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THE METABOLISM OF PIPERONYL BUTOXIDE IN THE RAT WITH ^{14}C IN THE METHYLENEDIOXY OR α-METHYLENE GROUP

L. FISHBEIN, H. L. FALK, J. FAWKES, S. JORDAN AND B. CORBETT
National Institute of Environmental Health Sciences, Research Triangle Park, N.C. (U.S.A.)
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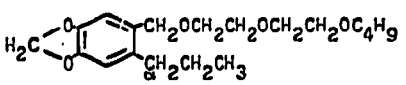
SUMMARY

The metabolism of methylenedioxy-^{14}C- and α-methylene-^{14}C-piperonyl butoxide in the rat was elaborated using both thin-layer and radioautographic techniques. Following single intravenous injection of the methylenedioxy-^{14}C isotope, 13 metabolites were detected in the bile and 11 in the urine, while the analogous administration of the α-methylene-^{14}C isotope resulted in 24 metabolites in the bile and 26 in the urine.

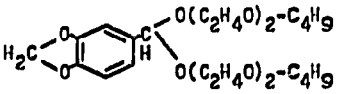
Co-chromatographic procedures have suggested the similarity (in R_F values) of a total of 14 biliary and 14 urinary metabolites arising from both isotopes. In addition, significant radioactivity from each isotope was detected in the lung and fat and identified as unchanged piperonyl butoxide in both cases. The rates of biliary elimination of metabolites from both isotopes indicated *initially* high levels of excretion followed by a *prolonged* period of elimination. The significance of this in addition to the tissue residues is discussed.

INTRODUCTION

In an earlier paper[1] we reported on the elimination of the methylenedioxyphenyl pesticide synergists piperonyl butoxide (I) and tropital (II) and their metabolites in rat bile and urine, resulting from single intravenous administration of the two compounds.



(I)



(II)

The purpose of this investigation was to explore further by thin-layer and radioautographic techniques the nature of the metabolites and radiocarbon distribution pattern in the animal of piperonyl butoxide labeled in the methylenedioxy-^{14}C* or α-methylene-^{14}C* side chain, in separate, but identical, experiments.

* Methylenedioxy-^{14}C and α-methylene-^{14}C piperonyl butoxide were obtained from New England Nuclear Corp., Boston, Mass., U.S.A., in specific activities of 26.0 and 27.0 mC/m*M*, respectively.

EXPERIMENTAL

Preparation of the plates

Thin-layer plates. Silica Gel GF (Analtech)* (250 mm, 8×8 in.) plates were washed by ascending chromatography with chloroform–methanol (1:1), then activated at 110° for 1h.

Preparative plates. Chromaflex TLC gradient plates (Kontes)** (a "wedge"-type channel plate whose ground channel decreases in depth from 1000 to 125 μ; outside dimensions are 200 × 200 mm) were prepared with Silica Gel PF_{254} (Brinkmann). The plates were washed with chloroform–methanol (1:1), then activated at 110° for 2 h.

Solvent systems

(A) Toluene–acetic acid–water (10:10:1).
(B) Ethyl acetate–acetic acid–methanol (70:10:20).
(C) *n*-Butanol–acetic acid–water (10:1:1).
(D) Benzene–acetone (39:1).

Chromogenic reagents

(1) Chromotropic acid[2].
(2) Ferric chloride–potassium ferricyanide reagent[3].

Bile and urine sampling

Single intravenous injections of both the ^{14}C-labeled piperonyl butoxide samples were given to adult male rats of the Sprague-Dawley strain averaging 300 g in weight. Bile samples were collected by fistula and urine samples by cannulation from each rat. Details on the handling of the animals, anesthesia, surgery and sample collection have been previously described[4]. Ten urine samples were collected from each administered isotope, one before i.v. injection, eight at appropriate intervals after injection and the last at the termination of the bile collection. All samples were frozen until the time of analysis.

Preparation of tissue homogenates

Tissues were homogenized in distilled water with a tri-R teflon tissue homogenizer, the final volume being adjusted to contain 100 mg solids/ml of homogenate. Radioactivity was measured in aliquots of the homogenate which had been added to hydroxide of Hyamine-10X (Packard Instrument***). The homogenate–hyamine solution was added to a dioxane scintillation solution***, then counted in a Packard Tri-Carb Model 3375. An internal standard was added to the sample (to determine the degree of quenching).

Purification of piperonyl butoxide

Non-radioactive. Technical grade piperonyl butoxide§ was purified by prepara-

* Obtained from Analtech, Inc., Wilmington, Del., U.S.A.
** Kontes Glass Co., Vineland, N.J., U.S.A.
*** Packard Instrument Corp., Darner's Grove, Ill., U.S.A.
§ Obtained from McLaughlin, Gormley and King Co., Minneapolis, Minn., U.S.A.

tive plate chromatography. A 20 % solution of piperonyl butoxide in benzene was applied with a Radin-Pelids streaker*, in amounts of approximately 75 mg per plate, to a gradient plate prepared with Silica Gel PF_{254}, then developed with benzene–acetone (39:1). After development, the impurities were scraped from the plate, the plate developed with methanol in a 90° dimension to flush the compound to one end of the plate where it was then scraped onto an elution column. The column was a 6-in. disposable pipette containing a plug of glass wool, then ¼ in. of Adsorbosil, CAB 1420, 140/200 mesh* and finally the silica gel scrapings from the above two-dimensional chromatogram of piperonyl butoxide. The column was eluted with methanol, and the eluate concentrated under nitrogen. (About 2–2.5 ml of eluate was obtained per preparative plate.)

Radioactive samples. Between 1900–2000 μC of the methylenedioxy-^{14}C isotope were mixed with purified non-radioactive piperonyl butoxide (1:25) and chromatographed on Silica Gel PF_{254} plates and developed with benzene–acetone (39:1) as above. The chromatographed sample was checked for purity by preparing an autoradiogram of a 5 μl re-chromatographed aliquot.

α-Methylene-^{14}C-piperonyl butoxide (7,775–8,000 μC) was purified by preparative chromatography as above. A two-fold purification followed by dilution with non-radioactive piperonyl butoxide (1:20) yielded a product of analogous isotope activity to that of the methylenedioxy-^{14}C-piperonyl butoxide.

Carbon dioxide analysis

Expired air from rats injected with labeled piperonyl butoxide was bubbled through two traps of 10 ml each of hydroxide of Hyamine 10-X**. Both traps were replaced with fresh solutions approximately every half hour. An aliquot of the solution was subsequently removed from each trap and counted (radioactivity was found primarily in the first trap with a slight amount carried over to the second trap).

Chromatography

Ten to twenty microliters of all bile and urine samples were applied on Silica Gel GF (Analtech) plates. The bile samples were developed two-dimensionally with toluene–acetic acid–water (10:10:1) and with *n*-butanol–acetic acid–water (10:1:1) for the 90° development. The urine samples were developed two-dimensionally with ethyl acetate–acetic acid–methanol (70:10:20) and with *n*-butanol–acetic acid–water (10:1:1) for the 90° development. Seven-day autoradiograms were then made of each plate and finally each plate was sprayed with chromotropic acid reagent, then heated at 120° for 5–10 min to develop the color and photographed using equipment and procedures previously described[1]. Separately developed plates were also sprayed with the ferric chloride–potassium ferricyanide reagent and the developed colors recorded (the reagent is positive for phenols).

