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Studies on the Disposition of 2,3-Dihydro-1H-imidazo[1,2-*b*] pyrazole in Rodents

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Abstract—2,3-*Dihydro*-1H-*imidazo* [1,2-*b*] *pyrazole* (*IMPY*, *NSC*-51143) *is a new ribonucleotide reductase inhibitor, presently undergoing clinical evaluation, that exhibits prolonged* in vivo *antitumor activity in experimental animals. Disposition studies were initiated to determine if the prolonged* in vivo *antitumor activity of IMPY could be explained by its pharmacologic properties. Plasma disappearance curves of radioactivity were biphasic after the i.v. administration of* 100, 250 *or* 500 mg/kg *to rats and* 250 mg/kg *to mice. The distribution phase was rapid in each case* ($t_{1/2}$ *of approximately* 30 min *or less*), *followed by a prolonged elimination phase. Radioactivity was distributed to all tissues of rats and mice including brain after an i.v. dose of* 250 mg/kg. *In rats,* 67.8% *of the administered radioactivity was excreted in the urine during the first* 24 hr. *By* 120 hr 74.3% *had been excreted via the urine compared with* 11.1% *in the fees, the latter by biliary excretion. The chromatographic profile of the urine collected from rats and mice* 4 hr *after drug administration indicated extensive metabolism. Thus, the prolonged plasma and tissue levels of parent IMPY and its metabolites can account for the prolonged duration of cytotoxic activity* in vivo.

INTRODUCTION

2,3-DIHYDRO-1H-IMIDAZO [1,2-*b*] PYRAZOLE (Imidazopyrazole, IMPY, NSC-51143, Fig. 1), an antitumor agent presently undergoing

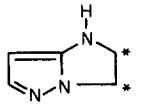

*Fig. 1. Structure of IMPY. *denotes* ^{14}C-*labelling.*

clinical evaluation, is an effective inhibitor of mammalian ribonucleotide reductase [1, 2]. However, unlike other ribonucleotide reductase inhibitors (guanazole and hydroxyurea) that require a frequent dosage schedule of once or twice daily for 10–15 days for optimal activity in mice [3, 4], IMPY is most effective when administered once every 8 days†. Furthermore, IMPY exhibits a more extended inhibition of DNA synthesis in L1210 cells *in vivo* of 18–20 hr as compared to the relatively short duration of <6 hr for guanazole and hydroxyurea [2]. The present study was initiated to determine if the sustained *in vivo* activity of IMPY is related to its disposition.

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MATERIALS AND METHODS

Chemicals

^{14}C-IMPY [2,3-dihydro-1 H-imidazo-2,3^{14} C-(1,2-*b*) pyrazole, 14.0 mCi/mmol] (purity >95%), synthesized by Research Triangle Institute, and IMPY were obtained from the Developmental Therapeutics Program, DCT, NCI. NCS tissue solubilizer was purchased from Amersham/Searle Corp., Arlington Heights, IL. Eastman 910 adhesive and ^{14}C-toluene (4.17×10^5 dpm/ml) were from Tennessee Eastman Co., Kingsport, TN and New England Nuclear Corp., Boston, MA, respectively. Scintillation fluid consisted of 6 g of 2,5-diphenyloxazole (PPO), 200 mg of *p*-bis[2-(5-phenyloxazolyl)] benzene (POPOP) (New England Nuclear), 1400 ml of toluene and 600 ml of methanol. Sodium heparin, 1000 units/ml, was obtained from the Upjohn Co., Kalamazoo, MI.

Studies with rats and mice

Plasma and tissue levels of IMPY equivalents were determined in male Sprague–Dawley rats (225–300 g, Taconic Farms, Germantown, NY) maintained on a diet of water and Purina Rat Chow (Ralston Purina Co., St. Louis, MO). Rats were anesthetized with sodium pentobarbital (45 mg/kg, i.p.), the abdominal aorta cannulated above the bifurcation of the common iliacs with a PE-50 cannula held in place with adhesive [5], and the incision sutured with the cannula in place. Following transfer to restraining cages [6], ^{14}C-IMPY (2–5 μCi/rat) diluted with non-radioactive IMPY to yield the final dose in a volume of 1 ml of distilled water was injected into the femoral vein over a 1 min period. The rats were allowed to recover from anesthesia and were provided free access to water. Blood samples were collected in heparinized tubes at various time points after drug administration. The tubes were centrifuged at 5000 ***g*** for 5 min and the radioactivity in the plasma determined as described below. Urine was collected at the end of the experiment by withdrawing urine from the bladder with a needle and syringe. In chronic excretion studies, rats were housed in glass metabolic cages with free access to food and water. Urine and feces were collected continuously and their radioactive content determined as described below. Bile was collected continuously from rats throughout some experiments following the cannulation of the common bile duct with PE-10 tubing. Total biliary ^{14}C-IMPY equivalents were determined by direct sampling of the bile.

