mediate not only because the addition of 2-chloroethylamine (VI) to the postulated 2-chloroethyl isocyanate (VII) explains very well the formation of 1,3-bis(2-chloroethyl)urea (II), but also because the delayed hepatoxicity of BCNU strongly resembles that of α-naphthyl isocyanate. In any event, the thermal decomposition of BCNU (I) to afford the original unnitrosated 1,3-bis(2-chloroethyl)urea (II) cannot be explained by the simple loss of a nitroso group directly from BCNU for the following reasons: first of all, such a homolysis is unprecedented; second, and most important, when 1-(2-chloroethyl)-1-nitroso-3-cyclohexylurea undergoes a like reaction in petroleum ether (b.p. 66°–75°) at 67° overnight, the product is 1,3-dicyclohexylurea [m.p. 224°, identical to an authentic sample (23) in every respect] and not 1-(2-chloroethyl)-3-cyclohexylurea, m.p. 130°–132° (24).

It has also been shown that 1,3-bis(2-chloroethyl)-urea (II) readily cyclizes in boiling water to 2-(2-chloroethylamino)-2-oxazoline (hydrochloride) (VIII) (25). However, we have no evidence that this has occurred at 43° in petroleum ether. Whether this is true *in vivo* remains to be seen.

## SUMMARY

1. BCNU is most stable at pH 4. In acid as well as in aqueous solutions of pH greater than 7 it is shortlived. Its half-life in plasma is about 20 min. *in vitro* and less than 15 min. *in vivo*.
2. The release of $Cl^-$ by hydrolysis of BCNU is too slow to account for its alkylating action.
3. BCNU is 80% bound to human plasma protein at 0°.
4. In the dog, after a single intravenous injection, BCNU enters the CSF immediately and then disappears rapidly from both the blood stream and the CSF. No more than 0.1% of unchanged drug is excreted in 4 hr. Nothing is known concerning its biotransformation *in vivo*.
5. Refluxing of BCNU at 43° in petroleum ether for 5 hr. converts it partly into 1,3-bis(2-chloroethyl)urea. The mechanism of this reaction is discussed.

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# Retention, Excretion, and Distribution of 2,3,5-Triiodobenzoic Acid and Its Metabolites in the Rat

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**The synthesis of $2(^{131}I)$,3,5-triiodobenzoic acid (TI*BA), starting with 2-amino-3,5-diiodobenzoic acid and sodium iodide ($^{131}I$) is described. The rate of excretion, biological half-life, and metabolites of this compound in rats were studied. Four metabolites and TIBA were found in the urine.**

In soybeans, TIBA affects plant morphology and flowering response (1, 2). When properly used it increases bean production through a better utilization of photosynthate (3). Since soybeans are used for animal and human consumption, the question arises as to the environmental health hazards of TIBA and its metabolites.

TIBA was originally synthesized by Wheeler and Johns (4). Anthranilic acid was reacted with iodine monochloride and the resultant product diazotized and reacted with potassium iodide. The synthesis of TI*BA has been de-

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scribed by Munakata and Nakai (5). They followed the procedure of Wheeler and Johns substituting sodium iodide ($^{131}$I) for potassium iodide.

Saz *et al.* (6–8) have reported that oral doses have been given to man without apparent toxic effects and were still being excreted 10 days after administration. Goldberg *et al.* (9) have reported the $LD_{100}$ for white mice to be 1.25 Gm./Kg. The $LD_{50}$ was 0.70 Gm./Kg. Crismer (10) reported a recovery of 40% in the bile and 12% in the urine of TIBA given i.v. to dogs. Knoefel and Huang (11) have reported that TIBA exerts its toxic action through its protein binding capacity and that TIBA was reabsorbed from the glomerular filtrate.