Preparation of autoradiograms

Seven-day autoradiograms were prepared by sandwiching 6.5 × 8.5 Kodak Medical X-ray (No-screen) Safety film between the chromatoplates and a glass plate, then taping the two plates together. The films were developed with Kodak X-ray

* Obtained from Applied Science, Inc., State College, Pa., U.S.A.
** Obtained from Packard Instrument Co., Darner's Grove, Ill., U.S.A.

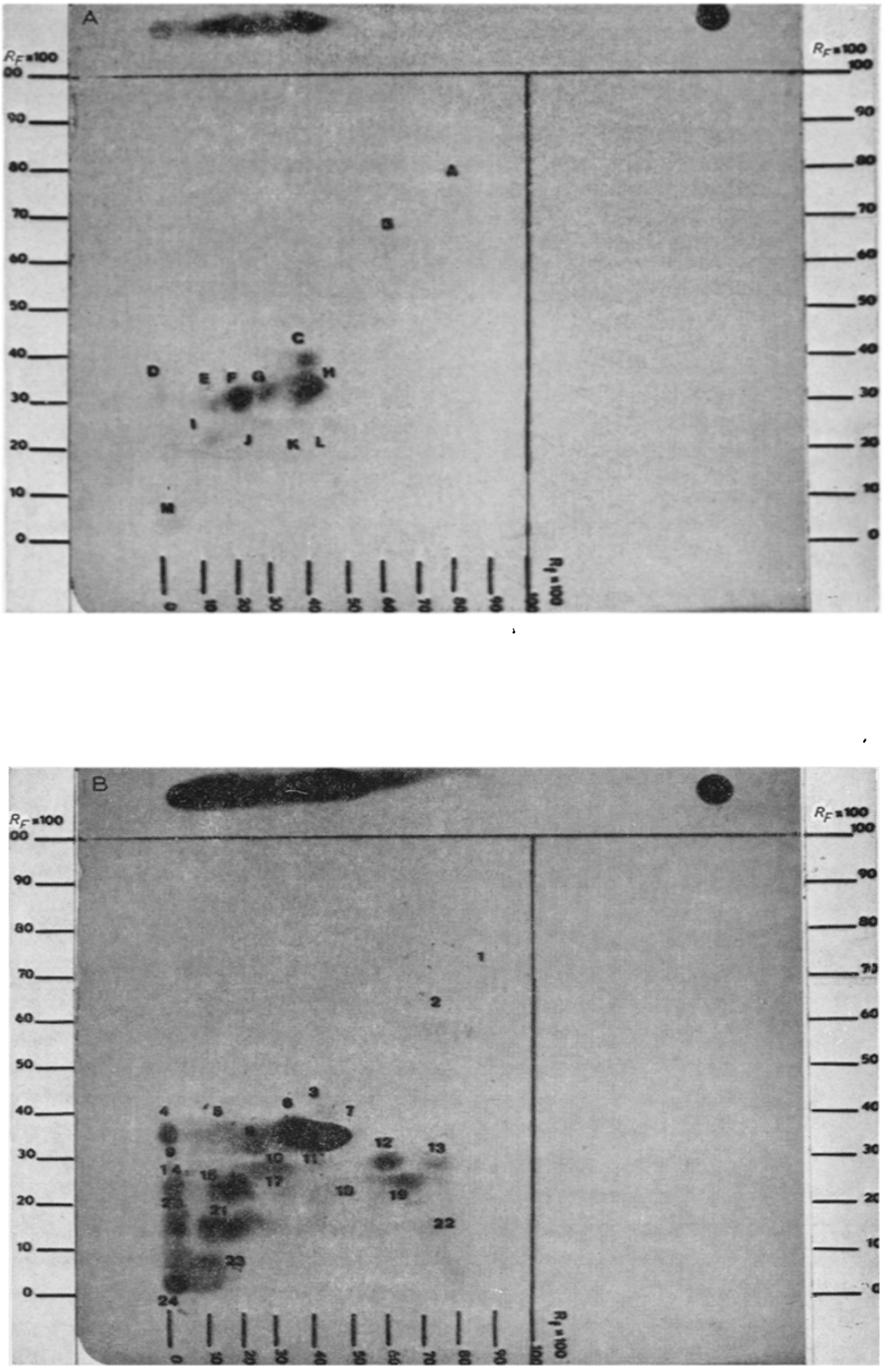


Fig. 1. (A) 168 h two-dimensional autoradiogram of rat bile (10 μl) after i.v. administration of methylenedioxy-^{14}C-piperonyl butoxide (50 μl, 183 μC). Plate developed (150 mm) first with toluene–acetic acid–water (10:10:1), then at 90° for 100 mm with *n*-butanol–acetic acid–water (10:1:1). (B) 168 h two-dimensional autoradiogram of rat bile (10 μl) after i.v. administration of α-methylene-^{14}C-piperonyl butoxide (50 μl, 167 μC). Plate developed (150 mm) first with toluene–acetic acid–water (10:10:1), then at 90° for 100 mm with *n*-butanol–acetic acid–water (10:1:1).

TABLE I

SUMMARY OF RAT BILIARY R_F[a] × 100 DIFFERENCES ON TWO-DIMENSIONAL CHROMATOGRAMS FOLLOWING SINGLE INTRAVENOUS ADMINISTRATION OF METHYLENEDIOXY-^{14}C- AND α-METHYLENE-^{14}C-PIPERONYL BUTOXIDE

Methylenedioxy-^{14}C bile					*α-Methylene-^{14}C bile*								
Metabolite designation	*R_F × 100*	*C.p.m.*[b]	*Detector and color*		*Metabolite designation*	*R_F × 100*	*C.p.m.*[c]	*Detector and color*		*Metabolite designation*	*R_F × 100*	*C.p.m.*[b]	*Detector and color*	
			Chromotropic acid	*Ferric chloride*				*Chromotropic acid*	*Ferric chloride*				*Chromotropic acid*	*Ferric chloride*
Radioactive metabolites														
A	68 (85)	140			1	68 (90)	209			13	25 (74)	417		
B	56 (70)	230			2	56 (76)	217			14	22 (0)	1898	Brown	
C	38 (43)	1884	Pink		3	38 (43)	605	Pink		15	22 (12)			
D	32 (0)	135			4	32 (0)	1,363			16	22 (19)	5107		
E	28 (17)	445	Pink		5	32 (5-25)	2,198			17	22 (30)			
F	28 (23)	2877		Blue	6	33 (35)	13,747	Pink		18	22 (49)	346	Brown	
G	28 (30)	1017	Pink		7	32 (42)	29,727	Pink		19	22 (65)	872		
H	32 (40)	2206	Pink		8	28 (23)	1,472		Blue	20	15 (0)	4282	Brown	Blue
I	17 (13)				9	28 (0)	263		Blue	21	15 (10)	6235		
J	22 (18)	1180			10	25 (28)	1,441			22	9 (79)	302		
K	22 (35)				11	25 (38)				23	7 (10)	3750		
L	22 (48)				12	25 (60)	1,010	Pink		24	0 (0)	2782	Pink	Blue
M	0 (0)	353	Pink	Blue										
Non-Radioactive metabolites														
	90 (90)		Brown			90 (89)		Brown						
	82 (88)		Brown			82 (86)		Brown						
	25 (60)		Pink											
	22 (46)		Brown											
	16 (32)		Green-brown			16 (32)		Green-brown						

[a] Adsorbent: Silica Gel GF. Developing solvents: first development: toluene–acetic acid–water (10:10:1) (unbracketed R_F values); 90° development: *n*-butanol–acetic acid–water (10:1:1) (bracketed R_F values).

