Physiologic Disposition and Intracellular Localization of Isometamidium

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Summary

Isometamidium leaves the blood rapidly in rats after i.v. injection and within a few minutes most of the compound is found in liver and kidney. It remains for long periods in subcutaneous sites of injection from which it is transferred selectively to liver, kidney, and spleen. After s.c. injection none is detected in urine or bile and only small fractions of the compound appear daily in feces. Little is absorbed after intragastric administration and most of it is recovered in feces as unchanged drug and homidium. In dogs and monkeys large fractions of i.v. doses accumulate in liver and kidney cortex; significant amounts of unchanged drug are found in these sites for as long as 8 weeks after treatment. Hepatic and renal cumulation accounts for the quick recovery from the acute actions of the drug which are prominent after i.v. injection, for the greatly reduced toxicity of the agent when given i.p. or s.c., and for the pathologic changes seen in liver and kidney cortex. The agent also causes local tissue injury at injection sites and degranulation of mast cells.

Intracellular distribution in vivo can be studied by fluorescence microscopy. In rats bright fluorescence is seen in hepatocytes, tubules of the renal cortex, ducts of salivary glands, islets of the pancreas, and in granular clusters (macrophages?) in spleen, lymph nodes, and thymus. In hepatic cells and renal cortical tubules fluorescence is exclusively cytoplasmic and in hepatic cells mainly granular. The agent sediments with mitochondria of liver fractions from treated rats. Lysosomes may also contain high concentrations. Essentially none is found in the cell sap and only small amounts are associated with nuclei and microsomal particulates. Isometamidium is bound by serum albumin, deoxyribonucleic acids, hepacin, and hyaluronic acid. Insoluble complexes form with the nucleic acids and mucopolysaccharides when there is charge neutralization of the cationic drug and the polyanionic molecules. Binding does not explain localization of the agent in rat liver mitochondria in vivo although it accounts for staining of nuclei in vitro and for fluorochroming of mast cell granules.

Introduction

Phenanthridinium compounds were first shown to have chemotherapeutic effects by Walls (37), and Browning et al. (4) who described the curative activity of 5-methyl-6-(p-aminophenyl)-8-amino-phenanthridinium in mice infected experimentally with Trypanosoma brucei and congolense. Field trials in Africa proved the agent to be useful in the treatment of T. congolense infections in zebu cattle (7, 19). The early observations prompted an extensive search for more effective derivatives and eventually the development of dimidium (6, 28) and ethidium (12, 42, 43) (Chart 1). Recent efforts have led to the introduction of compounds which are not only curative but have prolonged actions and are thus useful as prophylactic agents against cattle trypanosomiasis. One of these agents is metamidium (44), a mixture of 2 isomers of which IMDM (2, 3) is the more potent curative and prophylactic substance (Chart 1).

Phenanthridinium derivatives may have useful antitumor activity. Ethidium and related compounds are known to inhibit the incorporation of preformed purines into the nucleic acids of Ehrlich ascites tumor cells without apparent disturbance of de novo pathways of purine biosynthesis. Thus ethidium potentiates the inhibition of the ascites tumor caused by azaserine, a potent antagonist of de novo biosynthesis; the 2 agents are presumed to act together to effect a concurrent blockade of purine nucleotide supply for nucleic acid synthesis (17, 21, 22). Ethidium, metamidium, and IMDM have been shown to prolong the survival of mice bearing several different lines of transplanted leukemia (5) (J. H. Burechenal, unpublished observations). Other effects of phenanthridinium compounds that may be related to their antitumor activity include the capacity to inhibit bacterial nucleic acid polymerases (11, 29), to inhibit bacteriophage growth, to suppress Rous sarcoma virus in chicks (10), and to block selectively the synthesis of DNA in trypanosomiasis flagellates (24). This last action precedes inhibition of the synthesis of RNA and of protein. Finally the polyamine structure of the phenanthridiniums and the presence of centers of strong basicity such as the quaternary ring N-atom of the drug or properties found in other classes of antitumor substances (1): e.g., p-dimethylaminostyrylquinolinium derivatives (20), amidines (34), diquinadines (13, 23), and phthalalindes (1). Such considerations have prompted a trial of IMDM against human leukemia (4).

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1 Abbreviations: IMDM, isometamidium; Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin.
2 In progress at present in children with acute leukemia under the direction of Drs. J. H. Burechenal and M. L. Murphy.
Philips et al. have been able to study its tissue and intracellular localization. The acute actions of phenanthridinium derivatives have been reported by obtained evidence that cytoplasmic particulates, mainly mitochondria, are the principal sites of accumulation in hepatic and renal cells. In addition we found that IMDM is strongly bound to physiologic hydron concentrations by polyanionic macromolecules such as nucleic acids, mucopolysaccharides, and serum albumin.

Materials and Methods

The animals used were male Sprague-Dawley rats, 200-400 gm (CD line, Charles River Breeding Laboratories, Brookline, Massachusetts); dogs were adult mongrels of both sexes; and monkeys were male cynomolgus and rhesus, 3.6-4.8 kg. They were maintained as previously described with the exception that the dogs were fed a 1:1 mixture of water and Purina dog chow. Earlier publications list the various hematologic, biochemical, histologic, and toxicologic procedures used in the present work (28, 30).

The term, isometamidium, as used herein in units of dose or concentration, refers to the hydrated salt, 3-amino-5-ethyl-6-phenyl-8-m-amidinophenyl-diazocaminophenantridinium dimethanesulfonate trihydrate (mol. wt. 705, 65.3% of active cation). The agent was dissolved in 0.85% NaCl immediately before injection. Doses were given in rats in the constant volume of 0.01 ml/gm. Injections (i.v.) in rats were into femoral veins on April 12, 2017. © 1967 American Association for Cancer Research. cancerres.aacrjournals.org Downloaded from

Urine and feces were collected from rats which were housed individually in metabolism cages where drinking water and food were freely available. Flasks receiving urine were packed in ice. Each daily urine collection was diluted to 40 ml with water. Daily fecal collections were homogenized to a concentration of 2.5% in water (w/v). The various homogenates and samples of body fluids were either stored in the frozen state or chilled in ice when being used for analysis.

MAST CELL DEGRANULATION. Rats were injected i.p. with isotonic saline or with various doses of IMDM or Compound 48/80 (Wellcome Research Laboratories, Tuckahoe, New York) and were killed 20 min later. Intestinal mesenteries were spread over paper windows, fixed and stained in toluidine blue-formol solution, dehydrated, and mounted for microscopic study (25). Only those animals were scored positive which had extensive degranulation in >90% of mast cells throughout the mesenteric sample. The incidence of extensive effect was 0/8 in controls given isotonic saline, although in 3 of these controls about 50% of the mast cells had a few granules displaced outside of cell boundaries.