## EXPERIMENTAL

**Apparatus and Materials.**—A large volume liquid scintillation detector, the Purdue University Small Animal Counter (PUSAC), described by Dunavant and Christian (12), was employed for whole body counting. Crystal scintillation counting, using a 2-in. thallium activated, sodium iodide, well crystal, was used for feces, urine, and tissue counting. All activity determinations were corrected for background, counter efficiency, and decay as required.

Thin-layer chromatograms were made 250 $\mu$ thick with a model 200 (L-2) spreader.[1] Five plates, 20 × 20 cm., were prepared from 15-Gm. Silica Gel G[2] mixed with 37 ml. of water. The chromatograms were heated 0.5 hr. at 110° before use. Solvent chambers lined with Whatman No. 1 filter paper were used. Each chamber was saturated with solvent before use.

Thick-layer chromatograms, 1.0-mm. thick, were prepared using a model 200-11[1] variable thickness spreader. Five chromatograms were prepared from 85 Gm. of Silica Gel G and 150 ml. of water. The plates were activated at 110° for 1 hr. prior to use. Three solvents systems were used. They were as follows: (*a*) normal propanol–ammonium hydroxide (28%)–water (10:1:1), (*b*) normal butanol–acetic acid–water (4:1:1), (*c*) methanol–water (75:25).

All solvent systems were prepared fresh, except for solvent system (*b*) in which metabolite analyses were done with the solvent system 30 or more days old. Each solvent was allowed to proceed 12–15 cm. on the chromatogram.

Unlabeled TIBA was located with bromocresol green spray[3] (0.1% in *n*-butanol). All labeled compounds were identified from autoradiograms using No Screen medical X-ray film. After exposure, the film was developed using Kodak liquid X-ray developer and fixed with Kodak liquid X-ray fixer[4] following the manufacturer's recommendations. Reagent grade chemicals were used. Sodium radioiodide was purchased from Iso/Serve, Inc., Cambridge, Mass.

**Synthesis of TI*BA.**—Following a procedure described by Breckinridge *et al.* (13), which is a modification of the method used by Olivier and Combe (14), 2.5 Gm. of 2-amino-3,5-diiodobenzoic acid[4] was dissolved in 10 ml. of sulfuric acid with continual cooling to 0°. One gram of sodium nitrite was added slowly. The reaction was stirred for 2 hr. This mixture was poured over 33 Gm. of ice and air was bubbled through the solution for 1 hr. While cold, it was filtered through Whatman No. 1 filter paper by gravity. Sodium iodide ($^{131}$I), 54.5 mc., in 1 ml. of NaOH was added. A carrier solution of 1 Gm. NaI in 1 ml. of water was used to rinse the isotope container and this was added to the mixture. The solution was heated for 30 min. Free iodine was neutralized with sodium hydrogen sulfite. The resultant precipitate was separated by filtering by suction with a Büchner funnel and a double layer of Whatman No. 1 filter paper. The precipitate was dissolved with a minimum amount of hot ethanol. Crystallization was accomplished by adding distilled water to the warm alcoholic solution with stirring until a saturation point was obtained. The solution was reheated to effect complete solution, covered, and set aside for crystallization. Needle shaped, light orange crystals with a melting point of 223.6–225.5° were obtained. The specific activity was 9.80 mc./mmole. Yields of 80–86% of theoretical were obtained.

**Radio-Compound Purity.**—Dilutions of synthesized TI*BA in 10 $\mu$l. of ethanol, prepared to give a total of $10^8$ disintegrating atoms during a 15-hr. period, were chromatographed using thin-layer chromatography and solvent system (*b*), made fresh. Only one labeled compound, $R_f$ 0.69, appeared on the autoradiograph and this gave an $R_r$ value of 1.0 when compared with TIBA. Thin-layer chromatography with solvent system (*a*) gave three separate labeled compounds. The $R_f$ of TIBA was 0.60 and the two impurities were located at $R_f$ 0.39 and $R_f$ 0.73. Successive recrystallization decreased the amounts of the impurities significantly, but the impurities could not be completely removed, probably because of continued decomposition resulting from the recrystallization procedure. Repeated thin-layer chromatograms of the synthesized TI*BA compared with a radioiodide ion standard indicated an $R_r$ of 1.0 for the impurity at $R_f$ 0.73. The impurity at $R_f$ 0.39 was not identified.

