The Distribution of Tetraphenylporphinesulfonate in the Tumor-bearing Rat

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SUMMARY

The distribution of tetraphenylporphinesulfonate, following its parenteral administration to rats bearing the Walker carcinosarcoma, has been studied. It was found that the concentration in the tumor was greater than that in any of the other tissues studied, but several of these tissues also had appreciable concentrations. The concentration was found to be dependent upon dose, and the distribution was affected by time following administration.

Tetraphenylporphinesulfonate was found to be transported in the blood bound to the serum proteins. The concentration of this material found in the tumor was far greater than that obtained with hematoporphyrin in other studies.

Within 6 hours after certain porphyrins are injected into animals bearing tumors, the tumors exhibit the characteristic red fluorescence of porphyrins, whereas other tissues exhibit only their natural bluish fluorescence (1, 3, 5, 7, 9, 10, 12, 14). This has been interpreted as evidence for the selective uptake of porphyrins by tumor tissue. This phenomenon has been difficult to quantify, because most naturally occurring and commercially available porphyrins are nonhomogeneous mixtures whose composition varies from source to source (1, 12, 13) and which have solubilities and optical properties similar to those of endogenous porphyrins.

A porphine derivative, α, β, γ, δ-tetraphenylporphine (TPP), which does not occur naturally, was prepared. This material has spectral characteristics and solubilities which permit it to be distinguished from and measured in the presence of endogenous porphyrin and, although also not a single substance, has fewer components than other porphyrins whose uptake in tumor has been observed.

The uptake of TPPS by various tissues could be quantified by two specific optical methods developed for this purpose. TPPS was found to be more highly concentrated in Walker carcinosarcoma than in any other tissue of the rat, and this concentration was much greater than that of hematoporphyrin (14) after its parenteral administration.

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MATERIALS AND METHODS

Porphine.—α, β, γ, δ-tetraphenylporphine (TPP) was prepared from pyrrole and benzaldehyde by the procedure of Rothemund and Menotti (11). Tetraphenylporphinesulfonate (TPPS) was prepared from TPP by reaction with concentrated sulfuric acid by the method of Menotti (8).1 Sulfonation of TPP occurs on the phenyl rings, although it is not known at which position. The presence of sulfonic acid groups on the free β-positions of the pyrrole nucleus is ruled out by the spectroscopic observation that TPPS has an “etio” type spectrum, and not the “rhodo” type that sulfonic acid, possessing chromophoric properties, would produce if introduced into the porphine ring structure (4). The TPPS free base was obtained as flakes with a blue-green metallic color. It was readily soluble in water and methanol and only sparingly soluble in less polar organic solvents such as pentane and chloroform. The spectral characteristics were found to be markedly affected by changes in pH. The absorption spectrum at pH 9.0 and pH 2.0 is shown in Chart 1. The changes shown were observed as a gross change from red to green upon the addition of acid. The well known pH dependence of the fluorescence of porphyrins (4) also applied to TPPS. As shown in Chart 2, the excitation maximum shifted from 418 to 438 mμ, and the emission maximum from 650 to 678 mμ, when the pH of the medium changed from 9 to 2 (Chart 3).

1 I am indebted to Dr. Jack Goldstein for this preparation.
This preparation of TPPS was separated into four components by column chromatography. A 10-cm. glass column, 4 cm. in diameter, was packed with acid-washed celite (Johns Mansville Celite 545, acid-washed) which had been shaken with the aqueous phase of pyridine: chloroform: water (2:1:1) in a proportion of 6 gm: 5 ml. The pyridine-chloroform phase was used as the moving phase.

This chromatographic system was devised by Dr. Winslow Caughey, Department of Physiological Chemistry, Johns Hopkins University School of Medicine.

The first of the four fractions, a very small component, was determined to be tetraphenylporphine by its characteristic infrared spectrum. The other three fractions had identical ultraviolet and visible absorption spectra, fluorescent excitation and emission spectra, and no major differences were discernible in their infrared spectra which differed from that of TPP. Elemental analysis indicated that these fractions differed in their sulfur content; the second fraction had one sulfur atom per molecule of porphyrin, and the fourth fraction, which was the most abundant, had three sulfur atoms per molecule of porphyrin.

