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EFFECT OF ALKYL PHOSPHATES ON β-GLUCURONIDASE IN RATS: RELEASE OF β-GLUCURONIDASE FROM LIVER MICROSOMES INTO SERUM

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Abstract—Injection of alkyl phosphates elicits an elevation of rat serum β-glucuronidase activity without altering levels of serum lysosomal hydrorases and cholinesterase. Among alkyl phosphates tested, dibutyl phosphate and tributyl phosphate were much more effective and the activities increased 120- and 90-fold of control activity after 1 and 2 hr, respectively. Subfractionation study showed that after the injection of tributyl phosphate the increase in serum β-glucuronidase activity correlated with a depression in the activity of liver microsomal β-glucuronidase but not lysosomal one. Density gradient subfractionation study suggested liver microsomes as the main source of increased serum β-glucuronidase.

Alterations of serum β-glucuronidase activity have been reported in animals after administration of several drugs [1–5]. Williams reported the increase of the activity in rat serum caused by administration of pesticides and hepatotoxic agents [6]. We obtained the result that injection of several organophosphorus insecticides such as diazinon, DDVP and ethylthiometon remarkably elevate serum β-glucuronidase activity in rats [7]. Recently Stahl *et al.* showed that paraoxon and DFP elicit a massive and selective elevation of rat plasma β-glucuronidase [8]. From the observation that this response is neither blocked by atropine nor mimicked by neostigmine, they assumed that it is not cholinergically mediated.

In the previous paper [9], we reported that the extent of increase in serum β-glucuronidase activity of diazinon treated rats correlates well with the degree of decrease in enzyme activity in microsomal fraction of the liver.

In the present paper, we describe the structure-activity relationship of alkyl phosphates in elevating serum β-glucuronidase and the origin of the increased enzyme.

MATERIALS AND METHODS

Chemicals. Trimethyl phosphate (TMP), triethyl phosphate (TEP), tri-*n*-butyl phosphate (TBP), tri-*n*-amyl phosphate (TAP) and di-*n*-butyl phosphate (DBP) were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan), and diethyl phosphate (DEP) from Eastman Kodak Co. All these chemicals were of reagent grade. Tri-*n*-hexyl phosphate (THP) and tri-*n*-octyl phosphate (TOP) were prepared by reacting corresponding alcohols and phosphorus oxychloride in the presence of pyridine following the procedure described by Noller and Dutton [10]. Their purities were checked by thin-layer chromatography system of Lamotte *et al.* [11] and identified as expected phosphate esters by elemental analysis. Dimethyl, monomethyl, monoethyl and mono-*n*-butyl phosphate were isolated from the mixture of corresponding mono and dialkyl phosphate (Tokyo Chemical Industry Co.) through fractional crystalization of the barium salts by the procedure of Plimmer *et al.* [12]. The barium salts were converted to the free acids and extracted with ether. Their purities were checked by paper-chromatography system described by Plapp and Casida [13] and identified by elemental analysis. Triton WR-1339 was purchased from Nakarai Chemicals Ltd. (Kyoto, Japan). All other chemicals were of reagent grade.

Animals and treatment. Female Wistar rats weighing 150–200 g were used. Alkyl phosphates were dissolved in corn oil and injected i.p. The concentration was adjusted so that injection vol. was 1 ml/kg body wt. As a control corn oil was injected. After desired intervals, rats were sacrificed by decapitation and blood was collected from the carotid artery. In time course experiments, blood was collected from the tail vein under anesthesia with sodium phenobarbital (100 mg/kg, i.p.). Phenobarbital treatment did not affect the serum β-glucuronidase activity.

Subcellular fractionation of liver. Animals were fasted for 24 hr before sacrifice. Liver was homogenized in 3 vol. of 0.25 M sucrose containing 1 mM EDTA using a Teflon-glass Potter-Elvehjem type homogenizer. The homogenate was centrifuged at 1000 *g* for 10 min. The sediments were washed 2 times by homogenizing as above, the pellets being sedimented at 600 g for 10 min on each occasion and called nuclear fraction. Resulting supernatants were combined and successively centrifuged at 3300 *g* for 10 min, at 17,000 *g* for 15 min and 105,000 *g* for 30 min to obtain mitochondrial, lysosomal and microsomal fractions, respectively. The final supernatant was called supernatant fraction. Each sediment obtained was suspended in 0.25 M sucrose so as to adjust the vol. to 4 ml per g of the original tissue.

