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COMPARISON OF TWO METHODS FOR MEASURING PYRETHROID HYDROLYZING ESTERASES: EXTRACTION SELECTIVITY AND RADIOLABELED IDENTITY

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Two published methods are used widely for measuring pyrethroid hydrolyzing esterases. These two methods are based on measuring either the acid production (Jao and Casida, 1974)[1] or the residual unhydrolyzed ester (Suzuki and Miyamoto, 1978)[2]. These methods were compared using (1RS, trans)' permethrin, labeled at the carbonyl carbon of the acid moiety, and rat liver microsomes. No consistent results were obtained and the enzyme activity determined by the first method was lower than that of the second one. The selectivity of the extraction procedure in Method I and the identity of the labeled product were examined by tlc chromatography. Chloroform extract in acid production method contained more than 30% of the total acid released, thereby, introducing a large error in quantitation. Alkalinization of the reaction mixture prior to chloroform extraction using 1 ml buffer, pH 10, changed the extraction selectivity of acid permethrin (DCVA) in favour of the aqueous phase and consequently increased the accuracy of this method. No chemical hydrolysis of (1RS, trans) permethrin could be detected under conditions of pH 10 over 60 minute period.

Ester cleavage is an important step in the biological and environmental degradation of pyrethroid insecticides[3-5].

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In mammals, rapid hydrolysis appears to be a key detoxication step contributing to the low acute toxicity of some pyrethroids[6-8]. Therefore, an accurate measurement for the pyrethroid hydrolyzing activity is of special merit.

Different procedures were adopted for the measurement of pyrethroid hydrolysis. Two simplified methods are now used widely and these methods are based on measuring either the acid production during the course of hydrolysis (Jao and Casida, 1974)[1] or the residual unhydrolysed ester (Suzuki and Miyamoto, 1978)[2].

Reported in this paper are some data for comparing the above methods with respect to extraction selectivity and labeled product identity using the same substrate, (1RS, trans) permethrin and the same enzyme source, rat liver microsomes.

MATERIALS AND METHODS

Chemicals: ^{14}C-labeled (1RS,trans) permethrin (labeled at the carbonyl carbon, 58.2 mCi/mmol), unlabeled (1RS,trans) permethrin and DCVA (1RS, cis, trans)-3-(2,2-dichlorovinyl) -2,2-dimethylcyclopropanecarboxylic acid were provided by R. Robinson, FMC corporation, Middleport, N.Y. Labeled and unlabeled compounds were purified by tlc (Merck silica gel 60 F_{254} chromatoplates, 0.25 mm gel thickness) eluting with carbon tetrachloride-benzene (4:1). Radiochemical purity of the labeled material was in excess of 99% by the criteria described by Gaughan et al[9].

Esterase assay: Two methods were used for measuring pyrethroid esterase activity, and all measurements were made at 37°C. Rat liver microsomes were prepared according to Soderlund and Casida[10], and 1 mg protein equivalent of microsomal homogenate in 1 ml Tris-HCl buffer (pH 7.5; 0.05 M) was used. Protein determination was measured using the Biuret assay with crystalline bovine serum albumin as the standard[11].

Method I (Jao and Casida, 1974)[1] : ^{14}C-(1RS, trans)permethrin

was added in 10 ul ethanol (Final concentration 0.001 umoles) to 1 ml microsomal homogenate in a 15 ml conical centrifuge tube to initiate the reaction. After incubation with shaking for 10 minutes, reactions were terminated by vigorous shaking with 5 ml hexane. After extraction with hexane (2 x 5 ml), 5 ml of chloroform were added utilizing centrifugation in each case to separate the phases. Amounts of ^{14}C-DCVA liberated by hydrolysis were determined by liquid scintillation counting of duplicate aliquots (0.1 ml) of the aqueous phase in liquiscint.

Method II (Suzuki and Miyamoto, 1978)[2]: The same procedure was used in this method except for using liquid scintillation vials instead of conical centrifuge tubes and the reaction was terminated by cooling the reaction mixture in acetone-dry ice. To the frozen mixture, 1 ml of 0.05 M carbonate-borate buffer, pH 10, and 15 ml of scintillation cocktail (4 gm of 2,5-diphenyloxazole and 100 mg of 1,4-bis -2-(4-methyl-5-phenyloxazole) in one liter of toluene) were added and the vial was shaken vigorously to extract the unhydrolysed ester into the organic phase. The decrease in radioactivity was then measured in the liquid scintillation counter.

For zero time experiments, the enzyme was deactivated either by adding hexane (Method I) or by freezing in acetone-dry ice (Method II) before the addition of the substrate. All results reported are the means of at least four determinations.