Animals and tumor.—Female Sprague-Dawley rats weighing approximately 250 gm. were used in all experiments. Pellets of 1 cu. mm. of Walker carcinosarcoma 256 were implanted subcutaneously in the flank of the rat through a trocar. Eleven days after tumor implantation TPPS in physiological saline at pH of 7.4 was administered intraperitoneally in one set of experiments and intravenously in the others. Animals were sacrificed at different times following injection, and the tumors and other tissues studied were rapidly excised. There was no grossly visible necrosis or hemorrhage in any of the tumors studied. The few tumors which had grown 16 days or more and which showed evidence of central necrosis were eliminated from the study.

Methods of analysis.—One gram of tissue was finely minced and homogenized in 9 ml. of 0.25 M sucrose with a Potter-Elvehjem homogenizer. The homogenate was strained through cheese cloth to eliminate fibrous strands.

Fluorescence Method: A 1-ml aliquot of homog-
enate was further diluted 1:10 with 0.25 M sucrose. The concentration of porphyrin in this suspension was determined by measuring its fluorescence by a method specifically developed for the measurement of fluorescence in tissue suspensions. The exciting light entered the 1 X 1-cm. cuvette obliquely. The emitted light was viewed directly so that reflected light did not enter the photometer. The light source was an XBO162 Xenon arc lamp. The wavelength of the incident light was varied from 350 to 600 m\(\mu\) by means of a grating monochromator. A photomultiplier (type 1P 28) photometer (American Instrument Company) recorded the intensity of light emitted through a Corning 2404-color specification 2-29 red filter. The intensity of emitted red light was plotted against the wavelength of exciting light (Chart 4). The difference in fluorescence intensity between that at the wavelength of maximal excitation and at a wavelength which excited negligible red fluorescence, between 350 and 400 m\(\mu\) on this figure, was found to be directly proportional to the amount of TPPS present (Chart 5).

The excitation spectra of TPPS read by this fluorometer was similar to its absorption spectra and showed the same changes with pH. Identical spectra were produced by TPPS alone, TPPS added to tissue suspensions with negligible amounts of endogenous porphyrin and no other red fluorescent material, and TPPS as it occurred in tissue after parenteral administration. Proportional increments in peak height occurred when known quantities of TPPS were added to homogenates of tissue from injected animals. Since successive additions of equal quantities of TPPS produced a linear increase in the peak height at 418 m\(\mu\), the quantity of TPPS originally present in the homogenate could be calculated. The height of the 418-m\(\mu\) peak given by the addition of a known concentration of TPPS is determined by the concentration and tissue source of the homogenate to which it is added. The amount of TPPS present in a given suspension was therefore determined by first noting the height of the fluorescence intensity peak, and then increasing the TPPS concentration by at least two known amounts to determine the light output for a given concentration and to ensure that this output was linearly related to concentration. Approximately 2 \(\times\) 10\(^{-4}\) \(\mu\)g. TPPS in a 0.01 gm/cc suspension, equivalent to 0.02 \(\mu\)g TPPS/gm, could be detected by this method, and the highest values found in any of the tissues in this study fell within the range of linearity of the assay.

Absorption Method: One ml. of the 0.25 M sucrose tissue homogenate containing 0.1 gm/ml was diluted to 2 ml. with water, and 2 ml. of 10 per cent trichloroacetic acid was added. TPPS present, both in homogenates of tissues from animals that received parenteral TPPS and in homogenates to which known TPPS was added, was
completely precipitated with the protein, which had the red fluorescence and green color of TPPS in acid. The supernatant, which had no red fluorescence and no absorption at 418 m\(\mu\), was discarded.

The precipitate was stirred with 5 ml. of MeOH:NH\(_4\)OH (10:1) and filtered through Whatman \#1 filter paper. The TPPS was solubilized by this procedure. TPPS added to homogenates and TPPS present in tissues after parenteral administration behaved identically in this extraction. The absorption spectrum of the clear filtrate was obtained on the Bausch and Lomb 505 Recording Spectrophotometer. The absorption by TPPS at 418 m\(\mu\) was proportional to concentration. Recovery of known amounts of TPPS which had been added to homogenates and precipitated with trichloroacetic acid was shown by this measurement to be complete and reproducible (Chart 6).