Preparation of tritosomes. Tritosomes were isolated by the procedure reported by Vignais and Nachbaur [14]. Three and a half days prior to sacrifice, rats were injected with Triton WR 1339 i.p. in a dose of 850 mg/kg. Rats were starved 24 hr prior to sacrifice. Liver was homogenized in 3 vol. of 0.25 M sucrose containing 1 mM EDTA. The nuclear fraction was sedimented by centrifuging at 1000 *g* for 10 min and resulting supernatant was diluted with 6 vol. of the medium. After sedimentation of this supernatant at 1700 *g* for 10 min, resulting supernatant was centrifuged at 11,000 *g* for 20 min. The pellets obtained including mitochondria and lysosomes were resuspended in 5 vol. of the medium and centrifuged again at 11,000 *g* for 20 min. This procedure was repeated once more. Resulting sediments were resuspended in the medium so that protein concentration was 10–15 mg/ml. Two ml of this suspension was placed on the top of a 2 layer gradient formed by successively layering 4.5 ml of 41.4% (w/v) sucrose and 4.5 ml of 39.3% (w/v) sucrose. The tubes were centrifuged for 2.5 hr at 55,000 *g* in a swinging bucket rotor (Hitachi 40PS). The band floating on the top of 39.3% layer was collected and diluted slowly with equal vol. of water, and then centrifuged at 20,000 *g* for 20 min. Resulting sediments were suspended in 0.25 M sucrose and called tritosomes.

Hypotonic treatment of microsomes. Microsomal pellets obtained after Triton WR-1339 treatment were suspended in distilled water and then centrifuged at 105,000 *g* for 30 min. Resulting sediments were resuspended in 0.25 M sucrose and called hypotonically treated microsomes. As previously reported by us [9] or other group [15], the hypotonic treatment destroyed lysosomes and solubilized lysosomal β-glucuronidase without affecting total β-glucuronidase activity in original microsomal fraction.

Enzyme assays. β-Glucuronidase (E.C. 3.2.1.31) activity was assayed as previously reported [9] using *p*-nitrophenyl β-glucuronide as substrate. Acid phosphatase (E.C. 3.1.3.2) activity was assayed by the method of Gianetto *et al.* [16]. In determining both enzyme activities in liver subfractions, Triton X-100 was added in a final concentration of 0.1% (w/v). Glucose-6-phosphatase (E.C. 3.1.1.9) activity was assayed by the method of de Duve *et al.* [17]. Cholinesterase (E.C. 3.1.3.8) activity was assayed by the method of Voss and Sachsse [18] with slight modifications. The incubation medium containing 1 mM acetylthiocholine, 0.2 mM DTNB, 0.08 M phosphate buffer (pH 7.4) and serum in a final vol. of 3 ml was incubated at 37° for 20 min. The reaction was stopped by the addition of 0.5 ml of 7 mM neostigmine and thiocholine released was measured from the absorbance at 412 nm. *N*-acetyl-β-glucosaminidase (E.C. 3.2.1.30) activity was assayed by the procedure of Walker *et al.* [19] with slight modifications. The substrate, *p*-nitrophenyl *N*-acetyl-β-glucosaminide was prepared by the method of Findlay *et al.* [20]. The incubation medium containing 5 mM substrate, 0.05 M citrate buffer (pH 4.4) and serum in a final vol. of 1 ml was incubated at 37° for 1 hr. The reaction was stopped by the addition of 2.0 ml of 0.4 M glycine-NaOH buffer (pH 10.3) and *p*-nitrophenol released was measured from the absorbance at 420 nm. Hyaluronidase (E.C. 3.2.1.35) activity was assayed by the procedure of Bonner and Canty [21] with slight modifications. The incubation medium consisted of 0.4 mg potassium hyaluronate obtained from human umbilical cords, 0.033 M acetate buffer (pH 3.5), 0.1 M NaCl and serum in a final vol. of 0.3 ml was incubated at 37° for 6 hr covering with toluene. The reaction was stopped by the addition of 10 μl of 3 N NaOH and 0.3 ml of 0.27 M potassium tetraborate. After heating for 3 min in boiling water, *N*-acetylglucosamine released was measured from the absorbance at 585 nm following the method of Reissig *et al.* [22].