Purification was accomplished using a thick-layer chromatographic technique. Thirty-six spots of the synthesized TI*BA and unlabeled TIBA control were placed across a thick-layered chromatogram 1.5 cm. from the base of the plate. Each spot contained 3 mg. of TI*BA in 100 $\mu$l. of ethanol. After developing, the control TIBA was located with bromocresol green spray. The Silica Gel G in the corresponding area to the control presumed to contain pure TI*BA was scraped off and extracted with four 10-ml. portions of hot ethanol, and filtered through a sintered-glass funnel. The ethanolic solution was chromatographed using the thin-layer technique and solvent system (*a*). Only one labeled compound ($R_f$ 0.60) appeared on the autoradiogram of the chromatogram. To further prove the identity of the labeled compound, 250 mg. of unlabeled TIBA was added and the mixture, dissolved in hot ethanol, filtered through a sintered-glass filter and recrystallized. The crystals melted at 223–225° (the known melting point of TIBA) and contained the radioactivity. Accordingly, the

[1] Research Specialties Co., Richmond, Calif.
[2] Brinkmann Instrument, Inc., Westbury, N. Y.
[3] Research Specialties Co., Richmond, Calif.
[4] Eastman Kodak Co., Rochester, N. Y.

TABLE I.—WHOLE BODY RETENTION OF ORALLY ADMINISTERED 2,3,5-TRIIODOBENZOIC ACID

| Rat Group 1[a] | | Rat Group 2[b] | | Rat Group 3[c] | |
|---|---|---|---|---|---|
| Time, hr. | % Retained[d] | Time, hr. | % Retained[d] | Time, hr. | % Retained[d] |
| 0 | 100.0 | 0 | 100.0 | 0 | 100.0 |
| 12 | 78.0 | 4 | 83.3 | 6 | 85.9 |
| 24 | 56.8 | 8 | 62.8 | 12 | 59.0 |
| 36 | 37.7 | 12 | 46.8 | 18 | 41.9 |
| 48 | 30.1 | 16 | 33.5 | 24 | 34.1 |
| 60 | 24.3 | 20 | 26.8 | 36 | 16.8 |
| 72 | 22.1 | 24 | 23.1 | 48 | 14.9 |
| 84 | 19.1 | 36 | 13.5 | 60 | 11.4 |
| 96 | 17.2 | 42 | 12.1 | 84 | 9.1 |
| 120 | 16.0 | 48 | 11.2 | 96 | 8.9 |
| 144 | 15.0 | 60 | 10.2 | ... | ... |
| 168 | 14.4 | ... | ... | ... | ... |

[a] Seven, female, albino, Holtzman Rats; weight, 266–288 Gm.; temp., 21°; food and water *ad libitum*. [b] Six female, albino, Badger rats; weight, 165–175 Gm.; temp., 28°; food and water *ad libitum*. [c] Six, male, albino Holtzman rats; weight, 182–238 Gm.; temp., 28°; food *ad libitum*; 20 ml. water limit daily. [d] Per cent of administered dose.