IMDM ANALYSIS: METHOD 1. Four ml of 10% tissue homogenates or of 2.5% fecal homogenates, or 2 ml of plasma plus 2 ml of water, or 4 ml of dilute urine (10% of each daily collection) were introduced into small, glass-stopped bottles each containing 2 gm of NaCl. Ether (25 ml) and 1 ml of 30% NaOH were added in turn and, without delay, the bottles were shaken mechanically for 10 min. The contents were centrifuged briefly to separate the phases. Twenty-ml portions of the ether extracts were introduced into glass-stopped bottles containing 4 ml of 0.1 M acetate buffer, pH 4.7, and these were shaken for 10 min. The absorbance of the buffer layer was measured in the Beckman DU spectrophotometer at 378 m/,[1] the principal absorption maximum of IMDM; readings were also taken at 278 m/,[1] for reasons to be given later. Absorbance was measured promptly since there was a slow loss at room temperature (<3%/hr).

Under the stated conditions, starting with 4-ml portions of known aqueous solutions containing 10-104 μg of IMDM (10 determinations), 80.0 ±1.6% (±1 S.D.) was accounted for in the final acetate buffer extract. Using this transfer constant and the absorptivity in acetate buffer (O.D. = 1 for 21.2 μg/ml) the amounts in μg in the samples analyzed were calculated as follows: O.D. × 21.2 × 6.25.

Known amounts of the drug added before extraction to homogenates of rat liver, spleen, kidney, heart, and small intestine and to rat plasma, were recovered satisfactorily (100-103%). Recovery from brain and fecal homogenates and from urine was 80-86%. The sensitivity of the method, considered equivalent to an O.D. of 0.01 in the acetate buffer, was estimated to be 0.7 μg/ml of plasma, 3.3 μg/gm of tissue, 13 μg/gm of feces, and 13 μg/24-hr urine collection. Blanks obtained in extracts of samples from untreated animals were negligible (O.D. = <0.01 in acetate
buffer extract) except in rat small intestine and brain (see Results).

ANALYSIS: Method 2. In the above procedure unbreakable gels formed when blood hemolysates or more concentrated homogenates of liver and kidney were shaken with ether. The following procedure circumvented this. One ml of blood plus 4 ml of water or 5 ml of homogenates of liver (10%) or of kidney (20%) were placed in small bottles. Five ml of isopropanol, 20 ml of ether, and 1 ml of 30% NaOH were then added. The remainder of the procedure was as above. The transfer constant was found to be 84.5 ± 3.4% (11 determinations with 5-ml portions of aqueous solutions containing 10-102 µg of IMDM). Amounts in µg in the samples analyzed were calculated as: O.D. × 21.2 × 5.92.

The sensitivity of this procedure, calculated as for the first method, was 1.3 µg/ml of blood, 1.3 µg/gm of kidney, and 2.5 µg/gm of liver. Ten µg of the drug were added before ether extraction to homogenates containing 0.5 gm of liver or 1.0 gm of kidney or to hemolysates of 1.0 ml of blood. The recovery in % was, respectively, 89 ± 5 (4 rats), 87 ± 9 (4 rats), and 60 ± 6 (6 rats). Blanks were determined in blood, liver, and kidney of 6 control rats; in each instance the O.D. of the acetate buffer extract was <0.01.

PAPER CHROMATOGRAPHY OF FECAL EXTRACTS. Alkali and NaCl were added, as in analytic Method 1, to aqueous solutions of salts of IMDM or homidium and to fecal extracts; these were then extracted with ether. The extracts were flash-evaporated and the residues were dissolved in acetone. Spots calculated to contain 1–2 µg of the bases were applied to Whatman No. 3 paper. Ascending chromatograms were developed in dimethylformamide:water, 1:1 (System 1), triethylamine:water:dimethylformamide, 1:5:14 (System 2), and acetic acid:water:dimethylformamide, 1:5:14 (System 3). The dried chromatograms were studied with ultraviolet light (365 nm) for orange-pink fluorescent spots. Small pink zones, seen at the origin in chromatograms of fecal extracts, were not reported since they were present in all 3 systems with extracts from samples collected from untreated rats.

FLUORESCENCE MICROSCOPY. Rats were killed as described above and organs were removed promptly and immediately frozen in liquid nitrogen. Sections were cut at 8 µ at temperatures varying from −20°C to −10°C. They were mounted directly from the knife by contact with glass slides, which were at room temperature, and covered with glass slips. The sections were examined microscopically without fixation and without the addition of mounting medium.

The Leitz Labolux IIIA microscope was used with a high pressure Hg lamp (Osram HBO 200 W) as the light source. The incident light was filtered through absorbing glasses BG-12 and BG-38, together transmitting maximally at about 400 mµ, which is near the principal absorption peak of IMDM at 378 mµ, but 1% or less below 332 mµ or above 494 mµ. A blue-absorbing, barrier filter was placed in the microscope barrel. It passed <1% of light below 530 mµ and had a maximum transmission above 550 mµ. (As will be seen later the fluorescence emission from IMDM has a maximum at about 590 mµ.) Fluorescence observations were made with an oil immersion condenser (D 120 A).

The descriptions of fluorescence in tissue sections to be given below do not include the autofluorescence seen also in specimens from untreated controls: e.g., the dull green diffuse glow of many types of cells, the bright green of reticulum and elastica, and the scattered yellow-white dotlike granules found in most sections in small numbers.

Fluorescence was also studied microscopically in subcellular fractions from rat liver homogenates. For this purpose drops of various fractions were mounted under glass slips. The swing-out, deflecting mirror in the lamp housing of the microscope permitted instant change to a tungsten lamp source for dark-field illumination with visible light; this facilitated the identification of sources of fluorescence in the subcellular fractions.

RAT LIVER FRACTIONATION. The methods of DeDuve et al. (9) were followed in detail in preparing subcellular fractions of rat liver. Each of the fractions was studied in the fluorescence microscope and analyzed for IMDM by Method 2 and for total nitrogen, cytochrome oxidase, and total acid phosphatase (9).

BINDING STUDIES. Since IMDM stained and did not pass through dialyzing tubing, the determination of serum-binding by ultrafiltration was not possible. An alternative procedure was used which determined the effect of serum on the partitioning of IMDM between aqueous and nonaqueous phases. The partitioning was carried out in 10-ml, plastic-capped, glass vials which had been siliconized to prevent adsorption of the agent. Each vial received 1 ml of rat serum and 1 ml of IMDM in 0.1 M Tris, pH 7.5 (Sigma 121); 2 ml of n-hexanol were carefully layered over the aqueous phase. The hexyl alcohol had been previously redistilled and saturated with Tris buffer. The vials were placed upright and rotated slowly at 24°C in a plane tilted slightly from the horizontal so that the phases were gently swirled without intermixing. At intervals vials were removed and the absorbance of the alcohol layer was measured at 380 mµ, the extinction maximum for IMDM in solvent saturated with Tris buffer. Equilibrium was attained between 4 and 5 hr. Control partitions were also done with varying concentrations of IMDM in 0.05 M Tris. In these the O.D. of both layers was determined at equilibrium and the data were used to construct a curve relating absorbance in the solvent phase to concentration of diffusable IMDM in the aqueous phase. In serum experiments, this curve and the O.D. of the solvent layer were used to calculate the concentration of freely diffusible IMDM in both phases and, by difference, the concentration of bound agent in the aqueous phase.