[b] Counts taken from sample 92 min after i.v. administration of methylenedioxy-^{14}C-piperonyl butoxide.

[c] Counts taken from sample 112 min after i.v. administration of α-methylene-^{14}C-piperonyl butoxide.

developer and fixed with regular Kodak fixer using the manufacturer's recommended specifications.

RESULTS AND DISCUSSION

Bile metabolites

Following the single i.v. administration to the rat of methylenedioxy-^{14}C- and α-methylene-^{14}C-piperonyl butoxide, 13 and 24 radioactive biliary metabolites were detected by thin-layer chromatography and autoradiography (Figs. 1A and 1B, respectively). Table I depicts the summary of rat biliary metabolites in terms of their R_F values, their respective c.p.m. and detection with both chromotropic and ferric chloride–potassium ferricyanide reagents following two-dimensional chromatography. Unbracketed R_F values are those obtained with the first development and bracketed values obtained by the latter 90° development. Also shown in this table are R_F values and spot colors with chromotropic acid for *non*-radioactive biliary metabolites (a total of eight for both experiments also found with the above two-dimensional TLC).

A consideration of the isotopic material used suggests that most of the biliary metabolites resulting from the methylenedioxy-^{14}C isotope should also appear in the bile following administration of α-methylene-^{14}C-piperonyl butoxide as long as the propyl side chain remains intact.

To ascertain which of the isotopic metabolites were similar on both autoradiograms, two techniques were used, *viz.* (1) 10 μl of each isotopic bile sample were spotted on separate plates and each plate developed in two dimensions simultaneously in the same tank, and (2) 3 μl of the α-methylene-^{14}C isotopic bile was spotted separately on three TLC plates. Methylenedioxy-^{14}C biliary samples (3, 6 and 9 μl, respectively) were superimposed on the initial 3 μl α-methylene-^{14}C biliary spot and developed in two dimensions as above. Under these conditions, the following metabolites appeared identical (according to their R_F values): A = 1; B = 2; C = 3; D = 4; F = 8; M = 24 and H = 7.

It is interesting to note that all radioactive compounds in the methylenedioxy-^{14}C bile were detected with the chromotropic acid reagent when the metabolite concentration was equal or greater than 353 c.p.m. The only exception was metabolite F, which possessed a c.p.m. of 2,877. In the case of α-methylene-^{14}C metabolites, metabolites 12 and 18 also reacted positively with chromotropic acid reagent, but both appear as non-radioactive compounds in the methylenedioxy-^{14}C bile samples. Compounds at $R_F \times 100$, *e.g.* 90 (90), 82 (88) and 16 (32) also gave a positive chromotropic acid reaction but appeared as non-radioactive metabolites in both isotopic bile samples. These observations are difficult to interpret since chromotropic acid reagent reacts positively with the methylenedioxy moiety via intermediate formaldehyde formation thence chromogenic reaction with dihydroxy-2,7-naphthalenesulfonic acid. The possibility of two compounds present in one spot cannot be excluded. Table I also reveals a number of phenolic metabolites with the ferric chloride–potassium ferricyanide reagent, *e.g.* metabolites F and M and metabolites 8, 9, 20 and 24 found in the methylenedioxy-^{14}C and α-methylene-^{14}C bile samples, respectively.

Following i.v. administration of both isotopic piperonyl butoxide samples, bile samples were collected each minute for the first 5 min, then every 2 min for the next

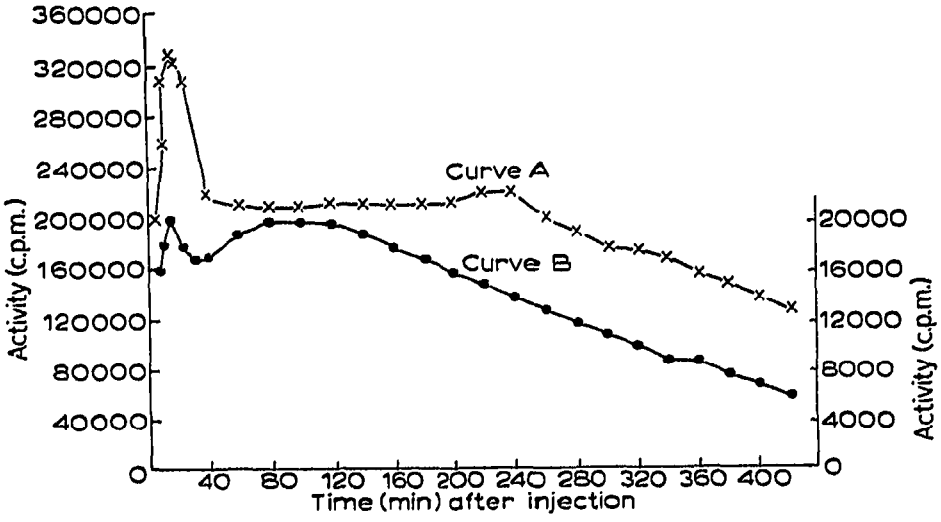


Fig. 2. Relationship of the total radioactivity (c.p.m.) with time of all the biliary metabolites following i.v. administration of α-methylene-^{14}C-piperonyl butoxide (curve A, scale 0–360 × 10^3 c.p.m.) and methylenedioxy-^{14}C-piperonyl butoxide (curve B, scale 0–20 × 10^3 c.p.m.).