Male CDF_1 mice (National Institutes of Health breeding colonies) were given i.v. injections via the tail vein with ^{14}C-IMPY (3–5 μCi per mouse). Blood samples were taken from the brachial artery. The concentration of ^{14}C-IMPY equivalents in the plasma was determined as described below.

Scintillation counting

Tissue levels of IMPY equivalents in rats and mice were determined by excising, blotting and weighing the organs, and homogenizing in 4 vol. of distilled water with a Polytron homogenizer (Brinkman Instruments, Westbury, NY). Homogenate samples of 100 μl were digested in NCS solubilizer at 49°C, and 18 ml of scintillation fluid was added. The samples were counted in a Beckman LS-230 liquid scintillation counter at room temperature. Plasma samples of 50 μl were digested in NCS solubilizer at 49°C for 1 hr, followed by the addition of scintillation fluid (18 ml) and counting. Urine samples of 100–200 μl were counted after direct addition to scintillation fluid. The feces were homogenized in 4 vol. distilled water and 100–200 μl samples were digested with NCS prior to addition of scintillation fluid (18 ml) and counting. Quenching was corrected by the internal standardization method with ^{14}C-toluene [7].

Thin layer chromatography

Plasma and urine samples containing ^{14}C-IMPY were chromatographed on Silica Gel G plates (Analtech Inc., Newark, DE) using the following solvent systems: I, butanol: acetic acid: water (4:1:1); II, benzene: ethanol (4:1) (all v/v). Plasma samples were deproteinized [8] before being applied to the plates while urine samples were applied directly. In all cases, samples contained at least 2000 dpm. Plates were placed in cassettes (20 × 25 cm, CGR Medical Corp., Baltimore, MD) and exposed to X-ray film (Kodak No-Screen, NS54T, Eastman Kodak Co., Rochester, NY) for 7–14 days before development. IMPY and its metabolites were quantitated by scraping the spots and determining the amount of radioactivity by liquid scintillation counting.

RESULTS

Plasma levels and tissue distribution

Plasma levels of total ^{14}C-IMPY equivalents during the first 6 hr after i.v. administration of 100, 250 and 500 mg/kg to rats are shown in Fig. 2. The curves were biphasic

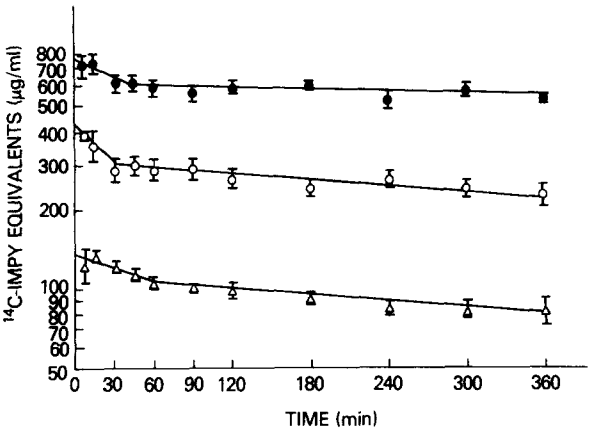

Fig. 2. Plasma levels of total ^{14}C-IMPY *equivalents in rats following i.v. administration* [500 mg/kg (●), 250 mg/kg (○), 100 mg/kg (△)]. *The plasma radioactivity was determined as described in Materials and Methods. Values are the mean ± S.E. of 3 or 4 rats and are expressed as micrograms of total IMPY equivalents per ml of plasma.*

with a distribution phase over the first 30–60 min followed by a phase of very gradual decline. The initial $t_{1/2}$ values were 33, 17 and 16 mm for the 100, 250 and 500 mg/kg doses, respectively. Plasma levels were proportional to dose over the measured time interval with the slope of the second phase decreasing slightly with increasing dose. Figure 3 shows the plasma levels in rats of