RESULTS AND DISCUSSION

From different experiments with the above two methods consistent results could not be obtained and usually method II gives higher activity than method I. For example; using 1 mg protein equivalent of rat liver microsomal homogenates gave 57.32 $\pm$ 1.68 per cent hydrolysis of *trans* permethrin measured by method I after 10 minute incubation time, However, higher activity (77.85 $\pm$ 1.45) was obtained by using method II. To examine the extraction selectivity and

the labeled product identity in method I, four groups of experiments were done. Three groups were for 10 minute incubation period and the fourth group was for zero time. In each group the radioactivity in 0.5 ml samples of hexane extract was measured using PPO scintillation fluid. The radioactivity in the whole aqueous phase after hexane extraction was counted in the zero time group and one group from the 10 minute groups, using 15 ml liquiscint. To another 10 minute group, 5 ml of chloroform were added in the usual way as method I and the radioactivity in duplicate aliquots (0.1 ml) of the aqueous phase was measured using liquiscint. Then the aqueous phase in this group was decanted carefully and the radioactivity in 0.5 ml samples of the chloroform extract was measured in PPO scintillation fluid. The aqueous phase of the last group (10 minute) was acidified to pH 1.0 using 2N HCl and extracted with ether (3 x 5 ml). Samples of the dry ether extract were counted for their radioactivity in PPO scintillation fluid. The hexane, chloroform and ether extracts were evaporated under nitrogen and 0.5 ml ether was used to wash material left on the walls into the bottom of the tubes. Finally the residue in the tip after complete evaporation was dissolved in 2 x 0.2 ml methanol-chloroform 1:1 for thin layer chromatography. The tlc plates were run two times in hexane and one time in benzene (saturated with formic acid)-ether 10:3. The labelled product identity was confirmed with authentic t-permethrin and DCVA; R_f values were, respectively, 0.64 and 0.48 (Figure, 1). Data for these groups of experiments are illustrated in Table (1) in which the radioactivity was corrected for dpm using ^{14}C-toluene. From this table, it seems that more than 96 per cent of t-permethrin at zero time was extracted by 2 x 5 ml hexane. Assuming that more than this proportion of the unhydrolyzed substrate was readily extracted by hexane, then the per cent radioactivity in the aqueous phase as it is or after acidification and extraction with ether (89.6 $\pm$ 0.18 and 89.28 $\pm$ 0.96 , respectively) seems to be ^{14}C-DVCA resulted from hydrolysis

after 10 minute incubation time and represents the per cent activity. These figures are very consistent since the radioactivity in the hexane extract from all 10 minute groups is about 9.76 per cent. However, by using chloroform the radioactivity in the aqueous phase decreased to only 57.32 per cent. The reduction in radioactivity in the aqueous phase due to using chloroform was found in the chloroform extract which contains 32.62 per cent of the total radiocarbon used. This proportion of radioactivity is quite the same as the difference between the per cent radioactivity in the aqueous phase with and without using chloroform. It is very probable that most of the radioactivity in the chloroform extract is ^{14}C-acid rather than ^{14}C-permethrin since the latter was completely extracted by hexane in zero time experimental group.

Tlc plates confirm the above results and Figure 1 represents an X-ray film to one of these plates. By scraping these plates for their radioactivity, chloroform appears to contain 90.128 per cent of its total radioactivity as ^{14}C-DCVA and only 8.712 per cent as ^{14}C-(1RS, <u>trans</u>)permethrin. On the other hand, the aqueous phase appears to retain some radioactivity as ^{14}C-(1RS, <u>trans</u>) permethrin and using chlorofom after hexane extraction helps to extract this radioactivity away from the aqueous phase. Then the question arises of how to change the extraction selectivity in a way to obtain most of the acid permethrin (DCVA) in the aqueous phase and the unhydrolyzed permethrin away from this phase. Then three groups of experiments were designed to increase the volume of the aqueous phase by using an extra volume (1 ml) of distilled water or Tris-HCl buffer (pH 7.5; 0.05 M) or to increase the volume and the alkalinity of the aqueous phase by adding 1 ml carbonate-borate buffer (pH 10; 0.05 M). These treatments were used with Method I and Method II just after the termination of the reaction. The extraction selectivity was measured by counting the radioactivity in the hexane extract, the aqueous phase and

the chloroform extract (Method I). The data of these experiments are shown in Table (2).