Protein determination. Protein was determined by the procedure of Lowry *et al.* [23] using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Effect of alkyl phosphates on serum β-glucuronidase activity. Table 1 shows the effect of a single i.p. injection of several trialkyl phosphates on β-glucuronidase activity in rat serum at 3 and 6 hr after the administration. Among them, TBP was most effective and showed 90-fold increase in activity. In other trialkyl phosphates, the potency decreased in the order of TEP, TAP, THP, TOP and TMP. Table 2 shows the effect of mono alkyl and dialkyl phosphates on the activity at 1 and 3 hr after the administration. Only DBP was effective and elicited 120-fold increase after 1 hr.

Figure 1 shows time course of serum β-glucuronidase activity after the injection of DBP, TBP and TEP (1 m-mole/kg body wt, respectively). In the case of DBP, the enzyme activity was rapidly increased and reached to a maximum at 1 hr. In the case of

Table 1. Effect of trialkyl phosphates on β-glucuronidase activity in rat serum*

Trialkyl phosphate	β-Glucuronidase (μmoles/hr/ml)	
	3 hr	6 hr
Control	0.17 ± 0.03	0.20 ± 0.03
Trimethyl phosphate	0.33 ± 0.03	—
Triethyl phosphate	7.42 ± 0.59	6.10 ± 0.79
Tributyl phosphate	15.40 ± 2.97	5.48 ± 1.01
Triamyl phosphate	2.47 ± 1.03	2.98 ± 2.79
Trihexyl phosphate	0.56 ± 0.07	0.70 ± 0.07
Trioctyl phosphate	0.54 ± 0.07	0.24 ± 0.07

* Trialkyl phosphates (1 m-mole/kg) were injected i.p. Values are means ± S.D. of four rats.

Table 2. Effect of mono and dialkyl phosphates on β-glucuronidase activity in rat serum*

Alkyl phosphate	β-Glucuronidase (μmoles/hr/ml)	
	1 hr	3 hr
Control	0.16 ± 0.03	0.17 ± 0.03
Monomethyl phosphate	0.21 ± 0.06	0.32 ± 0.07
Monoethyl phosphate	0.23 ± 0.06	0.20 ± 0.05
Monobutyl phosphate	0.31 ± 0.07	0.36 ± 0.07
Dimethyl phosphate	0.21 ± 0.05	0.24 ± 0.05
Diethyl phosphate	0.50 ± 0.16	0.65 ± 0.17
Dibutyl phosphate	20.20 ± 2.10	7.40 ± 1.05

* Mono and dialkyl phosphates (2 m-mole/kg) were injected i.p. Values are means ± S.D. of three rats.

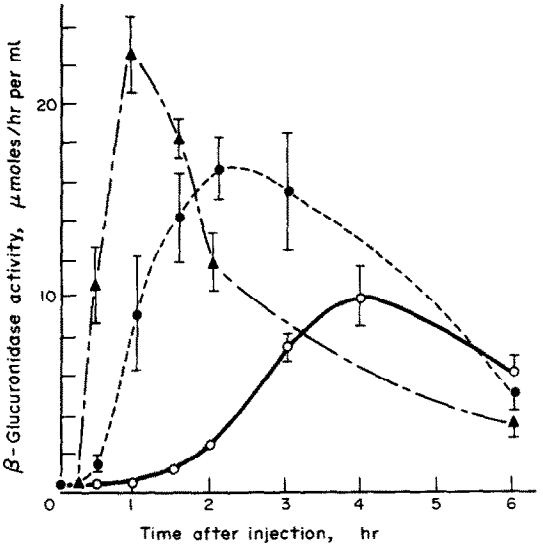

Fig. 1. Time course of β-glucuronidase activity in rat serum after administration of trialkyl phosphates. DBP, TBP or TEP was injected i.p. to rats in a dose of 1 m-mole/kg. Vertical lines show ± S.D. of the mean of four rats. ▲——▲ DBP, ●——● TBP, ○——○ TEP.

TBP and TEP, the enzyme activity was increased gradually and maximum activity was obtained 2 and 4 hr after the treatment respectively. By injecting TAP, THP and TOP, the enzyme activity was more slowly increased later than 4 hr (not shown). The slower elevation by the administration of TBP than DBP might suggest that TBP would be metabolized and DBP formed would act on animals. However the fact that DEP, a metabolite of TEP, was ineffective to increase the activity may indicate that trialkyl phosphates act as themselves.