Some tissues, notably liver, kidney, and spleen, had endogenous porphyrin which interfered with this assay. In the MeOH:NH\(_4\)OH extract, the absorption peak of endogenous porphyrin appears as a shoulder on the peak of TPPS. However, after conversion to acid pH by the addition of HCl, there is virtually complete separation of the absorption peaks, and TPPS can be determined on the basis of its peak in acid (Chart 7). This was routinely done in the assay of TPPS in those tissues in which the presence of moderate amounts of endogenous porphyrin was indicated by the presence of the shoulder on the TPPS peak.

Determinations of TPPS content of tissues by the fluorescence and absorption methods were in good agreement. The fluorescence analysis is simpler, can be performed quickly, and makes possible the observation of the fluorescence of TPPS as it is found in the tissue after a minimum of chemical manipulation. The absorption method is more specific for TPPS and permits its measurement in the presence of endogenous porphyrins. The two methods are complementary.

RESULTS

At autopsy, the tissues of all the animals were examined under ultraviolet light. The Harderian gland, which is known to possess a high concentration of protoporphyrin (2), was the only tissue of animals which had not been injected that showed red fluorescence. In all animals that had received TPPS, the tumor always had the most brilliant red fluorescence of all tissues. There was bright red fluorescence distributed irregularly in lung and faint red fluorescence in lymph nodes and pancreas. No fluorescence was apparent in liver, kidney, or spleen.

The distribution of TPPS in the tissues of six animals sacrificed 6, 24, or 120 hours after the intravenous injection of 10 mg. of TPPS is summarized in Table 1. These values may be compared with the results of analyses of tissues from a tumor-bearing rat that received no TPPS. The
concentrations of TPPS in \( \mu g/gm \) tissue as determined by the fluorescence method and as determined by the absorption method are presented side by side in this table. The agreement between the values is reasonable. In several instances, however, the fluorescence method gave values higher than the absorption method by an amount approximately equal to the endogenous porphyrin of that organ.

The TPPS content of tumors, as shown in Table 1, was always greater than that of any other tissue. However, the red fluorescence was detectable by the fluorescence method, and the values obtained were close to those obtained by the absorption method. The explanation of this discrepancy is obscure, but it tends to cast doubt on the validity of previous interpretations of relative tissue affinity for porphyrin that were made on the basis of visual inspection unaccompanied by quantitative analytic data.

The effect of time following the intraperitoneal injection of 10 mg. of TPPS on the concentration was studied over a large time span. The results are shown in Table 2. From this table it appears that a maximal concentration of TPPS is reached in the tumor after 36 hours. This remains high until the 3d day and then begins to diminish.

The variability in TPPS concentration in the tumors among animals sacrificed at the same time after injection can largely be attributed to differences in absorption from the peritoneal cavity. This variability as well as the lower tissue concentrations attained after intraperitoneal injection led to the use of the intravenous route in subsequent experiments.

The effect of dose on the TPPS uptake by tumor and several other tissues is shown in Table 3. After 11 days of tumor growth, pairs of rats

