within experimental error. Bjaastad and Brown (11) also found constant Z-values for varying camphor concentrations in polysorbate 20 solutions.

The behavior of the Z-value with concentration was also examined with the anionic surfactant, potassium laurate, and the cationic agent, dodecyltrimethylammonium chloride. The data from saturated camphor solutions were plotted for Fig. 2, and the data for saturated 2-heptanone solutions were plotted for Fig. 3.

Examination of Figs. 2 and 3 shows that the environmental polarity of the solubilized ketones begins to fall off above the CMC of both ionic surfactants (about 0.02 M). Dodecyltrimethylammonium chloride solutions exhibit the less polar cybotactic regions at high surfactant concentrations. In the potassium laurate solutions, however, the Z-value appears to attain a plateau. Klevens (2) has reported that potassium laurate achieves "full colloidal form" at about 0.15 M.

The Z-value method of measuring the polarity of the cybotactic region for micellar systems containing solubilizates appears to show promise. The polarity revealed by the method seems to conform to current micellar theories. Experimental refinements through the use of cells with shorter light paths may make possible the study of higher surfactant concentrations and solubilizates with higher molar absorptivities. Since the Z-value is an empirical measure, studies involving its use probably will not have great theoretical significance; however, further investigation of the method is warranted to gain more knowledge of solubilization in complex micellar systems.

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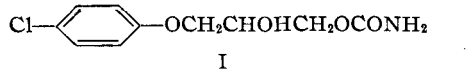
Preparation of Tritium-Labeled Compounds II

Chlorphenesin Carbamate by Exposure to Tritium Gas and Determination of Intramolecular Distribution of Tritium

By RICHARD C. THOMAS, DONALD R. BUHLER, and GEORGE J. IKEDA

Incorporation of stably bound tritium in purified chlorphenesin carbamate was 141 μc. per curie-day exposure. The intramolecular distribution of radioactivity was determined by a combination of biological and chemical degradation methods. Aromatic substitution predominated, accounting for 95 per cent of the tritium; two-thirds of this was located *ortho* to the chlorine substituent. The remaining 5 per cent of the radioactivity resided at the number one position of the alkoxy side chain.

CHLORPHENESIN CARBAMATE[1] [3-(*p*-chlorophenoxy)-1,2-propanediol 1-carbamate; I] is a new, centrally acting skeletal muscle relaxant recently discussed by Matthews *et al.* (1).



I

This report describes the preparation of labeled chlorphenesin carbamate by the tritium gas exposure method of Wilzbach (2) and determination of the intramolecular distribution of tritium in the resulting product.

This compound was prepared in a radioactive form to facilitate a study of its metabolism, part of which has recently been reported (3, 4). Exchange labeling by exposure to tritium gas was employed since previous experience (5) in this laboratory, as well as in others, with compounds of comparable structure indicated that incorporation of tritium would be adequate and purification would be feasible.

Advantage was taken of the rather unique pathway for metabolism of chlorphenesin carbamate in the rat (3) (Scheme I) to determine the complete intramolecular distribution of tritium in

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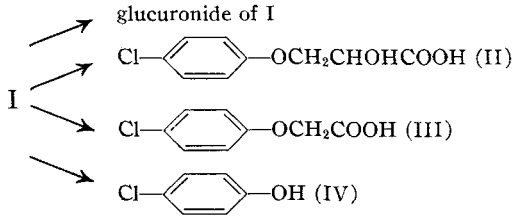
Previous paper: Thomas, R. C., and Ikeda, G. J., *J. Pharm. Sci.*, **55**, 112(1966).
[1] Trademarked as Maolate by The Upjohn Co., Kalamazoo, Mich.

this compound. The location of tritium on the side chain was determined by measuring the specific activities of the metabolites isolated from urine. Thus, the differences in molar specific activities of I and II, II and III, and III and IV represent tritium incorporation on the number one carbon atom (and amide nitrogen), the number two carbon atom, and the number three carbon atom, respectively. Bromination of one of the metabolites, *p*-chlorophenol (IV), was employed to locate tritium on the aromatic ring. The specific activity of the bromination product, 2,6-dibromo-4-chlorophenol (V), represents tritium incorporation *ortho* to the chlorine substituent, whereas the difference in specific activities of IV and V represents tritium incorporation *ortho* to the alkoxy side chain.