By comparing the selectivity of the extraction procedure after 10 minute incubation time (Table 2); it is appeared that increasing the volume of the aqueous phase by adding 1 ml distilled water or 1 ml Tris-HCl buffer, pH 7.5, increased the radioactivity in the aqueous phase by about 9 per cent over the per cent radioactivity measured in the aqueous phase without adding any of these (57 percent; Table 1). On the other hand, the reverse is true since doubling the volume of the aqueous phase decreased the radioactivity in chloroform extract by the same percentage. Furthermore, by increasing the volume and the alkalinity of the aqueous phase (addition of 1 ml carbonate-borate buffer; pH 10) increased the percentage radioactivity in this phase by an extra 21 per cent with an overall difference of about 30 per cent as compared to that in Table (1). Comparing the increase in percentage radioactivity in the aqueous phase with the decrease of that in chloroform extract, it is appeared that changing the volume and alkalinity of the aqueous phase due to using 1 ml carbonate-borate buffer, pH 10, changed the extraction selectivity of DCVA in favour of the aqueous phase rather than the chloroform extract. The activity calculated as the percentage radioactivity in the aqueous phase after alkalinization (87.249 $\pm$ 0.371) is a little bit lower than that calculated from whole aqueous phase without chloroform extraction (89.6; Table 1) which may be due to less unhydrolyzed permethrin retained by the aqueous phase after extraction. The percentage radioactivity in the hexane extract is very consistent and ranged from 7.51 per cent (using buffer, pH 10) to 8.71 per cent(using buffer, pH 7.5) after 10 minute incubation time which means that without alkalinization the acid solubility in hexane is minimal, and that alkalinization does not catalyze the

chemical hydrolysis of (1RS, trans) permethrin. Separate experiments showed that under condition of pH 10, no chemical hydrolysis could be detected over 60 minute period.

In conclusion, without alkalinization, a significant proportion of free DCVA could be extracted into chloroform, thereby, introducing large error in quantitation. Of course, the same quantitation would be valid for other pyrethroids with the same acid moiety and different alcoholic groups. However, the stability of the pyrethroid under alkaline condition has to be checked.

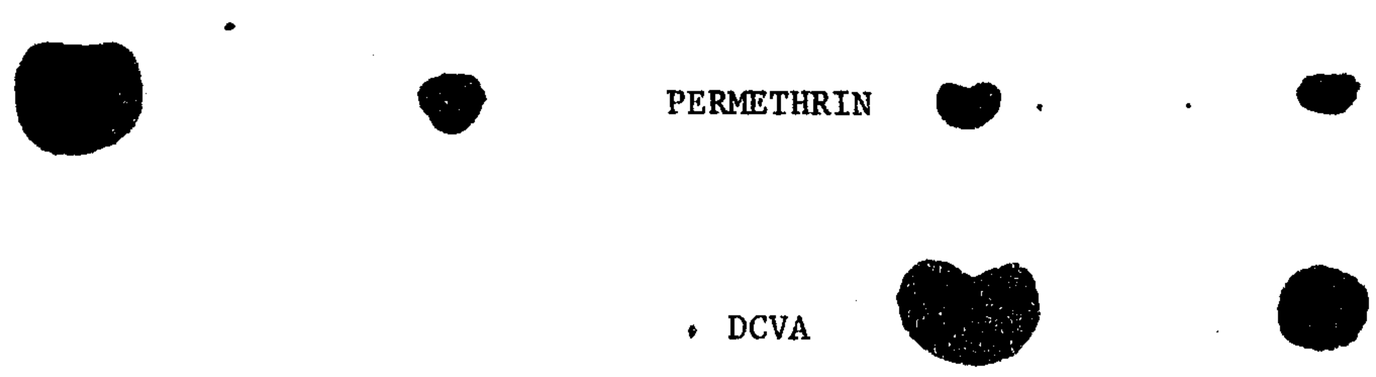
Listed in Table (2) is the effect of alkalinity on the quantitation of per cent activity of 1 mg protein equivalent of rat liver microsomes measured by the reduction of the radioactivity in the scintillation cocktail used in Method II. Also using 1 ml carbonate-borate buffer, pH 10, decreased the radioactivity in this cocktail by about 22 per cent as compared to using 1 ml distilled water, thereby, increase the quantitation of the per cent activity by same proportion.

Table 1- Selectivity of the extraction procedure(Method I) in dpm $\pm$ S.D. for permethrin hydrolyzing activity by rat liver microsomes using 0.001 umoles (1RS, trans) permethrin, 55667.403 dpm.

Incubation time (minutes)	*Hexane extract*	*Ether extract*	*Aqueous Phase without chloroform extration*	*Aqueous Phase after chloroform Extraction*	*Chloroform extract*
0	57930.5 $\pm$ 325.149	-	2207.28 $\pm$ 862.04	-	-
10	5435.5 $\pm$ 748.9	49676.1 $\pm$ 535.5	49877.6 $\pm$ 97.5	31908.5 $\pm$ 932.8	18160.6 $\pm$ 1156.14

Table (2): Effect of alkalinization on the extraction selectivity (Method I) and the quantitation of permethrin hydrolysis by 1 mg protein equivalent of rat liver microsomes using 0.001 umoles (1RS, trans) permethrin as a substrate.