Partitioning was also done with solutions of BSA (crystalline, General Biochemicals) in 0.05 M Tris and with rat serum which had been dialyzed overnight at 4°C against 500 volumes of 0.05 M Tris. At the end of the dialysis the serum was clarified by centrifugation. In these experiments IMDM was in 0.05 M Tris when added to dialyzed serum or solutions of BSA.

Binding was also studied spectrophotometrically. Absorption spectra were recorded between 325 and 600 mµ of solutions containing IMDM and dialyzed rat serum in Tris; the comparison cuvets were balanced optically with equal concentrations of buffered serum (Cary model 11 spectrophotometer). The spectra were compared with those of Tris solutions of IMDM.

Spectral changes produced by various macromolecules were determined with BSA, DNA (sodium salt, Type I, Sigma), RNA (Type IV, from liver, Sigma), heparin sodium (100 U.S.P. 4 The authors appreciate the kind help of Dr. L. F. Cavalieri who suggested this procedure.
TABLE 1

<table>
<thead>
<tr>
<th>Route</th>
<th>Dose (mg/kg)</th>
<th>Mortality*</th>
<th>Time of death</th>
</tr>
</thead>
<tbody>
<tr>
<td>i.v.</td>
<td>5</td>
<td>3/3</td>
<td>&lt;2 min</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>6/6</td>
<td>&lt;2 min</td>
</tr>
<tr>
<td></td>
<td>1.3</td>
<td>0/6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>0/6</td>
<td></td>
</tr>
<tr>
<td>i.p.</td>
<td>200</td>
<td>7/9</td>
<td>2-3 hr</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>9/15</td>
<td>1-4 days</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>11/15</td>
<td>1-4 days</td>
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<tr>
<td></td>
<td>25</td>
<td>1/9</td>
<td>1 day</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>0/6</td>
<td></td>
</tr>
<tr>
<td>s.c.</td>
<td>500</td>
<td>0/6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>0/6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>0/6</td>
<td></td>
</tr>
</tbody>
</table>

* Observed for 14 days after injection.

TABLE 2

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Incidence of Animals with Extensive Degranulation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Isometamidium</td>
</tr>
<tr>
<td>10</td>
<td>2/4</td>
</tr>
<tr>
<td>1</td>
<td>5/8</td>
</tr>
<tr>
<td>0.1</td>
<td>1/8</td>
</tr>
<tr>
<td>0.01</td>
<td>0/4</td>
</tr>
</tbody>
</table>

Subcutaneous injections as large as 500 mg/kg had no acute effects in rats even though these were at least 200 times greater than the minimum i.v. doses which could kill within 2 min. However, 24 hr after s.c. injection (Table 1) all animals had large, soft swellings at the site of injection. The lesions persisted throughout the period of observation and in half of the animals they ulcerated during the 2nd week. All of these rats had steady losses in body weight ranging from 11 to 36% by 14 days.

Four of the animals which had received either 500 or 250 mg/kg s.c. (Table 1) were killed for pathologic study at the end of the observation period; these had lost between 31 and 36% of initial body weight. Lesions in the subcutaneous tissues were edematous; they were bright orange-yellow when illuminated by visible light and a brilliant pink-orange with ultraviolet light (about 365 μm). These colors were like those of IMDM stains on filter paper or absorbent cotton. Most of the abdominal and thoracic viscera and the submaxillary-sublingual glands also had pink fluorescence under ultraviolet light; this was especially bright in the kidney cortex. Histologic study of tissues from the 4 rats revealed significant injury only in subcutaneous tissues at the injection sites where necrosis was found in muscle, connective tissue, and fat. The splenic red pulp in 2 animals, and peripancreatic lymph nodes in 1, had macrophages containing dark brown pigment. The liver of 1 animal had a small focus of necrotic cells containing orange granular material. A similar granularity was found in the mesenteric lymph nodes of another animal. Diminished spermatogenesis and abnormal germinal epithelial cells were seen in the testes of 1 of the 4 rats and the thymus was partially involuted in a single individual. No other microscopic changes were found in the abdominal and thoracic viscera, thyroid, salivary glands, sternal bone marrow, or skeletal muscle (except at injection sites).

One other pathologic effect of IMDM is shown in Table 2 which summarizes the incidence of acute mast cell degranulation in the mesenteries of animals receiving various i.p. doses of IMDM. The data show that doses as low as 1 mg/kg induced extensive degranulation within 20 min in most of the rats tested. The agent appeared to be less effective than Compound 48/80 which is a potent degranulator and histamine-releasor (26).

Physiologic Disposition. Chart 2 describes the distribution of IMDM in liver, kidney, and spleen after a single s.c. dose of
Isometamidium

CHART 2. Isometamidium in rat tissues. A single dose of 20 mg in 2 ml of isotonic saline was given s.c. in the flank of rats averaging 337 gm (309-364 gm). Analytical Method 1 was employed. Injection sites were not analyzed after Day 7 because they ulcerated and drained through the skin during the 2nd week after injection. Each symbol is from a single rat.

Approximately 60 mg/kg and its recovery from the injection site. At 1 hr after injection, there were already substantial quantities in liver and kidney. Concentrations increased to a maximum at 2 or 3 days and thereafter remained steady in kidney but decreased in liver. Smaller concentrations were found in the spleen. Here there was also relatively little change after 2 days. That tissue concentrations did not increase after the 3rd day may be attributed to pathologic changes at the injection site that retarded or blocked further clearance of the drug from the depot. Direct evidence for this was not obtained; for IMDM was not detected in the plasma at any time after the 1st day. Even during the 1st day only 1 plasma sample at 1 hr had as much as 7.5 µg/ml while the remaining samples, analyzed at 1, 4, or 24 hr, had 1.3 µg/ml or less.

Some attempt was made to identify IMDM in the tissue extracts of Chart 2. All were considered to contain the drug since spots, dried on paper, had fluorescent characteristics of IMDM under ultraviolet light. Absorbance of each extract was measured at 278 mµ, a maximum for IMDM, as well as at the 378-mµ peak used in the analyses (see Methods). The ratio of the 2 absorbances, A\textsubscript{278}/A\textsubscript{378}, in all extracts of injection sites varied between 0.75 and 0.78. In extracts of kidneys and livers, in which absorbance was sufficiently large to obtain meaningful ratios, i.e., in those estimated to contain drug concentrations of about 100 µg/gm, the ratios were found to be 0.75-0.85 and 0.77-0.86, respectively. The results, agreeing satisfactorily with the value of 0.76 for known solutions of IMDM in acetate buffer, indicated that most, if not all, of the specific absorption in tissue extracts was that of unchanged drug. This was also suggested by absorption and fluorescence spectra of liver extracts (Charts 3, 4).