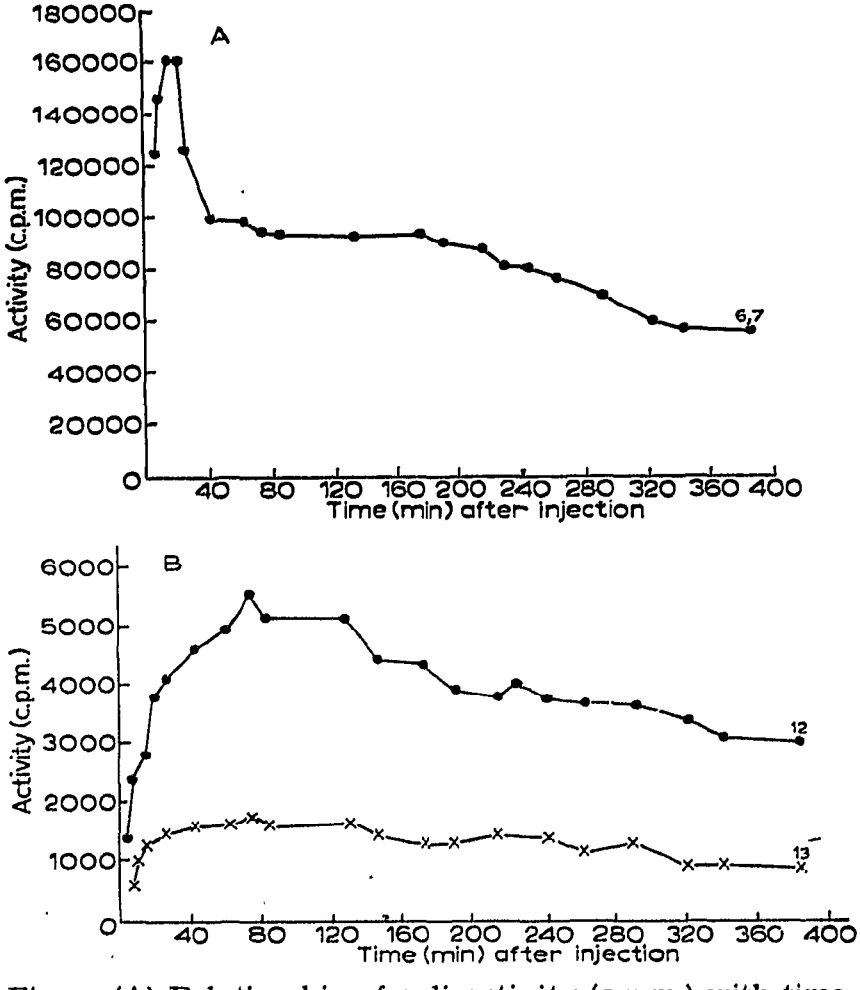


Fig. 3. (A) Relationship of radioactivity (c.p.m.) with time of the combined major biliary metabolites (numbers 6 and 7) following i.v. administration of α-methylene-^{14}C-piperonyl butoxide. (B) Relationship of radioactivity (c.p.m.) with time of two biliary metabolites of intermediary activity (numbers 12 and 13).

TABLE II

RADIOACTIVITY OF MAJOR RAT BILIARY METABOLITES FOLLOWING INTRAVENOUS ADMINISTRATION OF α-METHYLENE-^{14}C-PIPERONYL BUTOXIDE

Metabolite No.	*Sample collection time (min)*										
	6	*8*	*14*	*19*	*24*	*43*	*63*	*74*	*85*	*132*	*145*
6 and 7	126,215	147,475	162,814	162,213	127,940	100,957	99,315	94,838	94,720	93,998	81,282
12	1,376	2,437	2,789	3,822	4,133	4,628	4,952	5,551	5,167	5,128	4,450
13	583	600	1,153	2,247	1,470	1,615	1,609	1,762	1,595	1,659	1,463
(A) Total (c.p.m.) (6, 7, 12, 13)	128,174	150,516	166,756	168,282	133,543	107,200	105,876	102,151	101,482	100,785	87,195
(B) Remaining metabolites (c.p.m.) (all other)	39,206	50,118	82,931	81,729	93,064	57,362	63,501	50,799	50,993	56,884	51,889
All metabolites (c.p.m.)	167,381	200,630	249,687	250,010	226,607	164,562	169,377	152,950	152,475	157,669	139,084
Reference count (c.p.m.)	172,096	197,816	250,865	229,878	245,565	178,790	175,928	155,367	152,043	168,175	148,406
Recovery (%)	97.3	101.4	99.5	109.8	92.3	92.0	96.3	98.4	100.2	93.8	93.0
	174	*190*	*216*	*227*	*244*	*263*	*291*	*321*	*343*	*385*	
6 and 7	93,767	90,507	88,110	82,725	81,988	77,488	70,523	60,307	57,451	56,626	
12	4,370	3,927	3,802	4,052	3,792	3,709	3,688	3,384	3,088	3,033	
13	1,308	1,315	1,448	3,108	1,409	1,132	1,277	916	921	882	
(A) Total (c.p.m.) (6, 7, 12, 13)	99,445	95,747	93,360	87,885	87,184	82,329	75,488	64,607	61,460	60,541	
(B) Remaining metabolites (c.p.m.) (all other)	56,228	69,277	66,363	63,512	67,787	62,568	60,887	56,747	50,842	51,333	
All metabolites (c.p.m.)	155,673	165,026	159,723	151,397	155,052	144,896	136,375	121,354	112,302	111,874	
Reference count (c.p.m.)	160,488	154,623	174,652	165,776	170,414	154,742	141,948	128,053	125,076	107,785	
Recovery (%)	97.0	106.7	91.4	91.3	91.0	93.6	89.1	94.8	89.8	103.8	

30 min, every 5–6 min for the next 3 h and finally every 10 min to the conclusion of the experiment.

Within 4 min after administration, metabolites F and G as well as metabolites 6 and 7 appeared in the bile following administration of both isotopes. Within 13–14 min after injection, all metabolites detected appeared in each bile series.

Fig. 2 depicts the plot of the total activity *versus* time of all the biliary metabolites following i.v. administration of α-methylene-^{14}C- and methylenedioxy-^{14}C-piperonyl butoxide (curves A and B, respectively) and demonstrates the *prolonged* elimination of the metabolites (to 400 min) following initially high levels of excretion. The approximately ten-fold higher biliary elimination of the α-methylene-^{14}C isotope (curve A, 0–360 × 10^3 c.p.m. scale) compared to that of the methylenedioxy-^{14}C isotope (curve B, 0–20 × 10^3 c.p.m. scale) is also illustrative.

Figs. 3A and 3B illustrate the relationship of radioactivity (c.p.m.) with time for the combined major biliary metabolites (6 and 7) and metabolites of much less activity (12 and 13), respectively, following i.v. administration of α-methylene-^{14}C-piperonyl butoxide.

Table II summarizes the total (metabolites 6 and 7, 12 and 13, as well as the combined remaining metabolites) radioactivity of the biliary α-methylene-^{14}C-piperonyl butoxide metabolite recovery from 6.1 to 385.1 min. Metabolites 6 and 7 account for 50 % or more of the total biliary excretion.

Fig. 4 depicts a plot of the activity *versus* time of the major biliary metabolites (C and H, M, F and G) following the i.v. administration of methylenedioxy-^{14}C-piperonyl butoxide. Table III (in an analogous manner to that of Table II above) summarizes the total (metabolites C and H, F, G and M, as well as the combined remaining metabolites) radioactivity of the biliary methylenedioxy-^{14}C recovery from 2.3 to 417.6 min. The above five metabolites account for approximately 90 % or more of the total biliary excretion.