TABLE II.—ACCUMULATED PER CENT[a] EXCRETION OF ORALLY ADMINISTERED 2,3,5-TRIIODOBENZOIC ACID

| Rat Group 1[b] | | Rat Group 2[b] | | | Rat Group 3[b] | | |
|---|---|---|---|---|---|---|---|
| Time, hr. | Urine, % | Time, hr. | Urine, % | Fecal, % | Time, hr. | Urine, % | Fecal, % |
| 12 | 21.4 | 4 | 12.3 | ... | 6 | 14.2 | ... |
| 24 | 40.2 | 8 | 28.6 | ... | 12 | 37.2 | 0.8 |
| 36 | 53.8 | 12 | 38.3 | 0.5 | 18 | 51.2 | ... |
| 48 | 63.0 | 16 | 48.4 | ... | 24 | 57.1 | 2.2 |
| 60 | 68.4 | 20 | 54.5 | ... | 36 | 69.8 | 3.2 |
| 72 | 70.4 | 24 | 58.2 | 1.6 | 48 | 71.2 | 3.5 |
| 84 | 73.4 | 36 | 66.9 | 2.6 | 60 | 73.7 | 3.7 |
| 96 | 74.0 | 42 | 67.9 | ... | 84 | 75.0 | 4.0 |
| 120 | 76.7 | 48 | 68.5 | 2.7 | 96 | 75.3 | 4.1 |
| 144 | 77.6 | 60 | 70.2 | 2.9 | ... | ... | ... |
| 168 | 78.8 | ... | ... | ... | ... | ... | ... |

[a] Per cent of administered dose. [b] Same rat group conditions as listed in Table I.

purified TI*BA was considered to be free of labeled compound impurities.

**Whole Body Retention and Excretion.**—Three groups of rats were studied. All doses were oral using an oral administration needle. Each rat was given a dose of 0.2 $\mu$c. representing 0.10 mg. of TI*BA per 0.5 ml. All doses were in a 50% ethanol and distilled water solution. The results were tabulated in per cent retained and appear in Table I. The data suggest two exponential components. A short biological half-life component is indicated from 11.8–17.9 hr. This may represent blood concentration of TI*BA. A long biological half-life component, 395–403 hr., was also indicated. This component may indicate thyroid involvement. Excretion studies were run concomitantly with whole body retention studies using the same rats and groupings. The feces was collected at 12-hr. intervals and the urine was collected at 4–12-hr. intervals. Results are shown in Table II. Urinary excretion was the primary mode of excretion, accounting for 70–78% of the administered dose. Fecal excretion accounted for 3–4% of the administered dose.

**Distribution Study.**—Two groups of six rats each were starved for 24 hr. prior to and during the study. An oral dose of TI*BA, as before, was administered. Immediately after administration a whole body count was made. Four hours later the rats were anesthetized with ether and a 1-ml. blood sample was obtained from the tail. The animals were sacrificed by over-anesthetizing with ether and the thyroid and kidneys completely excised. An aliquot of muscle tissue (from the right rear leg) and liver was also obtained. Each sample was weighed immediately after removal and a radioactivity determination made in the crystal scintillation counter. Table III presents the data on the distribution of TIBA in the blood, liver, kidney, thyroid, and muscle tissue.

The distribution study clearly indicates thyroid concentration of the labeled iodine atoms. Table III indicates the thyroid concentration was 12–18 times the concentration found in the blood. This probably is due to a partial breakdown of the TIBA to iodide ion which in turn is taken up by the thyroid. However, TIBA itself or other metabolites may be concentrated in this particular tissue.

**Metabolite Studies.**—A total of 12 Holtzman rats were given orally 6.75 $\mu$c. representing 2.9 mg. of TI*BA in 50% ethanol and distilled water solution. The rats were not permitted food or water

TABLE III.—DISTRIBUTION OF 2,3,5-TRIIODOBENZOIC ACID 4 hr. AFTER ORAL ADMINISTRATION

| | % of Dose/Gm. of Tissue | |
|---|---|---|
| Tissue | Rat Group 1[a] | Rat Group 2[b] |
| Blood | 0.9 | 0.7 |
| Thyroid | 12.5 | 18.8 |
| Kidney | 1.2 | 0.5 |
| Muscle | 0.1 | 0.1 |
| Liver | 0.3 | 0.3 |

[a] Mean of six, female, albino Badger rats, 170–190 Gm. [b] Mean of six, male, albino Holtzman rats, 192–217 Gm.