Major Metabolites of Chlorphenesin Carbamate (I) in the Rat (3)

Scheme I

EXPERIMENTAL

Radioactivity Measurements—All counting was performed with a Tri-Carb[2] model 314X or 314EX2A liquid scintillation spectrometer at −8° under conditions suitable for measuring tritium. Appropriate aliquots of samples were dissolved in 15 ml. of diotol scintillator (6) [toluene–dioxane–methanol (350:350:210 by volume) containing 73 Gm. of naphthalene, 4.6 Gm. of 2,5-diphenyloxazole, and 0.08 Gm. of 1,4-bis-2-(5-phenyloxazolyl)-benzene per L.]. The absolute counting efficiency for each sample was determined by recounting following addition of an internal standard of tritium-labeled toluene and results then expressed as microcuries (μc.) or disintegrations per minute (d.p.m.). Paper chromatograms were scanned for radioactivity with a Forro[3] 2-π or 4-π radiochromatogram scanner.

Paper Chromatography—Chromatograms were developed by the descending method using Whatman No. 2 paper in the following systems: (*a*) 1-butanol–water (4:1 by volume); (*b*) 1-butanol–piperidine–water (81:2:17 by volume); (*c*) Bush B-3 (7), benzene–Skellysolve C–methanol–water (333:667:800:200 by volume); (*d*) Mattox BFW (8), paper impregnated with formamide–methanol (1:1 by volume), mobile phase butyl acetate–formamide–water (100:5:5 by volume); and (*e*) Zaffaroni type paper (9), impregnated with formamide–methanol (1:1 by volume), mobile phase benzene–chloroform (1:1 by volume) saturated with formamide.

[2] Packard Instrument Co., Downers Grove, Ill.
[3] Forro Scientific Co., Evanston, Ill.

Exposure to Tritium and Purification of Chlorphenesin Carbamate—A 2-Gm. sample of finely pulverized chlorphenesin carbamate was exposed to 4 c. of carrier-free tritium gas at room temperature in the dark for 32 days under approximately 0.2 Atm. tritium pressure. Following removal of the tritium gas, the crude sample was dissolved in ethanol and the resulting solution was evaporated to dryness *in vacuo*. Labile tritium was completely removed by repeating this procedure twice. The specific activity of the product at this stage was 76 μc./mg. This material was recrystallized twice from ethanol with the aid of charcoal to yield 1.55 Gm. of product having a specific activity of 27.3 μc./mg. Paper chromatography in the butanol–water, the butanol–piperidine–water, and the Bush B-3 systems, followed by scanning for radioactivity, showed 70% of the radioactivity apparently associated with chlorphenesin carbamate and the remaining 30% associated with an impurity less polar than the desired product. The sample was dissolved in methylene chloride and the solution was applied to a 2.8 × 122 cm. adsorbent magnesium silicate[4] column. The column was developed by the gradient technique using 2 L. of 20% acetone in Skellysolve B and 2 L. of 75% acetone in Skellysolve B while collecting 100-ml. fractions. The product emerged at approximately 45% acetone in Skellysolve B and comprised three major weight fractions. Each of these fractions revealed a single radioactive zone corresponding to chlorphenesin carbamate when subjected to paper chromatography as previously described. However, the three fractions had specific activities of approximately 9.5, 10.2, and 22.9 μc./mg., respectively, indicating the presence of a high specific activity impurity not resolved from the desired product by the three paper chromatography systems employed. This was confirmed in that paper chromatography in the Mattox BFW and the Zaffaroni systems revealed two radioactive zones, one of which corresponded to chlorphenesin carbamate, in the cases of the two fractions having the higher specific activities. The fraction having a specific activity of 9.5 μc./mg. revealed a single radioactive zone corresponding to the desired product. This fraction was diluted with an equal weight (0.590 Gm.) of nonradioactive chlorphenesin carbamate and recrystallized from ethanol–water to yield 1.00 Gm. of product having a specific activity of 4.5 μc./mg. and a melting point of 89–89.5° (capillary, uncorrected; authentic chlorphenesin carbamate had the same melting point). The ultraviolet and infrared spectra of the product corresponded to those of authentic chlorphenesin carbamate. The material showed a single radioactive zone corresponding to the mobility of the desired product in each of the five previously mentioned paper chromatography systems.