<table>
<thead>
<tr>
<th>Table 1</th>
<th>CONTENT OF TPPS IN TISSUES OF TUMOR-BEARING RATS AT DIFFERENT TIMES FOLLOWING INTRAVENOUS INJECTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microgram TPPS/gm tissue, wet weight</td>
<td></td>
</tr>
<tr>
<td>Tissue</td>
<td>F</td>
</tr>
<tr>
<td>Tumor</td>
<td>26.5</td>
</tr>
<tr>
<td>Kidney</td>
<td>4.3</td>
</tr>
<tr>
<td>Spleen</td>
<td>4.9</td>
</tr>
<tr>
<td>Liver</td>
<td>16.1</td>
</tr>
<tr>
<td>Colon</td>
<td>3.0</td>
</tr>
<tr>
<td>Duodenum</td>
<td>3.6</td>
</tr>
<tr>
<td>Stomach</td>
<td>3.7</td>
</tr>
<tr>
<td>Forestomach†</td>
<td>3.9</td>
</tr>
<tr>
<td>Heart</td>
<td>5.7</td>
</tr>
<tr>
<td>Lung</td>
<td>5.7</td>
</tr>
<tr>
<td>Muscle</td>
<td>2.7</td>
</tr>
<tr>
<td>Skin</td>
<td>2.6</td>
</tr>
<tr>
<td>Pancreas</td>
<td>8.0</td>
</tr>
<tr>
<td>Adrenal</td>
<td>5.7</td>
</tr>
<tr>
<td>Thymus</td>
<td>3.7</td>
</tr>
<tr>
<td>Lymph node</td>
<td>4.0</td>
</tr>
<tr>
<td>Submaxillary</td>
<td>4.9</td>
</tr>
<tr>
<td>Sublingual</td>
<td>3.1</td>
</tr>
</tbody>
</table>

* Less than 0.10 \( \mu g/gm \).
† These tissues were washed with water to remove digestive tract contents prior to extraction.
F = fluorescence; A = absorption.
The concentration of TPPS found in the tumor is many times that of hematoporphyrin which was found in the earlier study (14). After the intraperitoneal injection of 20 mg. of hematoporphyrin ca. 2.5 μg. hematoporphyrin were found per gm. of tumor tissue, and with the highest tolerable doses of hematoporphyrin only 6–10 μg/gm were found. More than 10 times that amount of TPPS localized in tumor after the injection of only 10 mg., and far greater concentrations occurred under optimal conditions.

### TABLE 3

**EFFECT OF DOSE ON TISSUE CONCENTRATION OF TPPS AFTER INTRAVENOUS INJECTION**

<table>
<thead>
<tr>
<th>ANIMAL</th>
<th>Mo. TPPS INJECTED</th>
<th>Microgram TPPS/gm TISSUE, WET WEIGHT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Tumor</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F</td>
</tr>
<tr>
<td>1</td>
<td>1.0</td>
<td>10.0</td>
</tr>
<tr>
<td>2</td>
<td>3.3</td>
<td>47.6</td>
</tr>
<tr>
<td>4</td>
<td>3.3</td>
<td>22.0</td>
</tr>
<tr>
<td>5</td>
<td>10.0</td>
<td>147.0</td>
</tr>
<tr>
<td>6</td>
<td>33.0</td>
<td>400.0</td>
</tr>
<tr>
<td>8</td>
<td>75.0</td>
<td>955</td>
</tr>
</tbody>
</table>

* Animal 9 died 6 hours after injection.
† Animal 10 died 10 hours after injection.
F = fluorescence; A = absorption.

received doses of 1, 3.3, 10, 33, and 75 mg. of TPPS by intravenous injection. They were sacrificed 24 hours later.

The three sulfonated fractions of the TPPS preparation obtained by column chromatography, as noted under “Methods,” were injected intravenously into tumor-bearing rats to determine whether these fractions distributed differently. The results of these experiments are shown in Table 4. There was a remarkably similar pattern of localization in tumor and other organs with all three preparations, and the actual amounts found in the tissues were about the same as those obtained. The degree of sulfonation of the least sulfonated TPPS fraction is adequate to confer as great tumor uptake as that of the most sulfonated TPPS fraction.

The blood of several animals that received TPPS injections was subjected to routine serum

of profound muscular weakness and tachypnea immediately following injection but died 6 and 10 hours later. Animals which received higher doses died immediately after injection.