Other organs were also analyzed in single rats from among the animals of Chart 2 at 4 hr and 1, 2, 4, and 7 days. These included brain, the submaxillary and sublingual salivary glands, thymus, lung, heart, testis, and gastrocnemius muscle. Positive measurements were obtained only in extracts of brain (17-23 µg/gm) and

CHART 3. Absorption spectra of isometamidium (IMDM) extracted from rat liver. Two rats received 20 mg of IMDM s.c. Three days later acetate buffer extracts were prepared by Method 1 from 400-mg samples of their livers. Extracts were also made from 400 mg of a liver from a saline-injected control and from 104 µg of IMDM in water. Spectra were recorded in a Cary model 11 spectrophotometer. The B and C extracts were from Rats 2 and 1 of Table 3.
Philips et al.

**Chart 4.** Fluorescence spectra of isometamidium extracted from rat liver. The extracts are those of Chart 3. Spectra were recorded in a Farrand spectrophotofluorimeter with high-pressure xenon arc, an ultraviolet-absorbing Corning filter, 3389, between cuvet and analyzing monochromator, a 1 P21 photomultiplier tube, and a Varian recorder.

**Chart 5.** Isometamidium in rat tissues. A single dose of 1 mg/kg was rapidly injected i.v. in lightly etherized rats, 272-336 gm. Each experiment was timed from the end of injection to the midpoint of the withdrawal of the blood sample. Liver and kidney were removed immediately thereafter. Each symbol is from a single animal. •, Concentrations in μg/gm of tissue; O, drug recovered in the whole organ in % of dose. See text for blood data.

of small intestine (8–33 μg/gm). These findings were of questionable significance since extracts of brain and small intestine of untreated controls gave blank values equivalent to 10–21 μg/gm (5 rats) and 13–18 μg/gm (3 rats), respectively.

Since only negligible concentrations of IMDM had been found in the plasma of animals of Chart 2, it appeared that the agent must be rapidly cleared from the circulation by organs such as liver and kidney. Direct evidence for this was shown by the experiment depicted in Chart 5. Within 1 min after i.v. injection 41% of the IMDM was already present in liver and by 10 min, 62%. Concentrations were maximal in kidney as early as 1 min after injection. In blood the agent was detected only in the 2 earliest samples, 1 and 1.5 min after injection, 1.2 and 1.7 μg/ml, respectively; none was detected thereafter.

A number of studies were done to measure the excretion of IMDM in urine and feces. In 1 experiment 3 rats were each given 20 mg s.c. and urine and feces were collected daily during the next 3 days. None of the agent was found in any of the daily urine collections, a remarkable result since the analysis was sufficiently sensitive for the detection in each daily sample of as little as 0.07% of the dose. That the agent had circulated from the injection site was shown by analysis of the livers and kidneys of 2 of the rats at 72 hr: the concentrations found were 114 and 131 μg/gm of liver and 131 and 161 μg/gm of kidney. Small amounts of the agent were present in extracts of each of the daily fecal collections equivalent to 0.7–2.3% of the dose/day (Table 3). Extracts of daily fecal samples from 3 control rats, which had received an injection of isotonic saline s.c., had no detectable blank.

Three other rats were given 20 mg of IMDM by intragastric intubation; urine and feces were collected daily for the next 2 days; and the animals were then killed for liver analyses. None of the agent was detected in the urine samples and none was found in any of the 3 livers. Presumably little or none had been absorbed and substantial quantities were, in fact, present in fecal collections. As shown in Table 3 the total found in the feces was equivalent to 30–38% of the dose given. Further study of these
extracts of the same feces revealed substances with RF values equivalent to 5-9% of the dose of IMDM. In acetate buffer, pH 4.7, at 285 m/μ, paper chromatograms of unstable 8-triazeno group and which has an absorption maximum to vary between 1.08 and 1.22; as stated previously the ratio of gastric intubation may have occurred for the most part in the expected from the IMDM present. The values obtained were like those of both homidium and IMDM (Table 4). Since homidium is 0.76. Complete spectra of these extracts had absorption maxima near 285 m/μ. This suggested the presence of homidium (Chart 1) which can be derived from IMDM by scission of the unstable 8-triazeno group and which has an absorption maximum in acetate buffer, pH 4.7, at 285 m/μ. Paper chromatograms of extracts of the same feces revealed substances with RF values like those of both homidium and IMDM (Table 4). Since homidium has negligible absorbance at 378 m/μ, the peak for IMDM, it would not have interfered with the analyses of the feces of Rats 4, 5, and 6 that are reported in Table 3. The maximal amounts of homidium present in these feces were estimated by using the absorption of the extracts at 285 m/μ in excess of that expected from the IMDM present. The values obtained were equivalent to 5-9% of the dose of IMDM.

Cleavage of IMDM to homidium in rats given doses by intragastric intubation may have occurred for the most part in the acidic environment of the stomach. When the compound was in solution in 0.1 M acetic acid at 23°C, it lost about 70% of absorption at 378 m/μ within 60 min. The same solutions gained absorption at 285 m/μ as expected if homidium were appearing by cleavage of the acid-labile triazeno group. Similar concentrations of IMDM at the same temperature in 0.1 M phosphate buffer, pH 7.5, remained unchanged. It is not likely that cleavage to homidium took place to any significant extent in animals given parenteral doses of IMDM. As discussed earlier the extracts of subcutaneous injection sites, of liver, and of kidney had absorption and fluorescence activation spectra which were essentially identical with those of IMDM. Such results could not have been obtained if significant concentrations of homidium had been present; for the spectra of the 2 substances are widely different. Furthermore, if homidium had been produced in vivo after parenteral injection of IMDM, it should have been excreted in urine (22). Extracts of urine of the rats of Table 3, which had been given s.c. doses of IMDM, had no specific absorption at 285 m/μ, though it was estimated that homidium could have been detected therein if present in the urine collections in amounts equivalent to as little as 0.1-0.2% of the dose of IMDM.

Since small quantities of IMDM were detected in the feces of s.c. injected rats (Table 3) a separate experiment was performed to determine if the drug was excreted in bile. For this 4 rats were injected with 20 mg s.c.; 2 days later they were anesthetized with α-chlorolose (200 mg/kg by intragastric intubation) and polyethylene catheters were inserted in the common bile ducts. Between 1.4 and 3.9 ml of bile were collected from each rat during the next 2 hr. They were then killed to obtain liver for analysis. Although the 4 livers contained a total of 2.0-2.4 mg of IMDM, none of the bile samples had detectable concentrations of the drug (1-ml portions analyzed).