TABLE IV.—RELATIVE PERCENTAGES OF METABOLITES FOUND IN THE URINE FOLLOWING THE ADMINISTRATION OF 2,3,5-TRIIODOBENZOIC ACID TO RATS[a]

| Metabolites, $R_f$ Values | Relative Abundance, % | S.E.$\bar{x}$[b] |
|---|---|---|
| 0.81 | 26.3 | ±3.2 |
| 0.77 | 31.3 | ±3.0 |
| 0.52 | 10.4 | ±3.6 |
| 0.48 | 20.4 | ±1.6 |
| 0.39 | 11.6 | ±1.8 |

[a] Thick-layer chromatographic separation with *n*-butanol–acetic acid–water (4:1:1). [b] Standard error of the mean as determined from 10 separate determinations, five from each of the two separate urine samples collected from two separate groups of five animals each.

24 hr. prior to or during the experiment. A 60-$\mu$l. aliquot of whole urine collected over a period of 18 hr. was spotted on each of three thick-layer chromatographic plates along with standards of TI*BA and radioiodide ion. The remaining urine was acidified with 1 *N* HCl using litmus paper as the indicator, allowed to stand for 30 min., and then a 60-$\mu$l. aliquot was spotted on chromatography plates.

A chromatogram was developed in each of the three solvent systems. In solvent system (*b*) (30 days old) four metabolites plus TIBA were found. Radioiodide ion standard gave an $R_r$ of 1.0 with a spot located at $R_f$ 0.52. TI*BA standard gave an $R_r$ of 1.0 for the spot at $R_f$ 0.77. The spot at $R_f$ 0.48 disappears in the hydrolyzed urine indicating a possible conjugate of TIBA. The spots at $R_f$ 0.39 and $R_f$ 0.81 were not identified.

A separation occurred in solvent system (*a*) indicating three metabolites plus TIBA. An $R_r$ of 1.0 for TIBA at $R_f$ 0.60 was obtained. An $R_r$ of 1.0 for iodide ion at $R_f$ 0.73 was obtained. A metabolite at $R_f$ 0.45 disappeared upon acid hydrolysis indicating a possible conjugate of TIBA. The third metabolite was at $R_f$ 0.52 and was not identified.

In solvent system (*c*) three metabolites were indicated plus a heavy concentration at $R_f$ 0.83. Iodide ion and TI*BA standards had $R_r$ values of 1.0 at $R_f$ 0.83. The other three metabolites occurred at $R_f$'s of 0.81, 0.77, and 0.73.

The metabolite experiment described above was repeated using 10 animals to obtain a quantitative evaluation of the metabolites present in the urine. The urine collected over a period of 18 hr. was pooled into two separate groups representing five animals in each group and chromatographed as previously described using solvent system (*b*). The separated labeled compounds were located on the chromatogram by autoradiography and the Silica Gel G containing each metabolite was quantitatively transferred to separate vials for counting in the well crystal scintillation counter. The relative percentage of each metabolite, based on the total activity found on the chromatogram, was calculated. The data are presented in Table IV.

## SUMMARY AND CONCLUSIONS


1. Radioactive labeled 2($^{131}$I),3,5-triiodobenzoic acid was synthesized and purified free of other radio-labeled compounds by thick-layer chromatographic separation followed by hot ethanolic extraction.
2. Whole body retention studies of TIBA showed a two component system—one with a biological half-life of 11.8–17.9 hr. and the second 395–403 hr.
3. Excretion studies showed that the primary route of excretion was urinary. Of the administered dose, 70–78% was excreted through the urine while only 3–4% was excreted through the feces.
4. Distribution studies in the blood, liver, kidney, muscle tissue, and thyroid after 4 hr. indicated an average thyroid concentration of 15 times the average blood concentration.
5. Metabolite studies using thick-layer chromatography indicated 4 metabolites plus TIBA in the urine. One of the metabolites was identified as iodide ion. Quantitative determination showed 31.3% TIBA and 10.4% iodide ion. Three unidentified metabolites were found to be present in the quantity of 26.3, 20.4, and 11.6%.


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