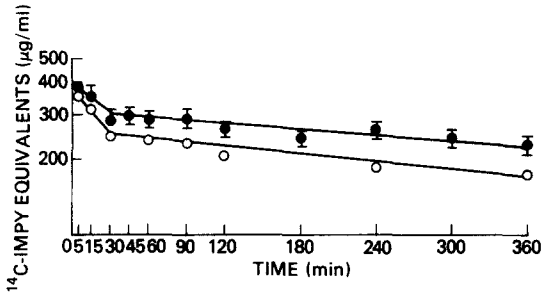

Fig. 3. Plasma levels of total (●) and unchanged (○) ^{14}C-IMPY equivalents in rats following i.v. administration of 250 mg/kg. Total and unchanged radioactivity was determined as described in Materials and Methods. Values are the mean ± S.E. of 4 rats for total equivalents and the mean of 2 rats for unchanged IMPY equivalents. Both are expressed as micrograms per ml of plasma.

total and unchanged IMPY equivalents for 6 hr following the i.v. administration of 250 mg/kg. Both the total equivalents curve and the unchanged IMPY curve were biphasic. The $t_{1/2}$ for the initial phase of the total ^{14}C-IMPY equivalents curve was approximately 17 min compared to 14 min for the parent IMPY curve. In both Figs. 2 and 3 the symbols represent the actual plasma levels of total ^{14}C-IMPY equivalents or parent drug while the solid lines are the best fit to this data using the MLAB program on a PDP-10 computer. The long-term plasma levels of total ^{14}C-IMPY equivalents and parent ^{14}C-IMPY were determined in mice at various time intervals from 2 to 24 hr after the i.v. administration of 250 mg/kg (Table 1). As can be seen in Table 1, parent IMPY accounted for a smaller percentage of the total ^{14}C-IMPY equivalents with time. Approximately 15% of the total equivalents was parent IMPY 24 hr after administration.

Table 1. Long-term plasma levels of radioactivity in mice after i.v. administration of ^{14}C-IMPY at 250 mg/kg

Time after administration (hr)	Total ^{14}C-IMPY equivalents (μg/ml)*	Parent ^{14}C-IMPY
2	191.5 ± 11.2†	115.5‡
4	167.1 ± 13.3	82.0
8	104.9 ± 14.1	32.9
16	128.5 ± 23.7	22.0
20	82.5 ± 3.2	8.5
24	63.8 ± 5.8	8.7

*Data are expressed as μg-equivalents of ^{14}C-IMPY or parent ^{14}C-IMPY per ml of plasma.
†Each value is the mean ± S.E. of four mice.
‡Each value is the mean of two determinations with the plasma of two mice pooled per determination.

The distribution of radioactivity (expressed as micrograms of IMPY equivalents per gram of tissue) in several tissues of rats 6 hr after i.v. administration of 100, 250 and 500 mg/kg doses is shown in Table 2. Tissue concentrations were approximately proportional to the dose of ^{14}C-IMPY administered. Radioactivity was distributed to all tissues; however, levels greater than those in plasma were found only for stomach, kidney and liver at the 100 mg/kg dose, for stomach and kidney at the 250 mg/kg dose, and for stomach at the 500 mg/kg dose. At all three doses, the levels of radioactivity in the above tissues were approximately 1.5 times greater than in plasma. Table 3 shows the tissue distribution

*Table 2. Tissue distribution of total radioactivity at 6 hr after i.v. administration to rats of ^{14}C-IMPY at 100, 250 and 500 mg/kg**

Dose (mg/kg)	Plasma	Stomach	Kidney	Liver	Spleen	SI	Lung	LI	Brain	Heart
100	81.83 ± 9.91†	158.87 ± 44.90	97.85 ± 10.15	117.67 ± 19.74	50.26 ± 13.45	34.17 ± 2.63	44.35 ± 3.52	31.84 ± 3.24	28.85 ± 9.37	33.22 ± 4.32
250	227.16 ± 19.15	324.15 ± 22.52	280.54 ± 16.03	225.44 ± 8.05	196.90 ± 94.70	128.46 ± 15.97	135.27 ± 3.94	99.69 ± 11.92	118.05 ± 5.14	111.90 ± 20.66
500	533.39 ± 0.24	812.32 ± 30.85	495.32 ± 29.95	473.50 ± 23.58	335.47 ± 24.68	145.97 ± 10.98	356.63 ± 15.01	159.37 ± 25.01	337.43 ± 10.10	300.78 ± 27.62

*Data are expressed as μg-equivalents of IMPY per g of tissue.
†Each value is the mean ± S.E. of three or four rats.

of radioactivity in various tissues at intervals from 6 to 24 hr following i.v. administration of 250 mg/kg. As was observed above, radioactivity continued to be distributed to all tissues with the stomach, kidney and liver maintaining levels greater than plasma at all time intervals examined (8, 16, 20 and 24 hr). The small and large intestines were the only other tissues found to contain levels greater than those in plasma at the later time points (16, 20 and 24 hr). Radioactivity appearing in the small and large intestines may be explained by biliary excretion as described below.