Fig. 4. Relationship of radioactivity (c.p.m.) with time of the major biliary metabolites (C and H, M, F and G) following i.v. administration of methylenedioxy-^{14}C-piperonyl butoxide.

Urine metabolites

Eleven and 26 metabolites have been detected in the urine of the rat following administration of methylenedioxy-^{14}C- and α-methylene-^{14}C-piperonyl butoxide,

TABLE III

RADIOACTIVITY OF MAJOR RAT BILIARY METABOLITES FOLLOWING INTRAVENOUS ADMINISTRATION OF METHYLENEDIOXY-^{14}C-PIPERONYL BUTOXIDE

Metabolism No.	*Sample collection time (min)*								
	2	*4*	*10*	*13*	*17*	*19*	*48*	*67*	*86*
C and H	339	1,794	3,028	3,866	3,955	4,063	3,616	3,758	3,800
F	149	1,474	4,676	5,563	5,694	6,662	5,922	5,386	5,563
G	—	—	1,169	2,493	2,872	2,816	2,090	1,336	1,398
M	250	—	—	557	528	405	517	896	998
(A) Total (c.p.m.) (C, H, F, G, M)	738	3,268	8,873	12,479	12,049	13,946	12,147	11,316	11,759
(B) Remaining metabolite (c.p.m.)	—	—	420	500	956	980	1,618	1,948	2,803
Total metabolite (c.p.m.)	738	3,268	9,293	12,979	13,005	14,146	13,765	13,324	14,562
Reference count (c.p.m.)	2,959	5,512	12,743	15,214	16,795	16,679	15,407	16,280	16,813
Recovery (%)	24.9	59.3	72.9	85.3	77.4	84.8	89.3	81.8	86.6
	97	*118*	*128*	*140*	*178*	*203*	*216*	*228*	*246*
C and H	4,161	4,575	4,537	4,601	3,818	3,606	3,292	3,015	3,020
F	5,676	5,474	5,315	5,111	4,378	3,189	2,960	2,862	2,918
G	1,255	1,225	1,182	1,231	1,169	1,225	1,032	853	736
M	939	955	1,005	1,149	1,299	1,364	1,329	1,367	1,512

(B) Remaining metabolite (c.p.m.)	2,432	3,098	3,008	3,145	2,733	2,648	2,514	2,505	2,010	
Total metabolite (c.p.m.)	14,463	15,327	15,047	15,237	13,392	12,032	11,127	10,262	10,802	
Reference count (c.p.m.)	14,463	16,769	15,881	16,227	15,360	12,874	12,033	11,847	11,419	
Recovery (%)	100.0	91.8	94.7	93.9	87.2	93.4	92.5	90.0	94.6	
	259	*273*	*286*	*317*	*330*	*343*	*358*	*368*	*402*	*418*
C and H	2,756	2,525	2,723	2,160	2,146	1,899	1,748	1,539	1,668	1,223
F	2,700	2,219	2,111	1,574	1,417	1,430	1,351	957	890	787
G	635	760	706	892	763	586	708	466	514	444
M	1,578	1,403	1,340	1,428	1,391	1,250	1,259	1,202	1,187	1,133
(A) Total (c.p.m.) (C, H, F, G, M)	7,669	6,907	6,880	6,054	5,717	5,165	5,066	4,164	4,259	3,587
(B) Remaining metabolite (c.p.m.)	2,063	1,801	1,835	1,848	1,802	1,617	1,632	1,827	1,246	1,276
Total metabolite (c.p.m.)	9,732	8,708	8,715	7,902	7,519	6,782	6,698	5,991	5,505	4,863
Reference count (c.p.m.)	11,263	10,193	9,991	8,300	7,965	7,535	7,336	6,456	5,856	5,464
Recovery (%)	86.3	85.4	87.2	95.2	94.4	90.0	91.3	92.8	94.0	90.0

