Further Studies on the Recovery of 2-Acetylaminofluorene from Rats Following Oral Administration

HELEN M. DYER, HELEN E. ROSS, AND HAROLD P. MORRIS

(National Cancer Institute, National Institutes of Health, Public Health Service, Bethesda, Md.)

Morris and Westfall (5) found, after a single feeding of 2-acetylaminofluorene (AAF) to rats, that tissue concentrations of the carcinogen were greatly decreased, or in some cases no longer detectable, well before the sixteenth hour, while considerable amounts of the material still remained in the stomach contents. They concluded that the AAF may have been rapidly "inactivated." It was also suggested by them that part of the carcinogen may have entered into chemical combination with some tissue constituent, resulting in an acetone-insoluble conjugate. Since their procedure recovered only acetone-extractable AAF or amino-fluorene (AF), such a conjugate would not have been detected. The present investigation was undertaken with the purpose of exploring the second possibility in an effort to find the large percentage of administered diazotizable material that could not be accounted for in the acetone extracts of the stomach, tissues, and excreta of the rat 5–24 hours after gastric instillation with AAF.

The fact that 7-hydroxy-2-acetylaminofluorene has been isolated from the urine of rats following oral administration of AAF (1) suggested the likelihood of an ester linkage with fatty acid if a hydroxy derivative of the carcinogen was present in the tissues. Suitable extracts of the tissues of rats that had received AAF either by stomach tube or by feeding were therefore saponified before analyzing for diazotizable material.

The expected ease of deacetylation, proof of which has recently been found for AAF by Morris, Weisburger, and Weisburger (4), made feasible the assumption that some AF was produced in the body following administration of AAF and that the amino group might enter into peptide linkage with free carboxyl groups of tissue proteins. Consequently, protein fractions of groups of tissues or of the whole animal, after administration of AAF, were hydrolyzed with HCl, KOH, or trypsin, preceding ether extraction and diazotization of the ether residues.

METHODS

METHOD OF ANALYSIS FOR AMINOFLUORENE

A slightly modified adaptation of the diazotization method of Westfall and Morris (11) was used. The modification is described as follows: three tubes containing the dried residue of an acetone or ether extract of the test material in a solution of 1 volume of glacial acetic acid, 0.5 volume of concentrated HCl, and 4 volumes of distilled water were heated for 1 hour in a boiling water bath. The tubes were then immersed in ice water, and the volumes of the solutions were made up to 3.0 ml. by the addition dropwise of a mixture containing the same proportions of the acids and water. To one of the three tubes was added 1 ml. of distilled water, and to two of the tubes was added 1 ml. of 0.03 M aqueous sodium nitrite. Each tube was shaken after the addition of the water or of the sodium nitrite and was quickly returned to the ice bath. After 1 minute (timing is important), 2 ml. of the contents of each tube (total volume was then 4 ml.) was pipetted with shaking into test tubes (fitted with glass stoppers) containing 5 ml. of the ammoniacal R salt solution (0.03 M R salt in 5 M NH₄OH). To each tube was added 0.5 ml. of 95 per cent acetone. The tubes were stoppered and the contents shaken. The red color, which is formed by coupling of the AF through the diazonium compound with R salt, was measured in a Coleman Universal spectrophotometer, Model 11, using a wave length of 525 mμ.1 The tube that received water instead of sodium nitrite was used as the blank for each determination. Although the method of analysis, with the procedure of heating in a boiling water bath for 1 hour in 1 N HCl, measures AF, the material administered was AAF, and that recovered was calculated as AAF.

Since the diazotization test was found to be negative with tissues of normal rats and positive with tissues of rats following administration of

1 This wave length corresponds to the maximal spectral absorption for the 2-fluorenyl-azo-1-naphthol-8,6-disulfonic acid sodium salt as demonstrated by the spectral absorption curve published by Westfall and Morris (11).