Table 4 shows the distribution of radioactivity in the tissues of mice at various times after the i.v. administration of ^{14}C-IMPY at a dose of 250 mg/kg. During the early time points radioactivity was well distributed to all tissues, including the brain. By 2 hr after administration, however, only the liver had a concentration of ^{14}C-IMPY equivalents greater than plasma. At the later time intervals (8 and 24 hr) the kidney and the liver were found to contain levels of ^{14}C-IMPY equivalents greater than those in plasma.

Excretion studies

The cumulative excretion of radioactivity in the urine and feces of rats following administration of ^{14}C-IMPY (250 mg/kg, i.v.) is shown in Fig. 4. The majority of the radioac-

Table 3. Tissue distribution of total radioactivity at various time intervals after i.v. administration to rats of ^{14}C-IMPY *at* 250 mg/kg*

Time (hr)	Plasma	Stomach	Kidney	Liver	Spleen	SI	Lung	LI	Brain	Heart
6	227.16	324.15	280.54	225.44	196.90	128.46	135.27	99.69	118.05	111.90
	±19.15†	±22.52	±16.03	±8.05	±94.70	±15.97	±3.94	±11.92	±5.14	±20.66
8	145.37	536.72	229.17	207.09	180.90	106.27	96.29	86.59	63.77	61.25
	±5.29	±76.03	±18.48	±19.54	±30.44	±18.28	±5.86	±16.36	±8.77	±11.00
16	60.38	234.23	100.61	135.29	51.26	67.45	40.07	80.06	20.97	24.19
	±3.99	±48.07	±8.86	±2.94	±12.96	±4.98	±4.83	±20.29	±2.92	±2.80
20	45.19	112.08	118.98	133.48	37.26	70.35	32.70	57.66	24.42	30.25
	±4.67	±24.58	±33.91	±19.78	±8.40	±5.63	±4.43	±2.37	±5.01	±7.06
24	39.04	95.79	59.51	108.19	56.98	71.37	32.44	127.20	12.93	19.54
	±5.64	±13.27	±9.42	±9.06	±18.55	±25.35	±2.92	±27.03	±1.72	±3.54

*Data are expressed as μg-equivalents of IMPY per g of tissue.
†Each value is the mean ± S.E. of three or four rats.

Table 4. Tissue distribution of total radioactivity at various times after i.v. administration of ^{14}C-IMPY (250 mg/kg) *to mice**

Time	Plasma	Brain	Heart	Lung	Spleen	Kidney	Liver
5 min	262.63	184.52	165.91	171.64	173.96	208.80	277.21
	±12.73†	±7.36	±3.36	±4.21	±6.04	±24.14	±11.52
10 min	287.43	211.48	174.82	205.96	207.96	161.80	297.33
	±27.72	±15.46	±18.16	±10.68	±20.46	±33.56	±36.26
15 min	257.18	186.04	162.55	171.57	184.00	148.62	256.34
	±10.66	±3.17	±9.11	±8.82	±2.77	±22.76	±6.37
30 min	228.90	158.42	143.93	150.97	174.93	143.58	237.27
	±8.92	±4.31	±10.31	±5.11	±18.08	±10.68	±8.49
1 hr	224.03	146.15	144.16	149.64	200.53	164.20	272.81
	±6.65	±9.95	±15.05	±21.66	±58.75	±26.26	±44.40
2 hr	191.53	129.53	125.25	134.43	125.43	199.70	266.67
	±11.23	±3.50	±5.74	±5.65	±5.48	±38.79	±27.42
4 hr	167.08	102.42	97.59	117.31	115.51	141.66	319.55
	±13.32	±10.85	±7.81	±9.44	±5.72	±33.62	±23.88
8 hr	104.85	38.30	51.17	81.50	149.06	177.88	388.79
	±14.05	±6.95	±9.90	±8.75	±53.15	±53.43	±27.17
24 hr	63.80	11.49	24.45	50.27	46.09	98.41	285.52
	±5.81	±0.99	±1.37	±4.77	±4.71	±22.94	±29.84

*Data are expressed as μg-equivalents of ^{14}C-IMPY per g of tissue.
†Each value is the mean ± S.E. of four mice.