Fluorescence Microscopy. In the preceding studies IMDM was found inhomogeneously distributed among different organs. A more remarkable inhomogeneity became apparent when tissues from rats, treated with 20 mg of the drug s.c. and killed either 24 hr or 7 days later, were studied by fluorescence microscopy. Brilliant yellow or orange fluorescence was found at 24 hr in epithelium of renal cortical tubules, in hepatic cells, and in duct epithelium of submaxillary and sublingual salivary glands. In liver the stroma was seen as a bright yellow network. Bile duct epithelium had pale yellow nuclei but the remainder of the portal tissues were blank. Cytoplasm of hepatic cells was bright yellow but nuclei were dark. (In touch preparations obtained from liver the fluorescence of cytoplasm in isolated hepatic cells was finely granular.) Glomeruli in the 24-hr kidney specimens had barely discernible yellow nuclei. Stroma of kidney cortex was bright yellow where it was adjacent to the basement membrane of tubular epithelium. A minority of cortical tubules had epithelium with intensely bright orange-yellow granular cytoplasm and bright yellow nuclei. The remaining cortical tubules were less brilliant. In the medulla all tubules had a dull yellow fluorescence but the nuclei were brighter than the cytoplasm; none of these was as bright as the least stained tubules in the cortex. Nuclei in most tubules in the papilla were faint yellow and less bright than those in the medulla (Fig. 1). Acinar cells of the submaxillary gland were faint orange, but those in the sublingual acini were not fluorescent. However, duct epithelium in both salivary glands was bright orange-yellow with nuclei brighter than cytoplasm (Fig. 2). The intensity of staining in these cells was like that in hepatocytes. Pancreatic acini were not visible but the islets selectively fluoresced a uniform yellow-

**Table 3**

<table>
<thead>
<tr>
<th>Route</th>
<th>Rat</th>
<th>0-24 hr</th>
<th>24-48 hr</th>
<th>48-72 hr</th>
</tr>
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<tbody>
<tr>
<td>s.c.</td>
<td>1</td>
<td>0.14</td>
<td>0.45</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.17</td>
<td>0.31</td>
<td>0.27</td>
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<tr>
<td></td>
<td>3</td>
<td>0.15</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>Intragastric</td>
<td>4</td>
<td>7.25</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>4.00</td>
<td>2.0</td>
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</tr>
<tr>
<td></td>
<td>6</td>
<td>4.8</td>
<td>1.4</td>
<td></td>
</tr>
</tbody>
</table>

* Calculated as equivalent to unchanged isometamidium.

**Table 4**

<table>
<thead>
<tr>
<th>Extract</th>
<th>System 1 (RF)</th>
<th>System 2 (RF)</th>
<th>System 3 (RF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isometamidium</td>
<td>0.01</td>
<td>0.19-0.21</td>
<td>0.44-0.51</td>
</tr>
<tr>
<td>Homidium</td>
<td>0.19-0.31</td>
<td>0.52-0.53</td>
<td>0.82-0.85</td>
</tr>
<tr>
<td>Rat 4</td>
<td>0.02</td>
<td>0.20</td>
<td>0.56</td>
</tr>
<tr>
<td>Rat 5</td>
<td>0.01</td>
<td>0.21</td>
<td>0.46</td>
</tr>
<tr>
<td>Rat 6</td>
<td>0.01</td>
<td>0.21</td>
<td>0.47</td>
</tr>
</tbody>
</table>

* Feces collected between 0 and 24 hr from rats described in Table 3.

**Acid-Labile Triazeno Group**

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orange with nuclei more so than cytoplasm (Fig. 3). Most of the spleen, thymus, and cervical lymph nodes were blank. However, clusters of yellow-orange granules were seen peripheral to follicles in lymph nodes, throughout the thymus, and in the red and white pulp of spleen. From their perifollicular distribution in nodes and their size, it was surmised that the clusters were macrophages laden with ingested drug. Similar clusters were seen in the lamina propria of the duodenum. Duodenal epithelium and outer muscle coat were faintly orange. Fibroblast nuclei in connective tissues were bright orange-yellow; these were observed in the septa between the sublingual and submaxillary glands, in perivascular connective tissue in heart, and in interstitial tissues of lung and pancreas. The remaining structures of heart, lung, and testis did not fluoresce.

In tissues taken from rats at 7 days the localization of fluorescence was similar to that at 24 hr. Fluorescence of hepatic cells was still cytoplasmic but was less intense and less uniform; many cells were not fluorescent. In renal cortex the epithelium of most tubules was now brilliant orange-yellow, brighter in cytoplasm than in nuclei. The medulla was largely nonfluorescent. Ducts of the salivary glands were still fluorescent although less intensely than at 24 hr. The irregular clusters of bright yellow-orange granules, thought to be in macrophages, were now more numerous in spleen, nodes, and thymus (Fig. 4). Their aggregation in spleen may have been responsible for the cumulation of drug in this organ (see Chart 2). Similar clusters were observed in the stroma of the base of crypts in duodenum. One other change was noted in the 7-day tissues: minute, scintillating, and brilliant red-orange pinpoint granules were present in abundance in the cytoplasm of hepatic cells and of renal cortical tubular epithelium. They were also seen in the macrophages described above, in a few cells in the renal papilla, and in connective tissues.

The above results showed that the intracellular location of fluorescence differed considerably in various tissues; e.g., it was found in cytoplasm of hepatocytes, and in both cytoplasm and nuclei of renal cortical tubules, but only in nuclei of epithelia of bile duct and renal papilla. There was reason to question whether such variations reflected true differences in intracellular distribution in vivo or whether they were artifacts of the experimental procedure. As examples of the latter possibility the following findings illustrate the rapidity with which diffusion artifacts can arise. Dry sections of liver and kidney from the 24-hr rats previously described were mounted with coverslips using isotonic saline and examined microscopically within 20 sec. There was a steady redistribution of fluorescence in liver which was complete within 2 min. At this time the nuclei of hepatic cells were bright orange with sharply outlined nuclear membranes and well-defined nucleoli and chromatin granules; there remained in cytoplasm only a diffuse, pale fluorescence with somewhat brighter granules and threadlike structures. The hepatic stroma was no longer fluorescent. All cells in portal areas, i.e., smooth muscle, leukocytes in blood vessels, and bile duct epithelium, had equally bright yellow-orange nuclei as brilliantly stained as those in hepatic cells. The redistribution of color in kidney was already over when observations were begun. All glomerular nuclei were brightly stained as well as smooth muscle nuclei of blood vessels. In renal cortex the stroma was no longer fluorescent. All tubular nuclei were now sharply stained but no more brightly than glomerular nuclei. Distribution of tubular staining was uniform throughout the cortex and not variable as in dry sections. Nuclei in medullary tubules were also uniformly and sharply colored but less intensely than in those of cortex; papillary tubular nuclei were still less intense although sharply defined. There was no stain remaining in the cytoplasm of any of the renal tubular epithelia.

The direction of diffusion under the conditions just described was from cytoplasm into nuclei within cells and across cell boundaries into nuclei of adjacent cells. It, therefore, seemed likely that the cytoplasmic fluorescence which had been seen in dry sections of liver and kidney was a near approximation of specific intracellular localization in these organs in vivo. Conversely some or all of the nuclear staining, which was present in dry sections of various organs, may have taken place by diffusion from cytoplasm during the time between death and freezing of tissue specimens.

IMDM was also shown to be an excellent fluorochrome for
mast cell granules. Mesenteries from untreated rats were spread as previously described and fixed for 15 min in solutions of 4% formaldehyde in water containing as little as 1 µg of drug/ml. They were then washed, dehydrated, and mounted in fluorescence-free medium. Granules in the mesenteric mast cells were found to have brilliant red-orange fluorescence (Fig. 5). Mesenteries taken from rats shortly after i.p. injection of IMDM and fixed in 4% formaldehyde contained numerous degranulated mast cells. The scattered granules were sharply stained like those present in intact cells (Fig. 6).