Received for publication October 6, 1950.
AAF or AF, the assumption is made that the chromogenic material measured was one of these compounds or closely related diazotizable derivatives and that it came from the carcinogen. Throughout the paper, therefore, the terms AF and AAF, or "specific" diazotizable material have been used to designate the organic solvent-soluble material which reacted with R salt to give a chromogen having the same absorption spectrum as that of 2-fluorenyl-azo-1-naphthol-3,6-disulfonic acid sodium salt.

Sensitivity of method.—The results of pilot recovery experiments of small amounts of AAF added to tissues before and after homogenization in acetone confirmed the findings of Westfall and Morris (11), who noted excellent agreement between replicate estimations but an absolute, constant error below 10 μg.—the source of which appeared to be in the preparation of the extracts. Preliminary recovery experiments, however, with AAF added to tissues and carried through the various procedures necessary for the hydrolysis of tissue constituents, demonstrated that recovery was not quantitative, especially with the hydrolysis of protein material in which the losses varied, with different tissues, from 35 to 75 per cent. This observation led us to set up, for each analysis, control flasks containing equal amounts of the particular tissue being analyzed with AAF added. The routine addition of 300 μg. of AAF to the control tissue was chosen, because that amount provided sufficient material for duplicates and blanks, even when partial disappearance of the added AAF occurred. When most of the AAF was recovered, however, it was necessary to make at least a 1:5 dilution of the final extract for analysis. Since the search was for unaccounted-for quantities of AAF ranging from 5 to more than 20 mg., the use of 300 μg. in the control flask is believed to be justifiable, especially since the individual analyses represented large fractions of the tissues of the entire rat. Moreover, the experimental tissues yielded completely negative results following the hydrolytic procedures. The results are therefore qualitative and significant.

Treatment of Rats

Male and female rats of the Buffalo strain were used. They were bred at the National Cancer Institute and were maintained, up to the experimental period, on N.C.I. pellets (8). Two types of experiments were set up: one in which a large single dose of AAF in propylene glycol was administered by stomach tube to rats that had been on fast, and the second type in which AAF was incorporated in the food for several weeks or months before the test period. Two different diets were used for the feeding of AAF, both of which have been found in this laboratory to be carcinogenic to the Buffalo strain of rats. One of the diets was a semi-synthetic type, containing, in addition to the carbohydrate and vitamins, 12 per cent commercial casein and 21 per cent lard in which was incorporated AAF to give 1.2 mg/gm of food. The second diet was prepared from natural foodstuffs and contained approximately 22 per cent protein from several sources and 10 per cent fat, the fat again having been used to dissolve AAF, this time to give 0.25 mg/gm of diet.

The test rats were placed in metabolism cages for fasting periods of 16—23 hours (and of 5 hours for 2 rats) following the administration, or the last day of ingestion, of the carcinogen. Feces and urine were thus available for analysis. Blood was removed by cardiac puncture with the rat under ether anesthesia. The animal was then killed, and the tissues were removed and weighed. They were homogenized in a Potter-Elvehjem homogenizer, or, in the case of the whole rat, the animal was skinned, the organs were then homogenized in a Potter-Elvehjem homogenizer and the muscle, skin, and skeleton in a Waring Blender following repeated freezing and mincing in a tissue grinder. The extraction solvent depended upon the particular procedure which was being followed, as indicated below.

Preparation of Tissues for Analysis

Acetone extraction of tissues.—Some homogenates were made in acetone according to the procedure followed by Westfall (10).

Ether extraction of materials.—Aqueous solutions such as blood, urine, hydrolysates of tissues and (or) saponified extracts were extracted with ether instead of acetone, acid extracts being neutralized before ether extraction. After evaporation of the solvent, the ether residue was analyzed.

Isolation of soluble proteins in acetate buffer.—The tissues were homogenized in water and extracted in ice-cold 0.5 M sodium acetate buffer of pH 5 following, with slight modification, the method used by Miller and Miller (2). The dried crude protein material was hydrolyzed as described below, and the hydrolysates were analyzed for the "specific" diazotizable material. Alcohol extracts and washes were evaporated at room temperature (or at reduced pressure), and the residues were saponified and then treated according to the procedure given under "Saponification."