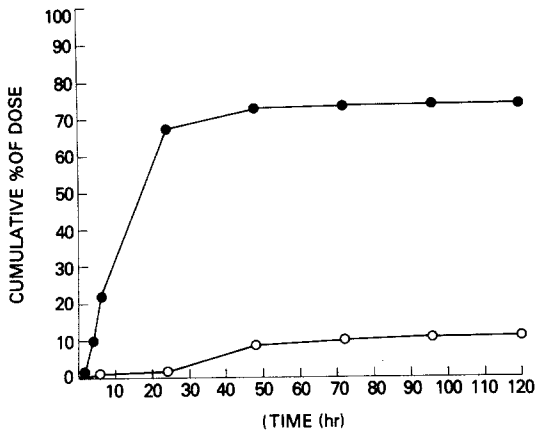

Fig. 4. Cumulative urinary and fecal excretion of ^{14}C-IMPY *equivalents in rats following i.v. administration of* 250 mg/kg. *Values are the means of serial determinations from* 3 *rats. Cumulative percentage of dose excreted in urine* (●) *and in feces* (○).

tivity was excreted within the first 24 hr in the urine, with 21.9% and 67.8% of the administered dose excreted by 6 and 24 hr, respectively. Successively smaller fractions of the dose continued to be eliminated during subsequent 24 hr periods, until less than 0.2% was excreted in the urine between 96 and 120 hr. Cumulative excretion of ^{14}C-IMPY in the feces was significantly less than via the urine (11.1% at 120 hr vs 74.3% via the urine). Fecal elimination of the dose was greatest between 24 and 48 hr. The radioactivity which appeared in the feces can be accounted for by biliary excretion, as 6.5% of a dose of 250 mg/kg administered i.v. to rats with biliary cannulas was eliminated in the bile within 6 hr.

Urine collected over 4 and 24 hr periods after the i.v. administration of ^{14}C-IMPY (250 mg/kg) to rats was chromatographed as described in Materials and Methods to determine the extent of metabolism. The chromatographic profiles indicated a major radioactive spot having an R_f of of 0.53 in system I and 0.32 in system II which was identical to that of native IMPY and accounting for 51.2% and 47.3% of the radioactivity in the 4 and 24 hr urines, respectively. The remainder of the radioactivity was distributed among distinct spots that differed in R_f from native IMPY. The chromatographic profile of mouse urine collected 4 hr after the i.v. administration of ^{14}C-IMPY at a dose of 250 mg/kg demonstrated that the drug was metabolized to a greater extent than in the rat, since only 15% of the urine radioactivity was accounted for by native IMPY. When ^{14}C-IMPY was incubated with normal rat urine for 24 hr at 37°C and chromatographed, only a single major spot with an R_f identical to that of parent IMPY was detectable. Thus, the distribution of radioactivity in the urine appears to be the result of the excretion of IMPY and IMPY metabolites. No attempt was made to identify the metabolites.

DISCUSSION

Studies on the antitumor properties of IMPY demonstrated that the schedule dependency differs markedly from that of other ribonucleotide reductase inhibitors. Guanazole and hydroxyurea require a frequent dosage schedule of once or twice daily for 10–15 days for optimal activity in mice [3, 4], while IMPY is most effective administered once every 8 days.* The optimal schedules for all three drugs can be explained by plasma half-life values and excretion rates. Unchanged IMPY accounted for the major species present in the plasma of rats from 5 min through the 6 hr measured after administration. In addition, greater than 60% of the initial plasma level of total ^{14}C-IMPY equivalents in rats was still present 3 hr after administration and 75% of this amount was unchanged ^{14}C-IMPY (Fig. 3). In mice, parent IMPY accounted for 50% of the plasma level of total ^{14}C-IMPY equivalents 4 hr after administration (Table 1). After 4 hr only 10% of the drug had been excreted in the urine of rats (Fig. 4). In contrast, the half-life of guanazole was observed to be 30 min and within 3 hr after administration less than 10% of the initial level remained in the plasma, while 70–80% was excreted in the urine [9]. With hydroxyurea approximately 70% of the dose appeared in the urine 4 hr after administration [10]. IMPY has been reported to have an I_{50} concentration of 3×10^{-4}M (33 μg/ml) for ribonucleotide reductase [2] to account for the inhibition of DNA synthesis. The plasma levels of parent IMPY in mice (Table 1) are sufficiently great to inhibit the reductase for 10 hr following administration. The levels of total equivalents in plasma remain approximately 2-fold greater than the I_{50} concentration at 24 hr and indicate that a metabolite(s) of IMPY also possibly acts to inhibit DNA synthesis. The plasma levels of parent IMPY were also sufficiently great in rats over the first 6 hr after administration to