Anal.—Calcd. for $C_{10}H_{12}ClNO_4$: C, 48.89; H, 4.93; Cl, 14.43; N, 5.70. Found: C, 48.53; H, 5.17; Cl, 14.57; N, 5.72.

Biological Degradation of Tritium-Labeled Chlorphenesin Carbamate—The isolation and characterization of the major urinary metabolites of tritium-labeled chlorphenesin carbamate in rats have been described (3). The specific activities of the

[4] Marketed as Florisil by the Floridin Co., Tallahassee, Fla.

isolated and purified chlorphenesin carbamate (I), *p*-chlorophenoxylactic acid (II), and *p*-chlorophenoxyacetic acid (III), recovered from the urine of rats which had received an oral dose (200 mg./Kg.) of the labeled chlorphenesin carbamate, were determined, and are listed in Table I.

The specific activity of I, recovered from the urine following hydrolysis of the glucuronide conjugate, was 5.8×10^7 d.p.m./m*M*. This was somewhat less than the value of 5.9×10^7 d.p.m./m*M* for the administered drug. The difference probably reflects a slight loss of tritium label *in vivo* (3).

The specific activity of the volatile metabolite, *p*-chlorophenol (IV), was estimated to be 5×10^7 d.p.m./m*M* on the basis of spectrophotometric determination of concentration in a chloroform solution. For an accurate determination of its specific activity, a nonvolatile derivative was prepared that could be further purified and would not lose exchangeable tritium during its formation. Thus, a portion of radioactive IV was condensed (as described in detail for its chemical degradation below) with chloroacetic acid under basic conditions to yield a small amount of crude III. The derivative was purified by recrystallization from methylene chloride to yield 1 mg. of III. This material was further purified by thin-layer chromatography on silica gel GF[5] using the system chloroform–methanol–formic acid–water (1000:100:4:96 by volume). The radioactive zone comprising III was eluted with 0.1 *N* HCl and the acid solution was extracted four times with chloroform. The concentration of III in the extract was determined by measuring its absorption at 227 mμ using the absorptivity for standard III at this wavelength (a = 62.76). The radioactivity was determined on duplicate aliquots of the extract and the specific activity of III then calculated. The value of 5.5×10^7 d.p.m./m*M* for the specific activity of IV determined in this manner is reported in Table I.

Chemical Degradation of p-Chlorophenol—A portion of the chloroform solution of radioactive IV, containing 7.1 mg. and 3.0×10^6 d.p.m. isolated from the urine of rats in the study described above (3), was mixed with 180 mg. of nonradioactive IV[6] carrier, and the chloroform solution was then extracted twice with 2-ml. portions of 1 *N* NaOH solution, and finally with 10 ml. of water. The combined basic extracts and washing were acidified with dilute H_2SO_4 and the solution was subjected to steam distillation. The distillate, containing the purified, diluted IV, was acidified with dilute H_2SO_4 and extracted five times with chloroform. The organic phase was washed with water to yield a chloroform solution of radioactive, diluted IV containing 2.74×10^6 d.p.m.