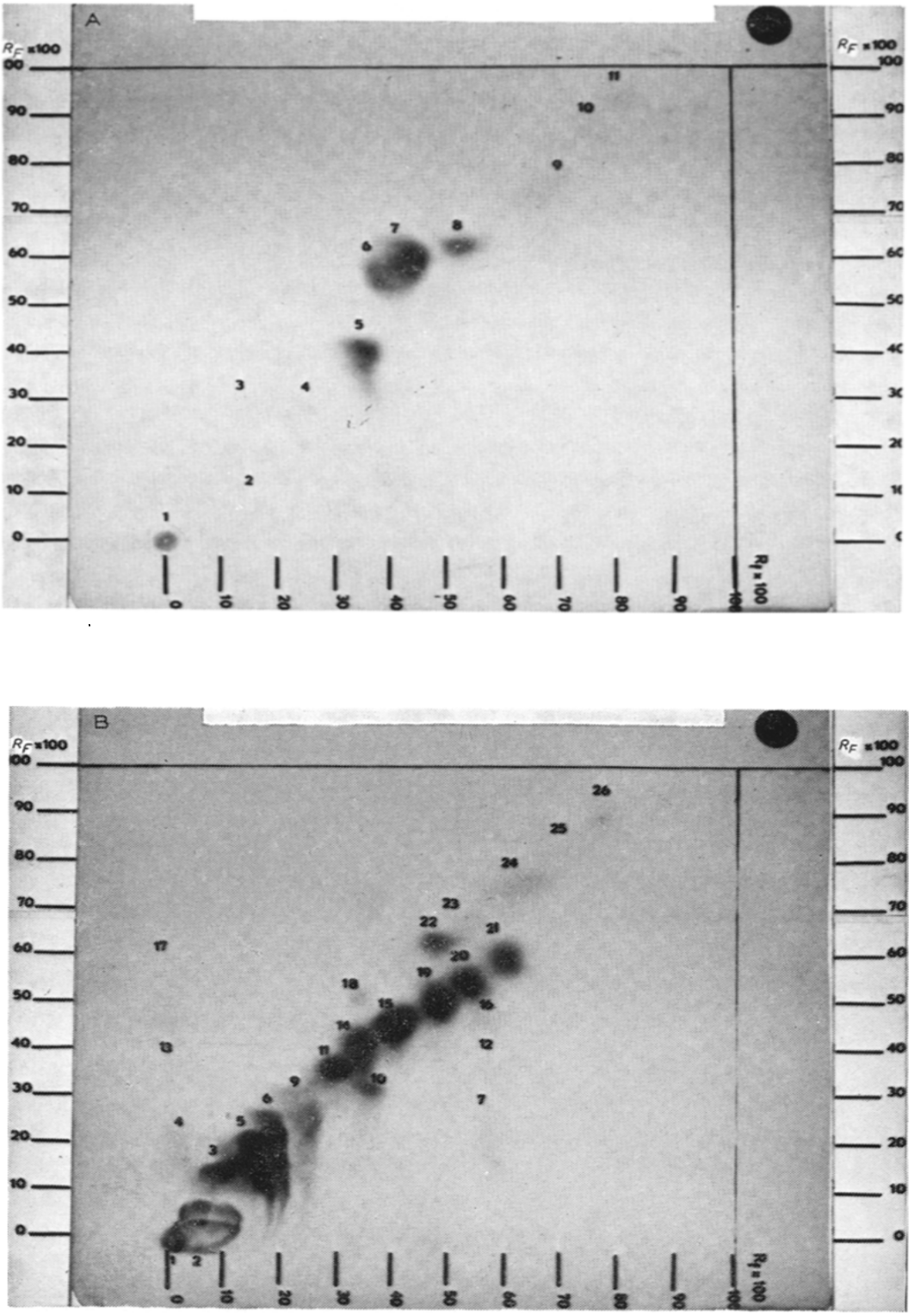


Fig. 5. (A) 168 h two-dimensional autoradiogram of rat urine (10 μl) taken 336 min after i.v. administration of methylenedioxy-^{14}C-piperonyl butoxide (50 μl, 183 μC). First development (150 mm) with ethyl acetate–acetic acid–methanol (70:10:20), then 90° development (150 mm) with *n*-butanol–acetic acid–water (10:1:1). (B) 168 h two-dimensional autoradiogram of rat urine (10 μl) taken 235 min after i.v. administration of α-methylene-^{14}C-piperonyl butoxide (50 μl, 167μ C). First development (150 mm) with ethyl acetate–acetic acid–methanol (70:10:20), then 90° development (150 mm) with *n*-butanol–acetic acid–water (10:1:1).

TABLE IV

SUMMARY OF RADIOACTIVE METABOLITES IN RAT URINE FOLLOWING SINGLE INTRAVENOUS ADMINISTRATIONS OF METHYLENEDIOXY-^{14}C- AND α-METHYLENE-^{14}C-PIPERONYL BUTOXIDE

Methylenedioxy-^{14}C-piperonyl butoxide		*α-Methylene-^{14}C-piperonyl butoxide*			
Metabolite spot No.	R_F[a] × 100	*Metabolite spot No.*	R_F[a] × 100	*Metabolite spot No.*	R_F[a] × 100
1	0 (0)	1	0 (0)	14	41 (31)
2	7 (15)	2	0 (5)	15	47 (36)
3	29 (13)	3	14 (4)	16	48 (51)
4	29 (24)	4	19 (0)	17	50 (0)
5	41 (31)	5	17 (12)	18	52 (33)
6	57 (36)	6	19 (15)	19	53 (43)
7	58 (43)	7	24 (51)	20	55 (51)
8	63 (51)	8	25 (17)	21	60 (57)
9	77 (67)	9	27 (22)	22	63 (41)
10	89 (73)	10	32 (32)	23	69 (49)
11	95 (77)	11	37 (24)	24	75 (57)
		12	39 (51)	25	82 (69)
		13	38 (0)	26	90 (73)

[a] Adsorbent: Silica Gel GF. Developing solvents: first development: ethyl acetate–acetic acid–methanol (70:10:20) (unbracketed R_F values); 90° development: *n*-butanol–acetic acid–water (10:1:1) (bracketed R_F values).

respectively, as depicted in two-dimensional autoradiograms shown in Figs. 5A and 5B. Table IV summarizes the R_F values of the urinary metabolites obtained on Silica Gel GF (Analtech). The developing solvents were ethyl acetate–acetic acid–methanol (70:10:20) for the first development and *n*-butanol–acetic acid–water (10:1:1) for the second 90° development.

Co-chromatographic autoradiographic procedures utilizing both isotopic urine samples (in a manner analogous to the biliary metabolite analysis described above), as well as a comparison of the autoradiograms from both isotopes and the tabular R_F summary (Table IV) suggests the following similar urinary metabolites (the asterisked number referring to the α-methylene-^{14}C metabolite): metabolites 1 and 1*; 2 and 5*; 3 and 6*; 4 and 9*; 5 and 14*; 8 and 22* and 11 and 26*.

Fig. 6 depicts the plot of the total activity *versus* time of all the urinary metabolites following i.v. administration of α-methylene-^{14}C- and methylenedioxy-^{14}C-piperonyl butoxide (curves A and B, respectively). Figs. 7 and 8 illustrate the relationship of activity *versus* time for the major urinary metabolites of methylenedioxy-^{14}C- and α-methylene-^{14}C-piperonyl butoxide, respectively. The analogous prolonged excretion of urinary metabolites for both isotopes is illustrative.

Table V summarizes the radioactivity (c.p.m.) recoveries with time of the urinary metabolites 1–9 following i.v. administration of the methylenedioxy-^{14}C isotope.

Table VI in a similar fashion depicts a summary of the radioactivity recoveries with time of the majority of urinary metabolites following i.v. administration of α-methylene-^{14}C-piperonyl butoxide.

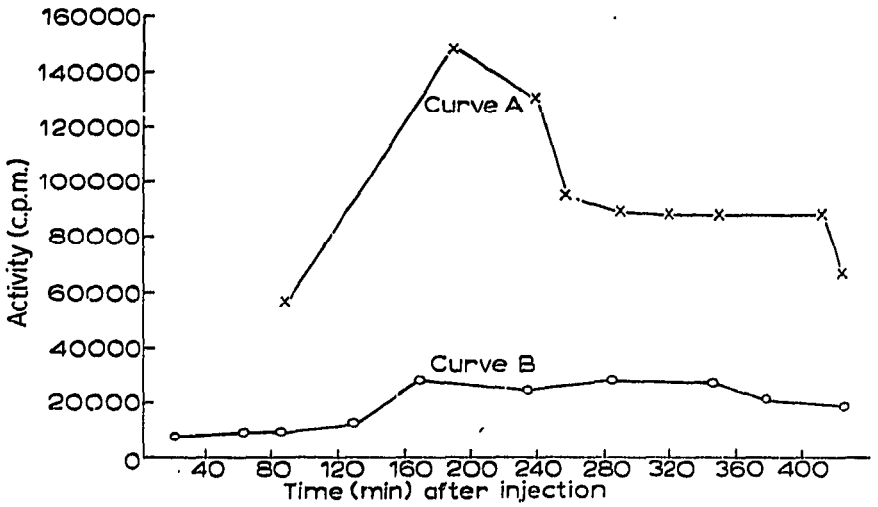


Fig. 6. Relationship of the total radioactivity (c.p.m.) with time of all the urinary metabolites following i.v. administration of α-methylene-^{14}C-piperonyl butoxide (curve A) and methylenedioxy-^{14}C-piperonyl butoxide (curve B).