SUBCELLULAR DISTRIBUTION. The observation that fluorescence was localized in the cytoplasm of hepatic cells prompted a study of the distribution of IMDM in subcellular fractions of liver. The tissue was fractionated at 48 hr after s.c. injection of 20 mg of the agent. Microscopic study of the nuclear fraction with visible light showed that it contained numerous nuclei, surrounded by rapidly moving granules, about 0.5–1.0 µ in diameter, and a few intact cells. When the illumination was changed to filtered light to detect fluorescence, the nuclei were found to be dark while most of the granules emitted light of colors that varied from yellow-orange to red-orange. In unbroken cells nuclei were also nonfluorescent and the cytoplasm was diffuse yellow-orange in which were dispersed orange-red granules like those just described (Fig. 7). When a single field was observed continuously, its nuclei gradually became orange. Fluorescent granules like those in the nuclear fraction predominated in the heavy and light mitochondrial fractions. The few nuclei which contaminated the heavy mitochondrial fraction were nonfluorescent. Amorphous aggregates with yellow fluorescence and a scattering of orange granules were present in the microsomal fraction. No fluorescence was seen in the supernatant.

Table 5 summarizes the analysis of the various fractions. A comparison of the data from control and treated livers shows that the agent had not altered the subcellular distribution of nitrogen, cytochrome oxidase, and total acid phosphatase. At least 60% of the IMDM and 59% of the cytochrome oxidase were recovered in the 2 mitochondrial fractions. Such results together with the microscopic findings indicate that the agent had been concentrated in or on mitochondria. Another 17% of the mitochondria was present in the N- and P-fractions as shown by their cytochrome oxidase activity (9); accordingly an equivalent portion of the IMDM could be accounted for in the mitochondria contaminating these fractions. The remainder of the IMDM was distributed between nuclei and microsomal aggregates. There was essentially none present in the supernatant.

Chart 6 presents the distribution of the specific activities of IMDM and the enzymes. The profiles illustrate the close relation between cytochrome oxidase activity and IMDM concentration. They also suggest that the L-fraction contained IMDM in an amount somewhat larger than that which could be solely associated with mitochondria. Since this fraction was enriched with lysosomes as shown by the high specific activity of total acid phosphatase (9), it is conceivable that IMDM is also cumulated by these structures.

Studies in Dogs and Monkeys

TOXICITY IN DOGS. Four animals were immediately affected by single, rapid i.v. injections of 4 mg/kg. Two died in apnea at 3 and 12 min; the other pair recovered from the acute response within 40 min and survived.

Eight other dogs, 4 males and 4 females, were given a total of 10 rapid i.v. injections of 2 mg/kg/day (on successive days excepting weekends). One received in addition 2 injections of 4 mg/kg/day. All responded acutely after each dose. Within 1 min they vomited; between 1 and 5 min they were dejecting, lacrimation, and salivating. Weakness and ataxia were evident by 1 min and by 5 min the animals were prostrate with fasciculation paralysis of all extremities. The eyes were in a fixed stare with lids flexed. Hind-leg flexor reflexes were absent though corneal reflexes and knee-jerks were active. The respiration was slow and shallow. By 10 min recovery was evident; the dogs began to move and respiration improved. Between 15 and 20 min they rose spontaneously and staggered about; by 30 min they appeared normal.

Except for the repeated episodes of severe, near-fatal, acute
intoxication, the dogs remained well during treatment. The daily intake of food and water, output of urine, body temperature, and weight were unchanged. Four of the animals developed warm, erythematous swellings at injection sites; these improved within 2 weeks after the last dose. Five of the dogs, which were observed for more than 2 weeks after the beginning of treatment, lost slowly 8–20% of their initial weight between the 2nd and 7th week. During this period they behaved normally except for some reduction in daily food intake. Thereafter, they ate well and regained weight.

In 3 of the group hematologic and blood biochemical measurements were made before and during treatment and at 1 day after the last injection. No significant changes were found in the following: hematocrit, reticulocytes, neutrophils, lymphocytes, platelets, plasma prothrombin time, blood clotting time, sulfobromphthalein clearance, blood glucose and serum chloride, alkaline phosphatase, and nonprotein nitrogen. Half of the group hematologic and blood biochemical measurements were made before and during treatment and at 1 day after the last injection. All 3 reacted acutely after each injection with weakness, ptosis, and dyspnea. They recovered and were normal by 20 min. Except for losses of 4 and 6% of body weight the 2 cynomolgus monkeys remained well during the 2 weeks of treatment (Monkeys 1 and 2, Table 6). The rhesus monkey lost 11% of body weight by Day 16, at which time it was dyspneic, depressed, and unable to stand upright. All 3 monkeys were killed and autopsied at 14–16 days after beginning treatment (2 or 3 days after the last injection).

The rhesus monkey had a severe fat nephroasis and a markedly fatty liver. The liver also contained scattered, necrotic hepatic cells and a few lymphocytes and plasma cells in portal regions. There were also erosions in the gastric mucosa, esophagitis with erosions, venous thrombi in adrenals, some decrease in nucleated elements in bone marrow with foci of hemorrhage and congestion in depleted regions, and acariasis in lungs. In Monkey 2 the liver had only a few necrotic hepatic cells, focal aggregates of macrophages with brown pigment, and occasional, pigmented Kupffer cells. Doubly refractile crystals, surrounded with inflammatory cells, were present in the kidney cortex (probably in distal tubules); a slight hydropnephrosis was also present. The lungs had focal hemorrhages and edema. Foci of edema were also seen in the lungs of Monkey 1; in addition there were a few collections of macrophages which were free of pigment. Other organs (listed in the dog studies) were normal in the 3 monkeys.

Tissue Analysis. The concentration of IMDM in the liver, kidney, spleen, and cardiac ventricular muscle was determined in most of the dogs and monkeys described above. Table 6 shows that abundant amounts of IMDM were accumulated by livers and kidney cortices. In dogs substantial fractions of the dose were still present in liver for as long as 8 weeks after treatment. The agent was retained unchanged in the tissue as shown by the fluorescence spectra (Chart 7). This was also shown in liver extracts, containing more than 0.1 mg/gm, by the fact that the absorption ratio, 278/378 m, ranged between 0.76 and 0.80 or close to the expected, 0.76, for IMDM.