The concentration of TPPS found in the tumor is many times that of hematoporphyrin which was found in the earlier study (14). After the intraperitoneal injection of 20 mg. of hematoporphyrin ca. 2.5 μg. hematoporphyrin were found per gm. of tumor tissue, and with the highest tolerable doses of hematoporphyrin only 6–10 μg/gm were found. More than 10 times that amount of TPPS localized in tumor after the injection of only 10 mg., and far greater concentrations occurred under optimal conditions.
protein electrophoresis (6) to study the transport of TPPS. The position of TPPS relative to the serum protein was determined by comparison of an unstained strip viewed under ultraviolet light with a strip stained with bromphenol blue. TPPS was found to migrate with the serum proteins; the red fluorescence was most intense at the albumin peak but was also present at the globulin peaks. This pattern of migration occurred in samples taken from 6 hours to 5 days after intravenous injection of 10 mg. of TPPS. The same pattern occurred when TPPS was added to control serum. When the minimum amount of TPPS that could be detected by its fluorescence, about 0.05 µg/ml, was added to serum, it appeared only with albumin. In this respect TPPS behaved like bilirubin and not like uroporphyrin I and III, coproporphyrin I and III, and protoporphyrin and hematoporphyrin, which were found to have electrophoretic movement completely independent of that of the serum protein bands (15).

There was no evidence that the TPPS found in the tumor and other tissues was different from the TPPS injected. The fluorescence emission record of homogenates was identical to that of TPPS in basic media. The general equivalence of the assays made by the absorption procedure, which detects both metallo- and free porphyrin, and the fluorescence method, which detects only free porphyrin, showed that there was little or no in vivo conversion to a metalloporphyrin. The absorption spectrum of TPPS extracted from tissue was indistinguishable from that of the injected TPPS. It could be readily distinguished from that of endogenous porphyrin, which was easily removed from extracts that contained TPPS by solvent extraction as follows: A MeOH:NH₄OH extract was evaporated to dryness and dissolved in buffered acetic acid (4 parts glacial acetic acid to 1 part saturated sodium acetate in water) and ethyl ether (3:10). When 3 per cent sodium acetate in water was added the solution split into two phases, and the absorption spectrum of TPPS was found in the aqueous phase.

**DISCUSSION**

There is great potential interest in materials capable of sufficiently selective concentration in tumors to permit external scintiscanning of a radioactive derivative for tumor diagnosis, or delivery of therapeutic radiation to the tumor. It has been suggested that several porphyrins were selectively localized in tumors, but quantitative evaluation of this special affinity has been lacking. TPPS has optical properties and solubility characteristics which make it more easily measurable than the naturally occurring porphyrins and their derivatives which have previously been used, and, as the data presented here show, is taken up by tumor tissue with considerably greater avidity than hematoporphyrin. These properties made possible the development of methods for a quantitative study of this phenomenon which has demonstrated that the uptake of TPPS is considerably higher in the tumor than any other tissue, but

**TABLE 4**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Method</th>
<th>Chromatographic Fraction (µg of TPPS per mg tissue, wet weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2 (moved fastest)</td>
</tr>
<tr>
<td>Tumor</td>
<td>Fluorescence</td>
<td>131.6</td>
</tr>
<tr>
<td></td>
<td>Absorption</td>
<td>130.0</td>
</tr>
<tr>
<td>Liver</td>
<td>Fluorescence</td>
<td>23.4</td>
</tr>
<tr>
<td></td>
<td>Absorption</td>
<td>24.5</td>
</tr>
<tr>
<td>Kidney</td>
<td>Fluorescence</td>
<td>28.8</td>
</tr>
<tr>
<td></td>
<td>Absorption</td>
<td>19.2</td>
</tr>
<tr>
<td>Spleen</td>
<td>Fluorescence</td>
<td>13.5</td>
</tr>
<tr>
<td></td>
<td>Absorption</td>
<td>10.8</td>
</tr>
<tr>
<td>Lung</td>
<td>Fluorescence</td>
<td>14.7</td>
</tr>
<tr>
<td></td>
<td>Absorption</td>
<td>11.2</td>
</tr>
<tr>
<td>Heart</td>
<td>Fluorescence</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>Absorption</td>
<td>3.2</td>
</tr>
</tbody>
</table>

The injections were of 10 mg. of each fraction. The animals were sacrificed 24 hours later, 12 days after implantation of the tumor.

that uptake is not limited exclusively to the tumor tissue. The possible diagnostic or therapeutic use of this material must take this distribution into account.

**REFERENCES**


The Distribution of Tetrakis[4-(2-hydroxy-5-sulfobenzoate)]porphyrazine in the Tumor-bearing Rat

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