Incubation time	*Addition of 1 ml of the following before organic extraction*	*Extraction selectivity (dpm ± S.D) of the initial organic extracts and the aqueous phase (Method I)*				*Quantitation of esterase activity (per cent)*	
		Hexane	*Chloroform*	*Aqueous*	*Total dpm*	*Method I*	*Method II*
Zero	Dist. water	58828.63 ± 1378.69	1436.17 ± 571.25	621.99 ± 107.31	60886.79	1.022 ± 0.176	-
	Tris - HCl buffer, pH 7.5	59767.46 ± 2191.91	1268.41 ± 85.04	650.65 ± 93.20	61686.52	1.055 ± 0.151	-
	Carbonate-borate buffer, pH 10	61695.674 ± 801.28	736.50 ± 282.53	802.57 ± 172.93	63234.74	1.269 ± 0.273	-
10	Dist. water	5347.73 ± 240.35	16886.25 ± 1027.70	43459.07 ± 836.87	65693.05	66.155 ± 1.274	55.966 ± 6.543
	Tris - HCl buffer, pH 7.5	5557.16 ± 376.16	15797.87 ± 96.71	42428.63 ± 697.47	63783.66	66.52 ± 1.093	63.370 ± 0.588
	Carbonate-borate buffer, pH 10	4845.81 ± 301.70	3379.71 ± 516.61	56281.58 ± 239.17	64507.1	87.249 ± 0.371	77.85 ± 1.453



Hexane extract (Zero time)	Hexane extract (10 min. incubation)	Ether extract (10 min.)	Chloroform extract (10 min.)

Fig.(1): TLC cochromatography of the initial organic extracts and an ether extract of the acidified aqueous phase (Method I) for (1RS, trans) permethrin hydrolysis by rat liver microsomes. The plate was run two times in hexane and one time in benzene (Sat. with formic acid) -ether (10 : 3; R_f = 0.64 and 0.48 for permethrin and DCVA, respectively).

REFERENCES

1) *L.T. Jao and J.E. Casida, Pestic. Biochem. physiol., 4, 465 (1974).*

2) *T. Suzuki and J. Miyamoto, Pestic. Biochem. physiol., 8, 186 (1978).*

3) *J.E. Casida; K. Ueda; L.C. Gaughan; L.T. Jao and D.M. Soderlund, Arch. Environ. Contamin. Toxicol., 3, 491 (1975/1976).*

4) *J.E. Casida; L.C. Gaughan and L.O. Ruzo, "In Advances in Pesticide Science" Vol. 2, H. Geissbuehler, Pergamon Press, NY (1979), P. 182.*

5) *J. Miyamoto, Environ. Health Perspec., 14, 15 (1976).*

6) *C.O. Abermathy and J.E. Casida, Science, 179, 1235 (1973).*

7) *C.O. Abermathy; K. Ueda; J.L. Engel; L.C. Gaughan and J.E. Casida, Pestic. Biochem. Physiol., 3, 300(1973).*

8) *D.M. Soderlund and J.E. Casida, "In Synthetic Pyrethroids", M. Elliott, Amer. Chem. Soc. Washington, DC (1977), P. 162.*

9) *L.C. Gaughan; T. Unai and J.E. Casida, J.Agric. Fd. Chem., 25, 9 (1977).*

10) *D.M. Soderlund and J.E. Casida, Pestic. Biochem. Physiol., 7, 391 (1977).*

11) *K.W. Cleland and E.C. Slater, Biochem. J., 53, 547(1953).*

مقارنة بين طريقتين لتقدير نشاط الانزيمات المحللة للبيروكسيدات

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نيويورك ـ الولايات المتحدة

لقد قورن مدى دقة طريقتين لتقدير نشاط الانزيمات المحللة للبيروكسيدات المشع بواسطة ميكروسومات كبد الفأر ـ الطريقة الاولى تعتمد على قياس الحامض المشع المنفرد والثانية تعتمد على قياس الكمية من المركب التى لم يحدث لها تحلل ـ وثبت باختبار اختيارية الاستخلاص ومدى مطابقة النواتج المشعة :

ان الطريقة الاولى بها خطأ حوالى ٣٠٪ ولقد عدلت هذه الطريقة بتغيير اختيارية الاستخلاص وعليه قد أمكن تقدير الحامض المنفرد كليا وتم تلاشى هذا الخطأ