Treatment of tissue residues.—The residues of

1 These diets will be described in detail in subsequent publications.
tissues remaining after extraction with acetate buffer were extracted with acetone. The acetone was evaporated, and the acetone-soluble residues were analyzed. Samples of the tissues remaining (final residues) after extraction with both acetate buffer and acetone were hydrolyzed with potassium hydroxide in the autoclave and with trypsin, by the same procedures as those used for the isolated, coagulated, acetate-extracted, protein material. In two experiments portions of the final residues were also hydrolyzed with HCl.

Saponification

To test for the presence of AAF conjugated with fatty acids, residues of acetone, ether, and alcohol extracts, after evaporation of the solvents, were subjected to saponification. After cooling, the saponified material was extracted with ether in separatory funnels, the ether extracts were washed with water until free of alkali, the solvent evaporated, and the ether residues analyzed as described. Recovery experiments with 300 μg of AAF added to the same weights of tissue and carried through the same extraction, evaporation, saponification, etc., procedures demonstrated that 85–100 per cent of such added material could be recovered.

Hydrolysis of Protein Material

Hydrolysis with KOH.—Since AAF could not be detected in the hydrolysates after refluxing protein material containing 300 μg of AAF with 4.5 N alcoholic KOH for 20 hours, according to the method of Miller and Miller (2), the hydrolysis was accomplished by autoclaving at 20 lb. pressure in the presence of KOH.

A study of the influence of alkali concentration and autoclaving time upon the recovery of AAF and upon the conversion of protein nitrogen to nonprotein nitrogen led to the use of a concentration of 1.0 N aqueous KOH for a period of 5 hours at 20 lb. pressure. In the presence of 0.2–1.0 gm. of proteins and of 5–10 gm. of tissue residues, the recovery of 300 μg of AAF added to the control flasks varied from as little as 25 per cent, with final residues of muscle, to 66 per cent with final residues of miscellaneous organs and with acetate buffer-soluble proteins. Under these conditions, control samples of 300 μg of AAF, in the absence of rat tissues, were recovered to the extent of 75 per cent.

Hydrolysis with HCl.—The acetate buffer-soluble proteins and the acetate acetone-extracted residues (final residues) of two rats that had received 20.3 and 19.5 mg of AAF in propylene glycol by stomach tube 23 hours before killing were hydrolyzed by refluxing for 24 hours in 20 volumes of 90 per cent HCl. The hydrolysates, after concentration in vacuo, were made slightly alkaline with NaOH and were extracted with ether in a separatory funnel. The recovery of 300 μg of AAF added to isolated protein material or to the protein-containing tissue residues was approximately 35 per cent when hydrolysis was carried out in this manner. Acetylaminofluorene, refluxed in 20 per cent HCl for 24 hours in the absence of protein material, then made alkaline and extracted as described, resulted in a recovery of 65 per cent of that added, thus representing considerably less than when protein was present to be hydrolyzed.

Hydrolysis with trypsin.—Because of the failure to recover AAF quantitatively from either acid or alkaline digests of protein material, trypsic digestion was tried, with 1–10 gm. of the protein material, and with digestion for 7 days at 40° C. with "Difco" standardized (1:850) trypsin at pH 8.3 ± 0.3. Toluene and chloroform were used as preservatives. The digests and preservatives were then extracted with ether in a separatory funnel, and, after volatilization of the ether, the residues were analyzed by the usual procedure. In the presence of large amounts of fatty material the ether residues were saponified preceding analysis.

Samples were removed before and after digestion for determination of total and nonprotein nitrogen. With some of the insoluble tissue residues, total nitrogen, before digestion, had to be determined with dry samples, since accurate sampling of the suspensions was impossible. The recovery with 300 μg of AAF added to the control flasks was between 84 and 98 per cent for all digests except those of the carcass and skin with hair. With these latter tissues there was present undigested residue, and the recovery of added AAF varied from 40 to 60 per cent.

