

*Best*

UNITED STATES DEPARTMENT OF AGRICULTURE  
Agricultural Research Administration  
Bureau of Entomology and Plant Quarantine  
Division of Fruit Insect Investigations

University of California

Hawaii Agricultural Experiment Station

Territorial Board of Agriculture and Forestry

Pineapple Research Institute

Hawaiian Sugar Planters' Association  
Experiment Station.

ORIENTAL FRUIT FLY INVESTIGATIONS

QUARTERLY REPORT

July 1 -- September 30, 1951

oOo

WORK PROJECT I-c-6. Physiology of the Oriental Fruit Fly. - C. C. Roan,  
Project Leader

### SUMMARY

The mass production of oriental fruit flies has proceeded normally. In mid-September the personnel and volume of production were reduced by one-half.

The preliminary work on methods for the production and detection of flies tagged with radioactive phosphorus has been completed. Flies produced from larvae feeding on a medium containing  $P^{32}$  are sufficiently radioactive to be detected up to one month after emergence and are much more uniformly marked than adult flies feeding on a solution containing  $P^{32}$ . The loss of  $P^{32}$  is more rapid from adult flies fed  $P^{32}$ . Female flies produced from larvae feeding on  $P^{32}$  show a more rapid loss than the males.

The characteristics of the brain cholinesterase of the oriental fruit fly appear to differ somewhat from other species that have been studied. There appears to be an optimal concentration for both acetylcholine and acetyl-beta methyl-choline. The in vitro  $IN_{50}$  concentrations for a series of compounds investigated are of the same order as those reported for the house fly. There is a marked competition between substrate and inhibitor in reacting with the cholinesterase. Topical applications of para-oxon rapidly inactivate the brain cholinesterase of the oriental fruit fly.

The carbamate, G-22008, is a very effective inhibitor of cholinesterase and appears to be a good competition in the presence of acetyl choline. Its action does not appear to be greatly affected by lower temperatures. A series of chemically related compounds is not available at the present time for comparative studies.

There appeared to be no difference in the cholinesterase activity or the action of selected inhibitors between flies provided with a protein or non-protein diet. These tests must be extended to in vivo conditions.

Line Project I-o-6-3. Mass Rearing of Fruit Flies.

This activity became the responsibility of the Physiology Project July 1, 1951. The work has proceeded in a routine manner except for some difficulty with the larval medium in the first week.

The techniques employed were those developed by the local staff of the University of California. The transfer of the cultures and equipment was accomplished by the California staff prior to July 1. This cooperation was of immeasurable value in eliminating any discontinuity of fly production.

Cultures of Ceratitis capitata, and Dacus cucurbitae larvae have been maintained at very low levels. Production figures and distribution of Dacus dorsalis were as follows:

Total production	2,245,234
Chemical Control	1,268,448
Commodity Treatment	228,210
Biology-Ecology	254,200
Area Control	134,800
Biological Control	40,000
Physiology	10,000
Stock culture	<u>95,000</u>
	2,030,658

Balance 214,576 distributed to University of Hawaii and Territorial Board of Agriculture.

Line Project I-o-6-4. Production of Fruit Flies Containing Radioactive Phosphorus.

Two additional lots of radioactive flies were produced. One experiment consisted of rearing larvae in a medium containing  $P^{32}$  by the method described in the previous report. In this experiment the radioactivity was increased to 0.34  $\mu\text{c}$  1 ml. In the second experiment three-day-old flies, held for 24 hours without food or water, were allowed to feed for 48 hours on a sugar solution containing 3.9  $\mu\text{c}$  of  $\text{KH}_2\text{P}^{32}\text{O}_4$  per milliliter. The normal diet was then restored and samples of flies withdrawn at intervals for assay of radioactivity.

The comparative amounts of radioactivity present in the various life stages of the oriental fruit fly reared in a larval medium containing  $P^{32}$  at two levels are shown in table 1. The amounts of  $P^{32}$  acquired by adult feeding are shown in table 2. A comparison of these data indicate that the larval rearing method produces a greater uniformity of marking. Nevertheless it is questionable if positive identification of flies produced from the two larval rearings would be possible.