As a check on the reliability of the dilution and distillation procedures, the concentration of dilute IV in the chloroform extract was calculated from a measurement of its absorption at 283 mμ using the absorptivity of authentic IV at this wavelength (a = 14.16). The solution was found to contain 160 mg. of diluted IV, thus giving a calculated specific activity of 2.2×10^6 d.p.m./m*M*. For an accurate determination of its specific activity, however, one-half of the chloroform solution containing 80 mg. (0.62 m*M*) and 1.36×10^6 d.p.m. of tritium-labeled diluted IV was added to 0.3 ml. of 5 *N* NaOH (1.5 m*M*) and the chloroform was removed *in vacuo*. Chloroacetic acid (79 mg., 0.75 m*M*) and 0.8 ml. of water were then added to the mixture which was heated under reflux for 6 hr. The cooled reaction mixture was then diluted with 10 ml. of water, acidified with dilute H_2SO_4, and extracted four times with ethyl ether. The ether solution was in turn extracted with 10 ml. of 0.2 *M* $NaHCO_3$ followed by water. The combined basic extract and washing were acidified with dilute H_2SO_4 and then extracted four times with ethyl ether. The combined ether extracts were washed with water and then evaporated to dryness *in vacuo* and the crude diluted III was recrystallized twice from methylene chloride to yield 54 mg. of crystals, m. p. 156–157° [capillary, uncorrected; lit. (3) value 157–158°]. The infrared spectrum of the purified material was identical with that of authentic III. The specific activity of diluted III (and therefore of diluted IV) was 2.13×10^6 d.p.m./m*M*, a value in excellent agreement with that (2.08×10^6 d.p.m./m*M*) calculated based on dilution of IV with the carrier.

Anal.—Calcd. for $C_8H_7ClO_3$: C, 51.49; H, 3.78; Cl, 19.00. Found: C, 51.54; H, 4.02; Cl, 19.50.

The second half of the chloroform solution of tritium-labeled diluted IV, containing 1.36×10^6 d.p.m., was extracted with 2 ml. of 1 *N* NaOH and then washed twice with 2-ml. portions of water. The combined basic extract and washings were adjusted to pH 7 with 1 *N* HCl and approximately 2 ml. of a bromine solution (800 mg. KBr dissolved in 5 ml. of water and containing 0.16 ml. of bromine) was added dropwise with stirring until a yellow color persisted (10). The white precipitate was removed by filtration, washed with water, and then recrystallized twice from methanol–water with the aid of charcoal to yield 74 mg. of crystals having a melting point of 89–90° [capillary, uncorrected; lit. (10) value 90°]. The infrared spectrum of the product was identical with that of authentic 2,6-dibromo-4-chlorophenol (V). Previous NMR studies carried out on nonradioactive V, similarly pre-

TABLE I—SPECIFIC ACTIVITIES OF PURIFIED URINARY METABOLITES AND THEIR DERIVATIVES FROM RATS FOLLOWING ORAL ADMINISTRATION OF TRITIUM-LABELED CHLORPHENESIN CARBAMATE[a]

Compd.	Specific Activity d.p.m. × 10^7/m*M*	% of Total Radioactivity
Chlorphenesin carbamate (I)[b]	5.8	100
p-Chlorophenoxylactic acid (II)	5.5	95
p-Chlorophenoxyacetic acid (III)	5.5	95
p-Chlorophenol (IV)[c]	5.5	95
2,6-Dibromo-4-chlorophenol (V)[d]	3.6	62

[a] Male Wistar rats received an oral dose of tritium-labeled chlorphenesin carbamate (200 mg./Kg.) with a specific activity of 5.9×10^7 d.p.m./m*M*. [b] Excreted as the glucuronide conjugate. [c] Specific activity determined as *p*-chlorophenoxyacetic acid prepared by condensation of the metabolite IV with chloroacetic acid. [d] The specific activity of V has been corrected for the dilution of IV with nonradioactive carrier during the preparation of V.

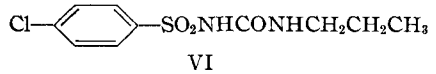
[5] Brinkmann Instrument Co., Great Neck, N. Y.
[6] Distillation Products, Inc., Rochester, N. Y.

pared, confirmed the structural assignment.[7] The specific activity of the purified, diluted V was 1.41 × 10^6 d.p.m./m*M*. For purposes of comparison, the specific activity of diluted V has been corrected for dilution of IV with nonradioactive carrier and the value of 3.6 × 10^7 d.p.m./m*M* is reported in Table I.