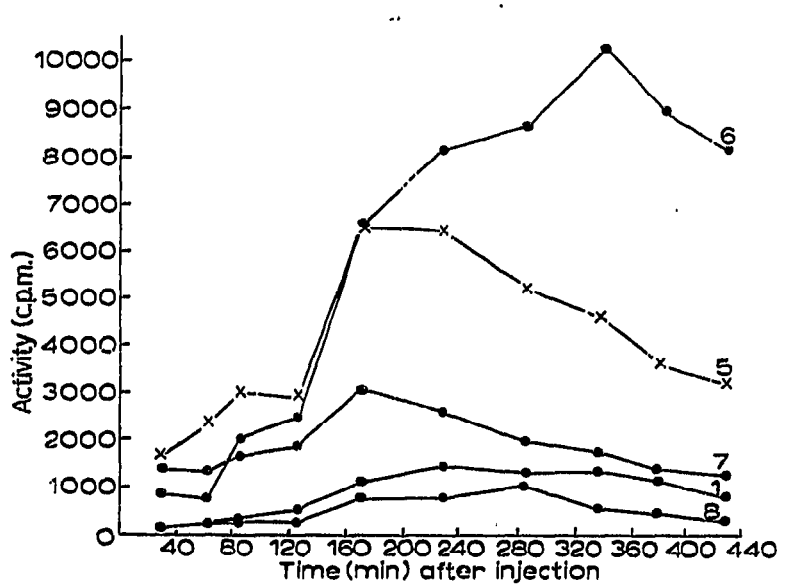


Fig. 7. Relationship of radioactivity (c.p.m.) with time of the major urinary metabolites (number 1, 5, 6, 7 and 8) following i.v. administration of methylenedioxy-^{14}C-piperonyl butoxide.

Tissue distribution of radioactivity

Table VII illustrates the percent radioactivity recovered from i.v. administered α-methylene-^{14}C- and methylenedioxy-^{14}C-piperonyl butoxide in rat tissues and excreta. Results from analogous experiments with methylenedioxy-^{14}C-tropital are included for comparison. Table VII illustrates the wide distribution of the isotopes in tissue and points out the unexpected large percentage of total radioactivity in both lung and fat for each isotope under study. Both whole lung tissue and peri-renal fat were analyzed by both thin-layer chromatography and autoradiography.

TABLE V

RADIOACTIVITY OF MAJOR AND MINOR RAT URINARY METABOLITES FOLLOWING SINGLE INTRAVENOUS ADMINISTRATION OF METHYLENEDIOXY-^{14}C-PIPERONYL BUTOXIDE

Metabolite No.	*Sample collection time (min)*									
	31	*63*	*86*	*128*	*171*	*238*	*286*	*336*	*380*	*426*
1	121	236	341	532	1,158	1,486	1,311	1,374	1,155	774
2	—	156	228	313	614	566	394	262	288	200
3	200	304	311	361	534	459	238	261	313	177
4	186	128	212	130	351	350	235	362	246	173
5	1,651	2,332	3,032	2,922	6,533	6,484	5,239	4,628	3,612	3,205
6	858	767	2,048	2,497	6,655	8,171	8,712	10,362	8,975	8,206
7	1,380	1,380	1,679	1,873	3,121	2,636	1,996	1,771	1,384	1,230
8	121	234	236	274	792	774	1,039	559	407	276
9	497	176	<100	<100	<100	<100	224	137	148	138
Total (c.p.m.)	5,014	5,713	8,087	8,902	19,759	20,926	19,389	19,716	16,528	14,376
Reference count (c.p.m.)	6,811	9,202	9,120	11,566	23,598	24,527	24,139	22,945	19.241	17,642
Recovery (%)	73.61	62.08	88.66	76.95	83.73	85.31	80.32	85.92	85.89	81.50

TABLE VI

RADIOACTIVITY OF MAJOR AND MINOR RAT URINARY METABOLITES FOLLOWING SINGLE INTRAVENOUS ADMINISTRATION OF α-METHYLENE-^{14}C-PIPERONYL BUTOXIDE

Metabolite No.	*Sample collection time (min)*								
	95	*182*	*235*	*253*	*282*	*311*	*343*	*408*	*419*
1, 2	3,164	14,637	6,171	4,498	6,156	4,532	5,103	4,713	3,858
3, 5, 6, 8, 9	10,737	41,838	45,417	34,358	29,718	28,132	28,446	27,000	23,034
11, 14	6,090	8,132	13,697	10,333	9,916	10,210	8,476	9,664	5,718
13, 17	868	8,708	1,222	993	909	459	342	440	657
15, 18	4,591	6,810	10,567	8,180	6,213	6,618	7,441	7,197	4,133
16, 12, 7	935	6,371	1,619	1,586	2,244	968	547	617	925
19, 22	2,922	5,918	9,131	6,497	5,338	5,419	5,582	5,470	3,309
20, 23	2,966	3,603	4,533	2,241	2,291	3,428	2,478	2,842	2,156
21, 24	2,531	3,578	3,678	2,498	2,139	1,105	1,982	1,910	1,133
Total (c.p.m.)	34,844	99,595	96,035	71,184	64,964	60,871	60,397	59,853	44,919
Reference count (c.p.m.)	40,882	114,648	104,578	82,008	78,576	76,627	64,864	72,622	56,981
Recovery (%)	85.2	86.9	91.8	86.8	82.6	83.8	93.1	82.4	78.8

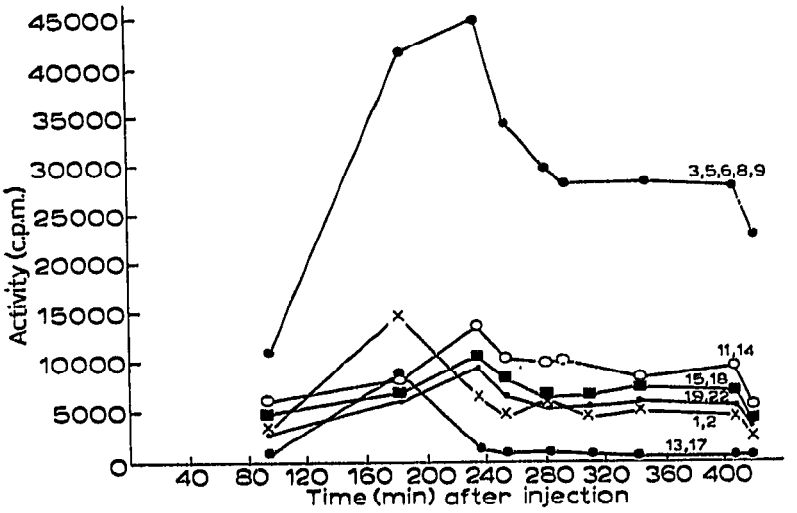


Fig. 8. Relationship of radioactivity (c.p.m.) with time of the major urinary metabolites following i.v. administration of α-methylene-^{14}C-piperonyl butoxide.