Recently Stahl *et al.* showed the elevation of serum β-glucuronidase activity mediated by non-cholinergic mechanism after the administration of organophosphorus insecticides [8]. We also tested the effect of alkyl phosphates on the elevation of β-glucuronidase comparing with the inhibitory action on serum cholinesterase. As indicated in Table 3, the increase of serum β-glucuronidase by TBP treatment was dose dependent. However, serum cholinesterase activity was not affected irrespective of the dose level. This suggests that the increase of serum β-glucuronidase activity was not mediated cholinergically.

It has been reported that β-glucuronidase is localized in both lysosomes and microsomes at subcellular level [17, 24–27]. Bârzu *et al.* reported that an organophosphorus insecticide, TETPP (asymmetric tetraethyl dithio pyrophosphate), labilizes lysosomal membrane *in vitro* [28]. In order to examine whether lysosomes take part in the elevation of serum β-glucuronidase, levels of two lysosomal enzymes were measured. Table 4 shows that these enzyme activities were same as control activity irrespective of the dose level of TEP. This result suggests that TEP did not attack lysosomes in the target cells *in vivo*.

Effect of alkyl phosphates on hepatic β-glucuronidase activity. We tested direct effect of alkyl phosphates *in vitro* on serum β-glucuronidase and obtained the result that all alkyl phosphates tested in this experiment had neither activating nor inhibitory action on β-glucuronidase activity of both normal and TBP treated serum in concentrations from 10^{-4} to 10^{-2} M. We also examined the nature of β-glucuronidase activity obtained from TBP treated serum. The results are summarized as follows*. (1) The enzyme activity was a linear function of serum concentration over 40-fold range. (2) Enzyme activity of the serum obtained by mixing normal and TBP treated serum had additive activity in all proportions. (3) Dialysis of the serum did not affect the enzyme activity after the treatment. From these observations the increase of the activity in serum was thought to be the release

Table 3. Effect of tributyl phosphate on β-glucuronidase and cholinesterase activities in rat serum*

Dose (m-mole/kg)	β-Glucuronidase (μmoles/hr/ml)	Cholinesterase (μmoles/min/ml)
Control	0.17 ± 0.03	2.47 ± 0.28
0.062	0.28 ± 0.11	1.95 ± 0.41
0.125	0.46 ± 0.37	1.73 ± 0.37
0.250	1.94 ± 1.09	1.94 ± 0.58
0.50	4.04 ± 0.88	1.69 ± 0.39
1.0	8.82 ± 3.64	1.99 ± 0.48

* TBP was injected i.p. 1 hr prior to sacrifice. Values are means ± S.D. of four rats.

* Y. Suzuki and H. Kikuchi, unpublished observations.

Table 4. Effect of triethyl phosphate on lysosomal enzyme activities in rat serum*

Dose (m-mole/kg)	β-Glucuronidase (μmoles/hr/ml)	N-acetyl-β-glucosaminidase (μmoles/hr/ml)	Hyaluronidase (μmoles/hr/ml)
Control	0.17 ± 0.03	1.27 ± 0.03	0.56 ± 0.18
1.0	12.71 ± 2.45	1.12 ± 0.11	0.57 ± 0.08
1.5	9.63 ± 1.32	1.45 ± 0.33	0.47 ± 0.05
3.0	10.83 ± 3.06	1.27 ± 0.14	0.52 ± 0.07

* TEP was injected i.p. 3 hr prior to sacrifice. Values are means ± S.D. of four rats.

of the enzyme from a tissue or tissues abundant in β-glucuronidase.

In female rats, it has been reported that β-glucuronidase is mainly localized in liver (70–80%), preputial gland (10%), spleen and kidney [25, 29]. Since we found that after the administration of TBP only the activity in liver among four tissues was decreased to 85% of the control, it was suggested that liver is the main source of the enzyme appeared in serum. Table 5 shows the results of subcellular distribution of hepatic β-glucuronidase activity 3 hr after the injection of TBP (1 m-mole/kg). β-Glucuronidase activity in microsomal fraction was significantly decreased in total and specific activities (28% of control). There were no significant changes in the activities in mitochondrial, lysosomal and supernatant fractions.