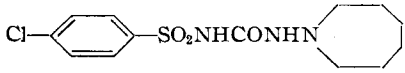
Anal.—Calcd. for $C_6H_3Br_2ClO$: C, 25.17; H, 1.05. Found: C, 25.52; H, 1.12.

RESULTS AND DISCUSSION

Incorporation of tritium into chlorphenesin carbamate was satisfactory, giving a specific activity of 9.0 μc./mg. (corrected for dilution with nonradioactive carrier). The incorporation of stably bound tritium was thus 141 μc./curie-day exposure, based on the weight of material exposed, the amount of tritium used, the length of the exposure, and the specific activity of the purified product. This falls within the range obtained (67–165 μc./curie-day exposure) for a series of seven sulfonylurea hypoglycemic agents (exposed as sodium salts), having structures somewhat similar to chlorphenesin carbamate (5). Two of the most closely related sulfonylureas, chlorpropamide (VI) and glypinamide (VII),



VI



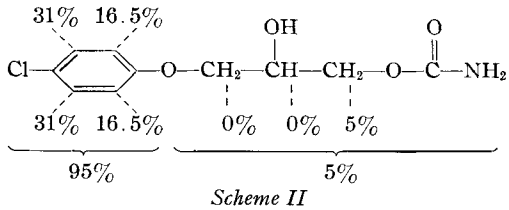
VII

incorporated 161 and 137 μc. of tritium per curie-day exposure, respectively.

The radiochemical purity of compounds labeled by exposure to tritium gas must be carefully determined, as first pointed out by Wilzbach (2). During the exposure, impurities having extremely high specific activities often are formed in trace amounts by weight. Although these high specific activity impurities have little effect on the chemical purity of the product, they can drastically reduce its radiochemical purity. In the present work, three paper chromatography systems showed the presence of one impurity in the tritiated chlorphenesin carbamate prior to column chromatography and no impurities in the major weight fractions eluted from the column. However, the specific activities of the column fractions varied indicating the presence of a second high specific activity impurity, resolved from chlorphenesin carbamate by the column, but not by the three paper chromatography systems. The presence of this impurity was then confirmed with two additional paper chromatography systems.

The data in Table I show the distribution of stably bound tritium in chlorphenesin carbamate exposed to tritium gas to be as shown in Scheme II. The side chain thus contains only 5% of the total molecular radioactivity. This must represent incorporation of tritium on the number one carbon or the amide nitrogen since this amount of tritium was lost upon oxidation of chlorphenesin carbamate to *p*-chlorophenoxylactic acid. Nuclear magnetic

[7] Determined in $CDCl_3$ with a Varian model A-60 instrument using tetramethylsilane internal standard by Dr. G. Slomp and his associates.



Scheme II

resonance studies (11), however, have demonstrated that the amide hydrogens of chlorphenesin carbamate are readily exchangeable with solvent, so tritium in this position would have been lost during purification of the labeled material. Consequently, the side chain tritium label must reside at the number one carbon atom.

The preferential incorporation of tritium into aromatic positions is in agreement with the results of Wilzbach (2) and Ache *et al.* (12) for toluene, Cacace *et al.* (13) for anisole, Meshi and Sato (14) for benzoylvaline, Krizek *et al.* (15) for diethyl benzylacetamidomalonate, and Golder *et al.* (16) for 2,6-di-*tert*-butyl-4-methylphenol. In the latter case, however, there was not a great difference between incorporation of tritium in aromatic positions and in the 4-methyl group. This may be due to shielding of the aromatic positions by the *tert*-butyl groups.

The aromatic ring, accounting for 95% of the molecular radioactivity of chlorphenesin carbamate, contained nearly twice as much tritium *ortho* to the chlorine substituent as *ortho* to the alkoxy side chain.