Treatment of Urine for Detection of the Glucuronide of Hydroxyaminofluorene

Neish (6) isolated the glucuronide of 2-hydroxyfluorene from rabbits' urine following daily administration of fluorene. In a search for the possible presence of the glucuronide of hydroxyaminofluorene, the urine, after ether extraction for the determination of unconjugated AF and AAF, was made approximately 0.2 N with HCl or with H2SO4 and was refluxed for 3 hours. After hydrolysis the treated urine was neutralized and again extracted with ether. The residue, after volatilization of the solvent, was analyzed. Recovery of the diazotizable material after addition of AAF to urine before refluxing was not quantitative, since not more than 60 per cent of 300 μg of added AAF could be accounted for.
RESULTS

No diazotizable material giving the color reaction with R salt was liberated from the hydrolysates of lipid or of protein material from rats' tissues following administration of AAF. It is evident, therefore, that the data presented in Tables 1–3 represent the recoveries from acetone extracts of the tissues and excreta of the rats. The last columns of Tables 1 and 2 list the different procedures used for the analyses.

Saponification of the residues from acetone, ether, or alcohol extracts of the tissues and excreta made possible the analysis of material containing large amounts of fat without the loss of AF or AAF as a result of their solubility in fat. Since tissues that contained moderate amounts of lipid material yielded the same amount of AF or AAF with and without saponification, it became apparent that saponification did not liberate significant amounts, if any, of the diazotizable material.

### TABLE 1

**RECOVERY OF ACETYLAMINOFLUORENE* FROM RATS AFTER A SINGLE ADMINISTRATION BY STOMACH TUBE**

<table>
<thead>
<tr>
<th>No. of rats</th>
<th>Weight of rat (g)</th>
<th>Amount of AAF administered (mg)</th>
<th>Total amount of AAF digested (mg)</th>
<th>AAF administered (mg)</th>
<th>Per cent recovered</th>
<th>Remainder of AAF</th>
<th>Procedures used</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>251</td>
<td>32.2</td>
<td>16</td>
<td>27.8</td>
<td>92.5</td>
<td>0.07</td>
<td>a, i, b, i, d, e</td>
</tr>
<tr>
<td>2</td>
<td>433</td>
<td>34</td>
<td>5</td>
<td>32.96</td>
<td>99.2</td>
<td>0.07</td>
<td>a, i, f, j, k, h, i, j, k, d, e, m</td>
</tr>
<tr>
<td>2</td>
<td>559</td>
<td>55.5</td>
<td>5</td>
<td>65.7</td>
<td>100</td>
<td>0.00%</td>
<td>a, i, c, i, j, k, d, e, m</td>
</tr>
</tbody>
</table>

* Could also have been aminofluorene or structurally related diazotizable derivatives.
† Per cent recovered, based on amount injected.
<table>
<thead>
<tr>
<th>INTERVAL FOLLOWING ADMINISTRATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAF from gastro-intestinal tract</td>
</tr>
<tr>
<td>AAF from feces</td>
</tr>
<tr>
<td>AAF from urine</td>
</tr>
</tbody>
</table>

### TABLE 2

**RECOVERY OF ACETYLAMINOFLUORENE* FROM RATS 17–22 HOURS† AFTER REPEATED INGESTION**

<table>
<thead>
<tr>
<th>No. of rats</th>
<th>Period of feeding (days)</th>
<th>Total amount of AAF ingested (mg)</th>
<th>Daily intake of AAF (mg)</th>
<th>Calculated average daily intake of AAF (mg)</th>
<th>Per cent recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>12</td>
<td>4.6</td>
<td>1.45</td>
<td>0.18</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>15.46</td>
<td>1.75</td>
<td>0</td>
<td>0.13</td>
</tr>
<tr>
<td>2</td>
<td>33</td>
<td>60.4</td>
<td>17.2</td>
<td>0</td>
<td>0.15</td>
</tr>
<tr>
<td>2</td>
<td>215</td>
<td>1,023</td>
<td>5.6</td>
<td>0</td>
<td>0.18</td>
</tr>
<tr>
<td>1</td>
<td>22</td>
<td>593</td>
<td>3.1</td>
<td>0</td>
<td>0.18</td>
</tr>
</tbody>
</table>