*Screening Data from the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute.

inhibit the reductase. Rat and mouse tissue levels of total IMPY equivalents (Tables 3 and 4) were also greater than the I_{50} through 8 hr after drug administration. Thus, the values for plasma and tissue distribution along with excretion rates can be used to explain the dosage schedules required for the optimal antitumor activity.

Chromatographic analysis of urine samples collected from rats and mice 4 hr after ^{14}C-IMPY administration indicated that the drug was extensively metabolized. Parent ^{14}C-IMPY accounted for 51% and 15% of the urine radioactivity in the rat and mouse, respectively. IMPY has also been observed to undergo considerable metabolism in the dog as metabolites of IMPY accounted for the majority of the urinary excretion products [11]. Whether the antitumor activity of IMPY is due to parent IMPY, to one or more of the *in vivo* metabolites, or to a combination of parent drug and metabolite(s) is not known. Regardless of the nature of the cytotoxic species, the prolonged duration of cytotoxic activity exhibited by IMPY may best be explained by the prolonged plasma and tissue levels of parent and metabolite moieties. If IMPY is the active species, then the duration of exposure to cytotoxic concentrations and the plasma levels of unchanged drug would be direcly related. However, if a metabolite(s) of IMPY is the actual cytotoxic moiety, the prolonged plasma levels of unchanged IMPY would still be related to the extent of cytotoxic exposure because IMPY would then be acting as a depot form and potential source of the cytotoxic species. In the latter case, however, rates of activation and deactivation of the cytotoxic metabolite(s) would have to be considered.

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REFERENCES

1. C. T. Beer, K. Kajiwara and G. C. Mueller, Synchronization of HeLa cells with 2,3-dihydro-1H-imidazo [1,2-b] pyrazole. *Biochem. Pharmacol.* **23,** 1115 (1974).
2. R. W. Brockman, S. C. Shaddix, J. W. Carpenter, N. F. DuBois and R. F. Struck, Studies on the mechanism of action of 2,3-dihydro-1H-pyrazolo [2,3-*a*] imidazole (NSC-51143) and evidence of its metabolism *in vivo*. *Proc. Amer. Assoc. Cancer Res.* **19,** 21 (1978).
3. M. Hahn and R. H. Adamson, Pharmacology of 3,5-diamino-1,2,4-triazole (guanazole). 1. Antitumor activity of guanazole. *J. nat. Cancer Inst.* **48,** 783 (1972).
4. B. Stearns, K. A. Losee and J. Bernstein, Hydroxyurea. A new type of potential antitumor agent. *J. med. Chem.* **6,** 201 (1963).
5. J. W. Still and E. R. Whitcomb, Technique for permanent long-term intubation of rat aorta. *J. Lab. clin. Med.* **48,** 152 (1956).
6. J. L. Bollman, J. C. Cain and J. H. Grindlay, Techniques for the collection of lymph from the liver, small intestine, or thoracic duct of the rat. *J. Lab. clin. Med.* **33,** 1349 (1948).
7. C. H. Wang and D. L. Willis, *Radiotracer Methodology in Biological Sciences*, p. 131. Prentice-Hall, Englewood Cliffs (1965).
8. A. M. Krstulovic. P. R. Brown and D. M. Rosie, Identification of nucleosides and bases in serum and plasma samples by reverse-phase high performance liquid chromatography. *Analyt. Chem.* **49,** 2237 (1977).
9. R. H. Adamson and M. A. Hahn, The disposition of the antitumor agent 3,5-diamino-1,2,4-triazole (guanazole) in mice, rats and dogs. *Xenobiotica*, **3,** 247 (1973).
10. R. H. Adamson, S. L. Ague, S. M. Hess and J. D. Davidson, The distribution, excretion and metabolism of hydroxyurea -C^{14}. *J. Pharmacol. exp. Ther.* **150,** 322 (1965).
11. L. Malspeis, J. J. V. DeSouza, A. E. Staubus and H. B. Bhat, Metabolism and pharmacokinetics of IMPY (NSC 51143) in the dog. *Proc. Amer. Assoc. Cancer Res.* **20,** 153 (1979).