marked with the more advanced tumors. It is of interest that, in our series, there is excellent correlation with tumor weight and liver concentration of stilbamidine—heavier tumors correlating with larger liver concentrations (unpublished results). We have also observed a marked increase in the concentration by the liver of stilbamidine in normal mice following heavy irradiation of the liver with colloidal chromic phosphate (P32) and following liver damage with carbon tetrachloride. The stilbamidine is mostly confined to the mitochondria in the irradiated livers, as in the livers of other mice. Mice treated with carbon tetrachloride were not tested in this way. These findings suggest that some important change occurs in the function of mitochondria in response to a variety of stimuli. Whether this response in the tumor animals is produced by some toxic product of the tumor tissue or whether it is of some entirely different nature is not known.

Although others have presented evidence for a chemical binding of stilbamidine with ribonucleic acid (26), our studies indicate that, in the liver cell at least, stilbamidine almost certainly is not attached to the ribonucleic acid. The evidence strongly suggests that there is no true chemical binding of stilbamidine with any substance of the mitochondria. It seems most likely that the stilbamidine is adsorbed to the membrane or particulate material of mitochondria. Evidence that mitochondria contain a membrane and inner particles has been presented by Dalton et al. (4). Studies with the electron microscope have also indicated a membrane and particulate bodies (28, 18). The membrane is composed of short protein fibrils and globular protein molecules, while the bodies consist of ribonuclear protein. Our studies and those of Hogeboom et al. (10) indicate that, in addition, the mitochondria contain a relatively large amount of protein (about 50 per cent) in a soluble form, and this is apparently not associated with the nucleic acid. The experiment described in Table 4 shows that the stilbamidine is not attached to the soluble protein, since very little was present in the supernatant which contained this protein.

In our liver fractionation experiments, the values for the distribution of nitrogen, ribonucleic acid, and deoxyribonucleic acid are in general
agreement with those reported by others (21, 23, 24); this indicates that our fractionation technic is comparable. However, our value for the percent agreement with those reported by others (21, 23, 24) for this indicates that our fractionation technic is comparable. However, our value for the percent agreement with those reported by others (21, 23, 24). desoxyribonucleic acid was observed in small amounts in other fractions than the nuclear sediment. The presence of some DNA outside of the nuclear fraction is probably due to disruption of some of the nuclei. The homogenate was not ejected through a hypodermic needle, since it has been considered that this would disrupt nuclei (21).

Other experiments carried out in this laboratory have shown that a high retention of stilbamidine in liver after injection is not unique to tumor-bearing mice. Studies to be the subject of another communication have shown that a high retention of stilbamidine in the liver and that this appears to be mostly concentrated in the mitochondria of the liver cells.

SUMMARY

The radioactivity present in the livers of normal and sarcoma-bearing mice after injection of stilbamidine-amidine-C14 disethionate has been shown to be present primarily in the mitochondria isolated by differential centrifugation in 0.25 m or 0.88 m sucrose. Subsequent disintegration of the mitochondria by sonic disintegration, or by release of gas pressure, released a large amount of the protein and dialyzable constituents into solution but only a small amount of the radioactivity. The particulate material remaining contained most of the stilbamidine and the ribonucleic acid of the mitochondria. However, experiments with ribonuclease and with trypsin and chymotrypsin on this fraction and on undisintegrated mitochondria indicate that the radioactivity was not associated with the ribonucleic acid or with the soluble, or perhaps even with the insoluble, protein of the mitochondria. Indeed, even though no clear evidence for the exact nature of the retention of the stilbamidine has been obtained, it appears most likely to be an association, possibly adsorption, with the membrane or particulate material of the mitochondria.

ACKNOWLEDGMENTS

The authors wish to acknowledge their appreciation to Professors Melvin Calvin and John H. Lawrence, to Dr. Bert Tolbert and Mrs. Patricia Adams for their interest and suggestions, and to Mr. Robert Self, Mr. Saburo Ikeda, and Mrs. Jean Siri for the preparation of cyanide, assistance in radioactivity counting, and the transplanting of tumors in the animals.

REFERENCES


25. SELL, R. E., and TOLBERT, B. M. Notes on the Preparation of Hydrogen Cyanide-C\textsuperscript{14} from BaC\textsuperscript{14}O\textsubscript{2}. UCRL Report #1299, 1951.


The Distribution of Stilbamidine in the Livers of Normal and Sarcoma-bearing Mice

Edward L. Bennett, Donald E. Pack, Barbara J. Krueckel, et al.

*Cancer Res* 1953;13:30-38.

Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/13/1/30

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.