### Table 6

<table>
<thead>
<tr>
<th>Animal</th>
<th>Total dose* (mg)</th>
<th>Day killed*</th>
<th>Concentration in %g/wet wt.</th>
<th>Recovery in % of dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
<td>Kidney cortex</td>
<td>Kidney medulla</td>
<td>Spleen</td>
</tr>
<tr>
<td>Dog 1♂</td>
<td>313</td>
<td>15 (2)</td>
<td>0.29</td>
<td>0.08</td>
</tr>
<tr>
<td>Dog 2♀</td>
<td>138</td>
<td>21 (8)</td>
<td>0.21</td>
<td>0.07</td>
</tr>
<tr>
<td>Dog 3♂</td>
<td>241</td>
<td>51 (40)</td>
<td>0.15</td>
<td>0.04</td>
</tr>
<tr>
<td>Dog 4♀</td>
<td>289</td>
<td>63 (40)</td>
<td>0.40</td>
<td>0.13</td>
</tr>
<tr>
<td>Dog 5♂</td>
<td>259</td>
<td>65 (54)</td>
<td>0.27</td>
<td>0.04</td>
</tr>
<tr>
<td>Dog 6♀</td>
<td>279</td>
<td>66 (65)</td>
<td>0.16</td>
<td>0.02</td>
</tr>
<tr>
<td>Dog 7♀</td>
<td>217</td>
<td>66 (64)</td>
<td>0.09</td>
<td>0.02</td>
</tr>
<tr>
<td>Dog 8♀</td>
<td>0</td>
<td>65 (54)</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Dog 9♀</td>
<td>0</td>
<td>66 (55)</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

* Dogs 1–3 and 5–7 and Monkey 1 received 10 i.v. doses of 2 mg/kg/day between 0 and 11 days; Dog 4, 10 doses of 2 mg/kg/day and 2 of 4 mg/kg/day between 0 and 23 days; and Monkey 2, 1 dose of 2 mg/kg/day and 9 of 4 mg/kg/day between 0 and 11 days. Dogs 8 and 9 were given 10 daily injections of saline between 0 and 11 days.

* Numbers in parentheses are days after last injection.
Isometamidium

**ACTIVATION SPECTRA**
(Detected at 590 mµ)

**EMISSION SPECTRA**
(Excited by 385 mµ)

![Spectra Diagram]

**Chart 7.** Fluorescence spectra of isometamidium (IMDM) extracted from dog tissues. Dog 4 received IMDM and Dog 9 was a saline-injected control as described in Table 6. Acetate buffer extracts were prepared from 400-mg samples of L, liver, KC, kidney cortex, and KM, kidney medulla. Spectra recorded as in Chart 4 except for detection by a 1 P28 tube. Compare spectra with that of IMDM, Curve A in Chart 4.

Extracts from the 2 monkeys the ratio was 0.77; in that of Dog 4, 0.88.

**Binding Studies**

The binding of IMDM was studied by hexanol partitioning with 6 pools of rat serum of which 2 had been dialyzed. At equilibrium IMDM was present in the serum phase in total concentrations of 16–23 µg/ml; in 5 of the experiments 94–97% of the agent was bound; in 1, 86%. Small amounts of a red precipitate formed in vials containing undialyzed sera but not in those with dialyzed preparations. In partitions with 2% BSA in the aqueous layer 14–18 µg of IMDM/ml were in the aqueous phases at equilibrium; 86–89% was bound (3 experiments). Partitions with 0.2% BSA were completed with 9–13 µg/ml in the aqueous phase of which 66–74% was bound.

Chart 8 depicts the changes in the absorption spectrum of IMDM produced by dialyzed rat serum. These included shifts in the 343 mµ minimum and 378 mµ maximum to 352 and 383–384 mµ, respectively, decrease in absorption at 378 mµ, and change of the 460–480 mµ shoulder to a minimum at about 470 mµ and a maximum at 500 mµ. Major spectral changes were also produced by BSA, DNA, RNA, heparin, and hyaluronic acid; examples are shown in Table 7. The effects of BSA were like those of dialyzed rat serum in which presumably albumin was the major binding component. The mucopolysaccharides and the nucleic acids could be distinguished in spectral effects from each other and from BSA. Complex changes were also observed in the ultraviolet absorption spectrum with heparin (200–325 mµ), 0.52 mg/ml plus 25 µg IMDM/ml: the minima at 260 and 300 mµ were replaced by a peak at 318 mµ, shoulders appeared at 245 and 290.

Insoluble complexes formed when the concentrations of the mucopolysaccharides or the nucleic acids were reduced with respect to that of IMDM. They were orange in visible light and fluoresced brilliantly in ultraviolet light; those from DNA were fibrous. Chart 9 shows that their formation increased in direct proportion to the amount of reactant added until maximal precipitation of IMDM occurred. With the addition of excess reactant, precipitation was less complete presumably because of formation of soluble complexes. Proportional precipitation also took place in titrations of IMDM (0.065 µmole) with hyaluronic acid up to the addition of 58 µg of the latter. At this point precipitation was incomplete incorporating only 88% of the IMDM. Further increments of the acid reduced the amount of precipitate formed.

Equivalents for precipitation of insoluble complexes calculated from duplicate titrations, like those shown in Chart 9, were found to be per µmole of IMDM cation: 0.66 and 0.71 µmole S with heparin, 1.17 and 1.18 µmole P with DNA, and 1.15 and 1.17 µmole P with RNA. Since these values were close to 1.0, it seemed likely that insoluble complexes were formed by charge neutralization. The heparin-equivalent, being less than 1.0, suggests interaction of IMDM with hexuronic acid moieties.
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**CHART 8.** Effect of dialyzed serum on the absorption spectrum of isometamidium. In B and C the comparison cuvets contained 50% and 5% of dialyzed rat serum, respectively.

**TABLE 7**

**CHANGES IN THE ABSORPTION SPECTRUM OF ISOMETAMIDUM**

<table>
<thead>
<tr>
<th>Reactant</th>
<th>(mg/ml)</th>
<th>ΔO.D. at 378 μm (%)</th>
<th>Maxima</th>
<th>Minima</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>0</td>
<td>378</td>
<td>343</td>
</tr>
<tr>
<td>BSA</td>
<td>7.3</td>
<td>−17</td>
<td>384, 500</td>
<td>333, 470</td>
</tr>
<tr>
<td>DNA</td>
<td>0.45</td>
<td>−14</td>
<td>388, 497</td>
<td>453</td>
</tr>
<tr>
<td>RNA</td>
<td>0.54</td>
<td>−14</td>
<td>390, 496</td>
<td>457</td>
</tr>
<tr>
<td>Heparin</td>
<td>0.52</td>
<td>−39</td>
<td>378, 515</td>
<td>357, 478</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td>0.55</td>
<td>−45</td>
<td>378, 515</td>
<td>357, 480</td>
</tr>
</tbody>
</table>

* 0.032–0.036 μmole/ml.

**Discussion**

Renal and hepatic cumulation of IMDM resembles that shown by other dibasic trypanocides and antitumor agents such as phthalanilides and diamidines (1, 41). The long retention of IMDM in these organs and in injection sites seems clearly associated with its prolonged prophylactic activity against trypano-

**CHART 9.** Titrations of precipitation of isometamidium (IMDM) by macromolecules. The heparin, DNA, and RNA were reacted with 0.046, 0.060, and 0.061 μmole IMDM, respectively. The linearly descending, initial portion of each curve was extrapolated to the abscissa to obtain the equivalent of reagent required to precipitate all of the IMDM.
isolated infections (18). Rapid cumulation by liver and kidney also explains the quick reversal of the acute actions of IMDM which are due to blockade of neuromuscular transmission and stimulation of cholinergic receptors (R. Wien, A. K. Armitage, and J. A. Edson, unpublished observations). Presumably the receptor sites involved are occupied by IMDM only while blood concentrations are relatively high. When these decrease with entry of the drug into parenchymatous organs, the binding of the agent to the receptors is reversed and recovery from paralysis and other acute reactions quickly ensues. If the agent is given i.p. or s.c., its concentration in blood is limited by kidney and liver uptake and acute toxicity is correspondingly reduced.