* This could also have been aminofluorene or structurally related derivatives.
† The rats were kept in metabolism cages during fasting periods.
‡ This represents the calculated average amount of AAF ingested during the 24 hours preceding transfer to metabolism cage.
§ Per cent recovery is based on the calculated amount AAF ingested during last 24 hours preceding fast in metabolism cage.
‖ See footnote 1 in Table 1 for guide to procedures.
¶ These rats were carrying primary subcutaneous tumors induced by AAF.
Although tryptic digestion failed to yield any diazotizable material giving the specific color reaction, all the protein materials became completely soluble during digestion except the acetate-acetone-extracted and the acetone-extracted residues of skin and skeleton and of whole rats in which there remained some insoluble residues consisting largely of bone and hair. Between 90 and 95 per cent of the total nitrogen of the soluble digests was converted to nonprotein nitrogen during digestion.

One series of tryptic digests was analyzed for amino acid nitrogen by the ninhydrin titration method of Van Slyke, MacFayden, and Hamilton (9). The α-amino acid nitrogen found was 36 per cent of the total nitrogen of a digest of the acetate-extracted proteins, 29.5 per cent in the digest of the acetone-extracted residue, and 18.9 per cent of the total nitrogen of the digest of the acetate-acetone-extracted final residue of whole rats.

While hydrolysis of the acetate-soluble protein material and the protein-containing residues of tissues, by autoclaving in potassium hydroxide and by refluxing in hydrochloric acid solution, resulted in the loss of considerable portions (varying in amounts with different tissues) of added AAF, no trace of AF or AAF was detected in any of the hydrolyzed material from rats to which AAF had been administered. These methods of hydrolysis also effected the solution of the substrates and the conversion of 90–95 per cent of the total nitrogen to nonprotein nitrogen.

Small amounts of some “specific” diazotizable material were recovered from the ether extracts of urine of rats during the collection periods of 16–23 hours following a single oral administration of AAF. Following this ether extraction, the urine that was made acid, refluxed, neutralized, and again extracted with ether yielded an additional small amount of diazotizable material which may have been present in the urine as the glucuronide, since this treatment would be expected to hydrolyze a glucuronide. The ether extracts of urine of normal untreated rats gave negative results in this test before and after acid hydrolysis. If the percentage losses in the experimental urines were proportional to those in which AAF was added to urine before refluxing in acid medium, the amounts of material excreted in this form in the urine would still represent insignificant fractions of the total amount of the AAF administered.

### Table 3

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Total Amount (μg.)</th>
<th>Per gm. Tissue (μg.)</th>
<th>Per cent of total recovered</th>
<th>Per cent of total administered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feces</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Urine</td>
<td>244</td>
<td>16.2</td>
<td>0.75</td>
<td>2.70</td>
</tr>
<tr>
<td>Gastrointestinal tract</td>
<td>7,800</td>
<td>9.8</td>
<td>0.049</td>
<td>0.17</td>
</tr>
<tr>
<td>Kidneys and adrenals</td>
<td>16</td>
<td>9.8</td>
<td>0.094</td>
<td>0.085</td>
</tr>
<tr>
<td>Lungs</td>
<td>8</td>
<td>5.8</td>
<td>0.12</td>
<td>0.44</td>
</tr>
<tr>
<td>Liver</td>
<td>40</td>
<td>5.8</td>
<td>0.12</td>
<td>0.44</td>
</tr>
<tr>
<td>Remainder of rat</td>
<td>869</td>
<td>4.1</td>
<td>0.060</td>
<td>0.60</td>
</tr>
<tr>
<td>Blood</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Could also be aminofluorene or structurally related derivatives.