To confirm the action of TBP on microsomes, lysosomes were prepared by using pretreatment technique with Triton WR-1339. As shown in Table 6, β-glucuronidase activity in tritosomes was increased 3 hr after the injection of TBP. When microsomes were treated hypotonically to remove contaminating lysosomes, β-glucuronidase activity in the microsomal preparation was decreased to 21 per cent of the control activity. These data suggests that the elevation of serum β-glucuronidase activity by TBP is mainly due to the release of the enzyme from liver microsomes. Acid phosphatase activity in the tritosomes and glucose-6-phosphatase in the microsomes did not change significantly after the injection of TBP. Uchiyama *et al.* reported that P = O analogs of organophosphorus insecticides such as sumioxon and DDVP did not affect drug metabolizing activity in microsomes [30]. So far as the biochemical data concerned, the effect of alkyl phosphates would be specific to β-glucuronidase in microsomes. Recently Swank and Paigen

Table 5. Subcellular distribution of β-glucuronidase activity in liver of control and tributyl phosphate treated rats*

Fraction	Total activity (μmoles/hr/g of liver)		Specific activity (μmoles/hr/mg of protein)	
	Control	TBP	Control	TBP
Homogenate	170.7 ± 6.3	149.5 ± 5.8	0.85 ± 0.09	0.72 ± 0.07
Nuclei	31.7 ± 4.1	25.8 ± 4.4	0.77 ± 0.05	0.64 ± 0.05
Mitochondria	49.8 ± 3.3	54.7 ± 8.3	1.59 ± 0.12	1.78 ± 0.21
Lysosomes	54.8 ± 3.8	51.4 ± 5.4	1.52 ± 0.12	1.43 ± 0.18
Microsomes	20.1 ± 1.1	5.6 ± 1.3	0.64 ± 0.03	0.18 ± 0.02
Supernatant	7.4 ± 0.8	9.2 ± 1.2	0.11 ± 0.02	0.14 ± 0.02
Recovery (%)	96.0	98.1		

* TBP (1 m-mole/kg) was injected i.p. 3 hr prior to sacrifice. Values are means ± S.D. of four rats. Recovery of protein was 93.5% for control and 95.2% for treatment.

Table 6. Effect of tributyl phosphate on β-glucuronidase, acid phosphatase and glucose-6-phosphatase activities in tritosomes and hypotonically treated microsomes*

Enzyme	Tritosomes		Hypotonically treated microsomes	
	Control	TBP	Control	TBP
β-Glucuronidase (μmoles/hr/mg protein)	3.97 ± 0.34	7.85 ± 0.43	0.77 ± 0.08	0.16 ± 0.03
Acid phosphatase (μmoles/30 min/mg protein)	7.07 ± 1.03	8.42 ± 0.90		
Glucose-6-phosphatase (μmoles/20 min/mg protein)			6.25 ± 0.67	7.46 ± 0.40

* TBP (1 m-mole/kg) was injected i.p. 3 hr prior to sacrifice. Tritosomes and hypotonically treated microsomes were prepared as described in the text. Values are means ± S.D. of six rats.

demonstrated that β-glucuronidase in microsomal membrane exists as complexes consisting of one catalytic protein of 280,000 mol. wt and one to four additional proteins of 50,000 to 55,000 mol. wt [31]. Tomino and Paigen speculated that these additional proteins named egasyn would have very hydrophobic regions and would be necessary for a core protein to bind microsomal membrane [32]. This unique mode of existence in microsomal membrane might be responsible for the specific release of this enzyme.

Concerning the pharmacological effects of alkyl phosphates, only narcotic action of trialkyl phosphates has been reported. Vandekar showed that trialkyl phosphates including TMP, TEP and TBP in sublethal doses produced anesthesia in rats and among them TBP provoked deep anesthesia in lesser doses [34]. TEP- and TBP-induced narcosis was further investigated by other groups [34–37] and it was clarified that narcosis is not related to cholinesterase inhibition. It is assumed that anesthetic drugs possess adequate lipid/water partition coefficient to enter nervous membrane. Speculating from this, alkyl phosphates composed of medium carbon chains such as DBP, TBP and TEP possess high affinities for liver microsomal membrane and detach β-glucuronidase directly or indirectly from the membrane. As it has been reported that trialkyl phosphates can act as alkylating agents [38], effect of alkylating action on microsomes might be concerned with the mechanism of enzyme release.