The distribution of tritium in several other disubstituted benzene derivatives, tritiated by the gas-exposure method, has been reported. Anthranilic acid (17) preferentially incorporates tritium *ortho* to the amino group, whereas salicylic acid (14) preferentially incorporates tritium *ortho* and *meta* to its hydroxyl group; 39% *ortho* and 48% in one or both of the two *meta* positions.

Previous studies with monosubstituted benzene derivatives, tritiated by exposure to tritium gas, have shown that tritium is preferentially incorporated in *ortho* and *para* positions regardless of whether the substituent is nucleophilic or electrophilic. Thus, nitrobenzene (13), chlorobenzene (13), toluene (2, 12), and benzoic acid (14) favor *ortho* incorporation, and anisole (13) favors *para* incorporation. Furthermore, Cacace *et al.* (13) have shown that total incorporation of tritium in the aromatic ring also is nearly independent of the chemical nature of the substituent present; nitrobenzene, chlorobenzene, and anisole, exposed to tritium under identical conditions, attain similar specific activities.

These results suggest that a homolytic, rather than a heterolytic, mechanism is involved in the incorporation of tritium into aromatic positions. Garnett *et al.* (17) proposed such a mechanism to explain the distribution of tritium in anthranilic acid. Ache *et al.* (12) suggested a relationship between incorporation of tritium in aromatic positions and homolytic aromatic substitution. As shown in Table II, a good correlation indeed exists, except for the *meta*/*para* substitution ratio in the case of nitrobenzene.

Pratt and Wolfgang (18) and Wexler (19) have systematically studied the exchange of tritium gas

TABLE II—COMPARISON OF TRITIATION[a] AND HOMOLYTIC AROMATIC SUBSTITUTION[b]

Reaction	% *ortho* Substitution				
	$PhCH_3$	PhCl	$PhOCH_3$	$PhNO_2$	PhCOOH
Tritiation	54, 56	50	40	58	58
Phenylation	71	62	67	62.5	...
Methylation	56.5	64	74	65.5	...
	meta/para Substitution Ratio				
Tritiation	1.92, 1.34	2.10	1.22	3.44	1.04
Phenylation	1.32	1.71	1.20	0.36	...
Methylation	1.56	2.27	1.36	0.21	...

[a] From *References 2, 12, 13, 14.* [b] Fieser, L. F., and Fieser, M., "Advanced Organic Chemistry," Reinhold Publishing Corp., New York, N. Y., 1961, p. 638.

with methane in the gaseous phase. They concluded that methane incorporates tritium by several mechanisms, all involving heterolytic reactions of positively charged transient species. It would be difficult to apply these mechanisms to more complex systems, particularly to a condensed phase system such as chlorphenesin carbamate.[8] However, it seems unlikely that an electrophilic reaction mechanism could explain the distribution of tritium on the aromatic ring of chlorphenesin carbamate or the other aromatic compounds discussed above.

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[8] It is interesting to note that the distribution of tritium in chlorobenzene is essentially the same whether it is tritiated in the gaseous phase or in the presence of excess liquid chlorobenzene (13).

Drug Standards

Outline of Details for Official Microbiological Assays of Antibiotics

By AMIEL KIRSHBAUM and BERNARD ARRET

Tables are presented listing pertinent data required for 57 different microbiological assays, including those for all antibiotics approved for human use in the United States.

In 1959, the authors published an "outline" for assaying antibiotics (1). Development of new methods, modifications and improvements of old ones, and development of methods for antibiotics which have since been discovered necessitate a revision and updating of that publication. As in the earlier article, pertinent information has been tabulated as a ready guide for the analyst performing microbiological assays of antibiotics. Complete details for the assays are not given since these tabulations are prepared for those already familiar with the basic methods. Procedural information can be found in the Code of Federal Regulations (2).

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PREPARATION OF MICROBIAL SUSPENSIONS

Since the earlier publication, a uniform procedure for preparation of microbial suspensions has been