TABLE VII

PERCENT RADIOACTIVITY RECOVERED FROM INTRAVENOUS ADMINISTRATION OF α-METHYLENE-^{14}C, METHYLENEDIOXY-^{14}C-PIPERONYL BUTOXIDE AND METHYLENEDIOXY-^{14}C-TROPITAL IN RAT TISSUES AND EXCRETA

Tissues	*Isotope*[a]				
	Piperonyl butoxide				*Tropital*
	α-Methylene-^{14}C		*Methylenedioxy-^{14}C*		*Methylene-^{14}C*
	V-4	*V-7*	*V-5*	*V-6*	
Lung	25.07	17.15	16.38	14.73	12.30
Liver	1.33	1.34	4.42	3.61	2.58
Spleen	0.01	<0.01	0.04	0.05	0.09
Kidneys	0.13	0.14	0.28	0.29	0.18
Heart	0.46	0.11	0.11	0.09	1.01
Thymus	0.81	0.17	0.11	0.21	0.06
Bladder	<0.01	—	0.01	0.01	0.01
Muscle	11.61	3.41	3.08	5.13	5.74
Fat	9.22	14.00	11.67	18.10	12.33
G.I. tract	1.08	0.75	0.63	0.89	0.72
Blood	0.67	3.11	3.87	2.88	Not available
Bile	25.52	46.70	3.40	3.33	7.00
Urine	5.21	5.10	0.95	0.74	13.00
CO_2	<0.001	—	40.00	41.61	0.80[b]
Total recovery (%)	91.12	91.98	84.95	91.67	(55.82)[b]

[a] α-Methylene-^{14}C-piperonyl butoxide: 50 μl (3.72 × 10^8 c.p.m.) (167 μC) administered. Collection time: 7 h 35 min. Methylenedioxy-^{14}C-piperonyl butoxide: 50 μl (4.07 × 10^8 c.p.m.) (183 μC) administered. Collection time: 7 h 56 min. Methylenedioxy-^{14}C-tropital: 50 μl (5.3 × 10^6 c.p.m.) (3.2 μC) administered. Collection time 7 h 55 min.

[b] Sample lost during collection.

TABLE VIII

TWO-DIMENSIONAL THIN-LAYER CHROMATOGRAPHY[a] (R_F VALUES × 100) OF RAT LUNG AND PERI-RENAL FAT FOLLOWING INTRAVENOUS ADMINISTRATION OF METHYLENEDIOXY-^{14}C- AND α-METHYLENE-^{14}C-PIPERONYL BUTOXIDE

Plate No.	*Solvent system*	*Piperonyl butoxide standard*	*Lung*		*Piperonyl butoxide standard*	*Peri-renal fat*
			Methylene-dioxy-^{14}C	*α-Methylene-^{14}C*		
1	A	66[b]	o, 66[b]	o, 66[b]		
	B	(94)	(o), (94)	(o), (94)		
2	A	66[b]	66[b]	66[b]	75	75
	C	(19)	(19)	(19)	(19)	(19)
3	C	18	18			
	C	(18)	(18)			
4	A	66[b]		66[b]		
	D	(83)		(83)		
5	B				94	94
	C				(19)	(19)

[a] Absorbent: Silica gel GF (Analtech). Solvent systems: (A) toluene–acetic acid–water (10:10:1); (B) ethyl acetate–acetic acid–methanol (70:10:20); (C) benzene–acetone (39:1); (D) *n*-butanol–acetic acid–water (10:1:1). Unbracketed R_F values are for the first dimensional development. Bracketed R_F values are for the 90° development.

[b] Solvent front 150 mm. All other solvent fronts are 100 mm.

Table VIII depicts the results of two-dimensional thin-layer chromatography on Silica Gel GF of rat lung and peri-renal fat homogenates obtained from both isotopically labeled piperonyl butoxide treated animals. Four solvent systems were used to identify the radioactive spot as unmetabolized piperonyl butoxide in lung tissue (along with two metabolites in minor amounts), and in peri-renal fat, using two solvent systems.

Table IX depicts the percent radioactivity found in rat lung homogenates on two-dimensional radioautograms following the i.v. administration of both ^{14}C-labeled piperonyl butoxide samples and shows that the overwhelming amount of radioactivity in both cases is accounted for by unmetabolized piperonyl butoxide (96–98%).

Ancillary studies are in progress involving the elaboration of metabolites following the *oral* administration of piperonyl butoxide to the rat and preliminary TLC findings have also demonstrated the presence of free piperonyl butoxide in whole lung homogenates.

CONCLUSION

It is impossible at this stage to identify the large number of biliary and urinary metabolites detected following the i.v. administration of both methylenedioxy-^{14}C- and α-methylene-^{14}C-piperonyl butoxide.

CASIDA[5] has shown that tropital is oxidized to piperonylic acid following i.v. administration thence converted to piperonyl conjugates with the following amino acids: alanine, glutamate, glutamine, glycine and serine.

TABLE IX

PER CENT RADIOACTIVITY OF METABOLITES IN RAT LUNG HOMOGENATES ON TWO-DIMENSIONAL RADIOAUTOGRAMS FOLLOWING INTRAVENOUS ADMINISTRATION OF METHYLENEDIOXY-^{14}C AND α-METHYLENE-^{14}C-PIPERONYL BUTOXIDE

	Methylenedioxy-^{14}C			*α-Methylene-^{14}C*		
	$R_F \times 100$	*C.p.m.*	*% activity*	$R_F \times 100$	*C.p.m.*	*% activity*
	0 (o)	405	2.7	9 (o)	455	1.5
	66 (o)	149	0.1	66 (o)	169	0.6
	66 (94)	14,179	96.2	66 (94)	29,044	97.9
Total c.p.m. (20 μl)		14,733			29,668	
Total c.p.m. (100 μl)		73,665			148,340	
C.p.m. whole homogenate (100 μl)[b]		71,730			130,540	
C.p.m./mg (corr.)		12,366			24,175	

[a] Absorbent: Silica Gel GF (Analtech). Developers: first dimension: toluene–acetic acid–water (10:10:1) (unbracketed R_F values); 90° development: ethyl acetate–acetic acid–methanol (70:10:20) (bracketed R_F values).

[b] Sample dissolved in hyamine and counted in a Packard Tricarb Scintillator Model No. 3375.

Conditions may exist for the metabolism of piperonyl butoxide, *e.g.* via a piperonylic acid and/or propyl piperonylic acid derivative, followed by conjugation as above; this facet is currently under investigation.

CASIDA and co-workers[6,7] have also shown that following *oral* administration to mice and rats, piperonyl butoxide is largely attacked at the methylenedioxy grouping. The release of formate-^{14}C on scission of the hydroxymethylene-^{14}C-dioxyphenyl group would lead to both the formation of $^{14}CO_2$ and also the introduction of formate-^{14}C into the general metabolic pool, hence leading to the potential formation of large numbers of tagged metabolites. We are investigating this aspect in our studies. It should also be noted that neither urine nor bile contained *unchanged* piperonyl butoxide.

The prolonged excretion of biliary metabolites shown in Figs. 2, 3A, 3B and 4 following the i.v. administration of both isotopes re-emphasizes the conclusions reached in earlier studies with piperonyl butoxide[1] and tropital[1,8]. Of paramount importance is the finding of free piperonyl butoxide in the lung following both i.v. and oral administration of labeled piperonyl butoxide. Equally important is the finding of the high percentage of radioactivity in the lung following i.v. administration of methylenedioxy-^{14}C-tropital.

These studies serve to emphasize certain hazards which may be encountered on repeated and prolonged inhalation or contact with pesticide synergists which needs further exploration.

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