REFERENCES

1. G. Weisman, J. W. Uhr and L. Thomas, *Proc. Soc. exp. biol. Med.* **112,** 284 (1963).
2. T. F. Slater and A. L. Greenbaum, *Biochem. J.* **96,** 484 (1965).
3. K. Saito and E. Suter, *J. exp. Med.* **121,** 739 (1965).
4. R. Nilius and F. W. Rath, *Z. ges. exp. Med.* **144,** 157 (1967).
5. G. Plomteux, M. L. Beaumariage, Z. M. Bacq and C. Heusghem, *Biochem. Pharmac.* **16,** 1601 (1967).
6. C. H. Williams, *Toxic. appl. Pharmac.* **14,** 283 (1969).
7. M. Uchiyama, Y. Suzuki and K. Takanaka, *Hoigaku No Jissai To Kenkyu* [Studies and practice of legal medicine (in Japanese)] **14,** A162 (1970).
8. P. Stahl, B. Mandell, J. S. Rodman, P. Schleisinger and S. Lang, *Archs. Biochem. Biophys.* **170,** 536 (1975).
9. Y. Suzuki, H. Kikuchi and M. Uchiyama, *Chem. pharm. Bull., Tokyo* **23,** 886 (1975).
10. C. R. Noller and G. R. Dutton, *J. Am. chem. Soc.* **55,** 424 (1933).
11. A. Lambotte, A. Francina and J. C. Merlin, *J. Chromat.* **44,** 75 (1969).
12. R. H. A. Plimmer and W. J. N. Burch, *J. chem. Soc.* **1929** 292.
13. F. W. Plapp and J. E. Casida, *Analyt. Chem.* **30,** 1622 (1958).
14. P. M. Vignais and J. Nachbaur, *Biochem. biophys. Res. Commun.* **33,** 307 (1968).
15. C. H. Walkinshaw, H. M. McClure and J. L. Van Lancker, *Lab. Invest.* **13,** 524 (1964).
16. R. Gianetto and C. de Duve, *Biochem. J.* **59,** 433 (1955).
17. C. de Duve, B. C. Pressman, R. Gianetto, R. Wattiaux and F. Appelmans, *Biochem. J.* **60,** 604 (1955).
18. G. Voss and K. Sachsse, *Toxic. appl. Pharmac.* **16,** 764 (1970).
19. P. G. Walker, M. E. Woollen and D. Pugh, *J. clin. Path.* **13,** 353 (1960).
20. J. Findlay, G. A. Levvy and C. A. Marsh, *Biochem. J.* **69,** 467 (1958).
21. W. M. Bonner and E. Y. Cantey, *Clinica. chim. Acta.* **13,** 764 (1960).
22. J. L. Reissig, J. L. Strominger and L. F. Leloir, *J. biol. Chem.* **217,** 959 (1956).
23. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
24. C. Walkinshaw and J. L. Van Lancker, *Lab. Invest.* **13,** 513 (1964).
25. M. Wakabayashi and Y. Shirai, *Kobe J. med. Sci.* **12,** 71 (1966).
26. W. H. Fishman, S. S. Goldman and R. DeLellius, *Nature, Lond.* **214,** 457 (1967).
27. H. Ide and W. H. Fishman, *Histochemie* **20,** 287 (1969).
28. T. Bârzu, B. Cuparencu and A. Hantz, *Biochem. Pharmac.* **22,** 185 (1973).
29. J. Conchie, J. Findlay and G. A. Levvy, *Biochem. J.* **71,** 38 (1959).
30. M. Uchiyama, T. Yoshida, K. Homma and T. Hongo, *Biochem. Pharmac.* **24,** 1221 (1975).
31. R. T. Swank and K. Paigen, *J. molec. Biol.* **77,** 371 (1973).
32. S. Tomino and K. Paigen, *J. biol. Chem.* **250,** 1146 (1975).
33. M. Vandekar, *Nature, Lond.* **179,** 154 (1957).
34. H. W. Chambers and J. E. Casida, *Toxic. appl. Pharmac.* **10,** 105 (1967).
35. H. W. Chambers and J. E. Casida, *Toxic. appl. Pharmac.* **14,** 249 (1969).
36. D. R. Brown and S. D. Murphy, *Toxic. appl. Pharmac.* **18,** 895 (1971).
37. D. R. Brown and S. D. Murphy, *J. Pharmac. exp. Ther.* **179,** 396 (1971).
38. R. F. Hudson, in *Structure and Mechanism in Organophosphorus Chemistry*, p. 264. Academic Press, New York (1965).