The acute effects of IMDM must be anticipated as a hazard in the clinical use of the substance. As shown by others phenanthridinium derivatives elicit a wide variety of responses which are typical of agents containing quarternary nitrogen groups and which involve stimulation or blockade of cholinergic effectors (15, 40). In addition the agent may cause significant release of histamine and other active constituents through its degranulating effect on mast cells. Presumably such acute actions could be avoided or minimized by slow i.v. infusions of the agent. Chronic histopathologic effects must also be anticipated. These may consist of severe local damage, if the agent is directly injected into tissues, and of lesions in hepatic cells and renal cortical tubular epithelium. From the studies reported herein renal and hepatic disturbances may be expected to be slow in onset and of long duration.

The present work has shown that in rats the agent is localized in the mitochondria of hepatic cells and probably also in those of the tubular epithelium of the renal cortex. Even though both tissues are richly populated by these organelles, concentrating activity cannot be ascribed to mitochondrial function; for IMDM was not observed in the mitochondria of any of the other tissues studied by fluorescence microscopy. Moreover, a dicarboxylic fluorescent phthalanilide, which is also concentrated heavily in hepatocytes and renal cortical epithelium, has been shown to be selectively localized in the nuclei of these tissues (29). Possibly the mechanism of accumulation is akin to the specialized transport system involved in the cumulation and secretion of organic bases by renal cortical tubules (27). The assumption of a mechanism capable of transporting IMDM into cells seems reasonable; it is otherwise difficult to understand the rapid entry of the cationic substance which is, furthermore, firmly bound to serum proteins.

Once inside hepatocytes why does the agent concentrate in mitochondria? The organelles may also have a transport mechanism which can pump IMDM inward and thus dynamically remove the agent from the cell sap. Furthermore, potential binding sites at other loci, such as nuclei, may be masked in living cells. Either the pump mechanism, or masking, or both together could be lost during cell death and the agent would then promptly transfer from mitochondria to other loci. Presumably this occurred when fluorescence was seen to move from cytoplasmic granules into nuclei of hepatic cells after dry, cryostat sections were covered with saline. Two other observations seem pertinent. (a) Rat liver mitochondria have an energy-dependent mechanism for uptake of strong bases such as guanidines (31). (b) Acridine orange, although highly selective for the staining of RNA and DNA in fixed tissue, is excluded from these moieties in living HeLa cells. After entry the dye is by an energy-dependent process selectively cumulated in acid-phosphatase-positive granules presumed to be lysosomes. If such cells die, the acridine is promptly transferred from the lysosomes to RNA- and DNA-containing structures (33).

The mitochondrial localization in mammalian cells has its counterpart in the selective uptake of phenanthridines and diamidines by the kinetoplast of trypanosomes (16, 36), an organelle which has recently been shown ultrastructurally to be a modified mitochondrion (8, 35). There is no evidence implicating mitochondrial dysfunction as the primary mechanism of chemotherapeutic action of these agents in trypanosomes (24), or for that matter, in mammalian cells (17, 21, 22). On the contrary, phenanthridines are considered to act by primary inhibition of DNA-synthesis. Even in the present studies it is difficult to associate pathologic effects with mitochondrial concentration of IMDM. Although severe lesions were seen in the liver and kidney cortex in 1 monkey, it is remarkable that damage was so infrequent in these tissues. Apparently mitochondria have adequate function even when heavily laden with IMDM.

The binding of phenanthridines to DNA may account for antiviral actions (10) and for inhibition of RNA- and DNA-polymerases (11, 39) and of DNA-synthesis in trypanosomes (24) and tumor cells (17, 21, 22). A similar suggestion has been offered for the action of phthalanilides and diamidines (1). Unfortunately, the binding of such substances is not restricted to DNA since they can react effectively with polyanionic molecules in general: proteins, nucleic acids, and mucopolysaccharides (1). To be certain that the chemotherapeutic effects of a compound like IMDM are, in fact, due to DNA-binding it would seem essential to demonstrate its presence in the DNA of inhibited cells in vivo. DNA-binding does, of course, explain the fluorochroming of nuclei which takes place in tissue sections in vitro. Binding to heparin accounts for the effective staining of mast cell granules which are known to contain an abundance of this mucopolysaccharide (32).

Acknowledgments

The authors are indebted to R. Wien, A. K. Armitage, and J. A. Edson, Biological Research Laboratories, May and Baker, Ltd., Dagenham, Essex, England, for unpublished reports of studies of the acute pharmacology of isometamidium chloride (SAMORIN, May and Baker). They also wish to thank Dr. A. W. Nineham and his colleagues of May and Baker, Ltd., for liberal supplies of the isometamidium salt and of homidium chloride and for extensive information about composition and physical-chemical properties.

References

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15. Goodwin, L. G., Goss, M. D., and Lock, J. A. The Chemo-
Figs. 1-7. All photographs were taken with an ultraviolet light source; fluorescent material, white in the photographs, was orange-yellow in the original (except as indicated). Tissue sections (Figs. 1-4) were fresh-frozen, unfixed, and covered with a glass coverslip over the dry tissue as described in the text. Figs. 5 and 6 were mounted in fluorofree mountant. Fig. 7 was from a drop of the fraction, mounted with a coverslip.

Fig. 1. Cross section of rat kidney showing bright fluorescence of cortical tubules (top) and diminished intensity in the direction of the papilla (bottom). Animal killed 24 hr after receiving 20 mg of isometamidium s.c. Rats from Figs. 2-4 received same dose, and except for rat in Fig. 4, were killed 24 hr later; Fig. 4 was killed 7 days after injection. × 35.

Fig. 2. In the submaxillary salivary gland fluorescence is localized in the ducts. × 300.

Fig. 3. In pancreas fluorescence is localized in the islets. × 300.

Fig. 4. In spleen, bright fluorescent material is contained in macrophages of red pulp (upper right). At left smaller amounts of orange-fluorescent material is present in a follicle; some of the fluorescence represents autofluorescent yellow granules seen normally. × 180.
FIG. 5. Mast cell from imprint made of mesentery of normal rat and fluorochromed with isometamidium according to method described in text. Oil immersion. X 700.

FIG. 6. Degranulated mast cell in mesenteric spread of rat 20 min after injection of 25 mg/kg of isometamidium given i.p. Oil immersion. X 700.

FIG. 7. Single, intact hepatic cell from fraction of rat showing granular appearance of fluorescent material in cytoplasm. X 720.
Physiologic Disposition and Intracellular Localization of Isometamidium

Frederick S. Philips, Stephen S. Sternberg, Alice P. Cronin, et al.


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