<table>
<thead>
<tr>
<th></th>
<th>AAF recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Per cent</th>
<th>Per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

DISCUSSION

Whether or not the failure to account quantitatively for the AAF added to proteins after acid or alkaline hydrolysis is a result of destruction of the carcinogen or of combination of the amino group with metabolites present in the hydrolysate is not known. Since the method used for analysis requires the presence of the nitrogen of the amino group for diazotization, deamination of the carcinogen would result in failure of recovery. Moreover, any linkage of the amino group that could not be broken by hydrolysis would result in failure to detect the material. Neish (7) has shown that AF condenses with pyruvic acid in vitro to yield a cinchoninic acid derivative, 6-methyl-2,3-(fluoren)-2'-1'-pyridine-4-carboxylic acid. Since the nitrogen of this compound is present in the pyridine ring, it would not be diazotizable. Another reaction that would result in the formation of non-diazotizable material has been suggested by the observation of Neish (8) of a reaction in vitro between AF and dihydroxymalonic ester to yield ethylfluorene dioxindole carboxylate-2',3'-indeno-4,5-isatin and the oxime of this product.

The failure to detect any of the administered
AAF in hydrolysates of proteins or of lipids and the small fraction of the total administered AAF accounted for in the acetone extracts of the tissues and excreta by the diazotization method suggest the necessity and rationality of following the metabolic path of this carcinogen through the body by means of tracer atoms. While this work was in progress, Morris and associates (4) studied the distribution of radioactivity in the tissues of the rat with carbon-14 in the 9 position and with carbon-14 in the methyl group of orally administered AAF. The somewhat larger amounts of radioactivity accounted for in the body of the rat outside the gastrointestinal tract after oral administration of 3-C14-labeled AAF, compared with the recoveries of the diazotizable material, may possibly be due to the greater sensitivity of their test. But it seems to be more reasonable to conclude that the nitrogen from the administered AAF is present in the tissues in a nondiazotizable form and/or in extremely water-soluble material. Since a different distribution in the body of the 9-labeled carbon and the nitrogen of the molecule is possible, the results of studies now in progress with AAF containing N14 will be a most important means for following the metabolism of this carcinogen.

SUMMARY

The diazotization method of Westfall, with slight modifications, was used to determine the presence of 2-acetylaminofluorene (AAF), 2-aminofluorene (AF), or of closely related derivatives in acetone extracts of the tissues and in ether extracts of hydrolysates of protein and lipid fractions of the tissues of rats, following single doses of AAF by stomach tube or after repeated feeding of the carcinogen.

The method of analysis yields reliable recoveries of AAF from acetone extracts of tissues to which AAF is added before homogenization, but not more than 5 per cent of that which disappeared from the gastrointestinal tract during 5 to 23 hours of fasting could be recovered from the acetone extracts of the tissues and excreta of treated rats.

The recovery of 300 μg. of AAF added before the saponification of fat was excellent, but saponification of extracts of lipid material from rats’ tissues did not increase the amount recovered over that from unsaponified lipid extracts, except where large amounts of fat interfered with the glacial acetic acid solution of the carcinogen.

Moderate to large losses of AAF were encountered in control recovery experiments with the procedures used in the acid or alkaline hydrolyses of protein material. With 300 μg. of AAF added to the control tissues the poorest recovery, however, and that with only two fractions of tissues, was 75 μg. (25 per cent), whereas the hydrolysates of protein material from the experimental rats yielded completely negative results. Tryptic digests of protein material from the tissues of treated rats also failed to give any color in the test, although pilot control recovery tests demonstrated almost quantitative recovery.

It is concluded that orally administered AAF does not combine as such or as AF with the proteins or saponifiable lipids of the tissues, at least, in combinations that are reversible upon hydrolysis, or in amounts that represent significant fractions of that part of the administered AAF which cannot be recovered from acetone extracts of the tissues and excreta of the rats.

The experimental results indicate that much of the administered AAF is changed by the tissues of the rat into a nondiazotizable form.

REFERENCES

8. ———. The Chemistry of 2-Aminofluorene. II. Reaction with Dihydroxymalonic Ester. Ibid., pp. 357—60.
Further Studies on the Recovery of 2-Acetylaminofluorene from Rats Following Oral Administration

Helen M. Dyer, Helen E. Ross and Harold P. Morris


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/11/5/307

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.