Samples of flies were periodically removed from all three cages and assayed for radioactivity to determine how long after ingestion it would be possible to detect such flies and to determine the rate of loss of radioactivity by metabolic processes. The data from this experiment are shown in figure 1. The rate of loss of  $P^{32}$  from flies which acquired this material by larval feeding is considerably less than in the case of adults feeding on  $P^{32}$  solutions. It was of interest to note that a very high percentage of the phosphate ingested by the adults was utilized.

In the above samplings the flies were separated according to sex and it was observed that the females were losing  $P^{32}$  more rapidly than the males. Figure 1 shows the differences in the rate of loss by males and females reared from larvae feeding in a radioactive medium. Due to the variation in amounts of  $P^{32}$  acquired by adult feeding such comparisons were impractical.

An 80 mg. sample of eggs collected from flies reared from larvae feeding in a radioactive medium gave 1000 cpm. While this amount of radioactivity is inconsequential from the standpoint of progeny from the radioactive flies it is an important factor in contributing to the loss of  $P^{32}$  by parent female.

On the basis of these tests it would appear possible to make positive identification of various lots of flies released at different time intervals, preferably of the order of 14.3 days. The most reliable method would be the use of flies produced from larvae reared in a medium containing  $P^{32}$  since they are more uniform in regards to the amounts of  $P^{32}$ .

Neither the flies produced from larvae feeding on a medium containing  $P^{32}$  or adults feeding on a solution of  $P^{32}$  showed any indication of adverse effects from exposure to the different levels of radiation. These flies were equal to the normal laboratory strain in percentage emergence from pupae, in fecundity, in fertility, in viability of larval progeny, and in longevity under laboratory conditions.

The use of  $P^{32}$  did not complicate normal larval rearing procedures unduly since laboratory personnel came in contact with the materials only during the process of mixing the  $KH_2P^{32}O_4$  with the larval medium and at the time the larvae were removed for pupation. The use of rubber gloves and quantitative techniques minimized operator exposure and prevented laboratory contamination.

Table 1. Radioactivity of various life stages of the oriental fruit fly reared in a carrot medium containing  $KH_2P^{32}O_4$  at the rate of 0.1 and 0.34 microcuries per milliliter.

No.	0.1 $\mu$ c/ml.			0.34 $\mu$ c/ml.		
	Counts per minute <sup>2/</sup>			Counts per minute <sup>2/</sup>		
	3rd Instar larvae	Pupae	Adults <sup>3/</sup>	3rd Instar larvae	Pupae	Adults <sup>3/</sup>
1 <sup>1/</sup>	3,800	4,800	1,900	5,300	4,800	3,200
2	4,600	5,200	2,900	5,100	5,100	3,500
3	3,400	5,200	2,100	5,800	5,600	2,600
4	4,100	4,100	2,800	5,300	5,400	3,700
5	4,400	4,500	2,200	5,600	5,000	3,800
6	4,200	4,200	2,900	4,700	4,500	3,000
7	4,100	3,800	2,400	5,800	5,200	3,300
8	3,700	3,400	2,400	4,900	4,700	3,500
9	4,100	3,100	2,500	5,300	5,400	3,000
10	4,700	5,300	2,600	5,300	5,000	3,400

- 1/ Succeeding life stages are not necessarily the same individuals.  
 2/ Corrected for background and decay to the date the larvae in each experiment were counted.  
 3/ Freshly emerged adults.

Table 2. Radioactivity of adult oriental fruit flies after feeding for 24 and 48 hours on a sugar solution containing  $KH_2P^{32}O_4$  at the rate of 3.9 microcuries per milliliter.

Counts per minute per individual <sup>1/</sup>			
24 hours <sup>2/</sup>		48 hours <sup>2/</sup>	
Females	Males	Females	Males
2,800	2,400	4,200	5,600
2,300	2,800	4,900	3,900
5,000	3,400	5,700	4,000
3,600	3,500	6,300	4,800
3,300	3,300	3,700	5,000
1,920	3,800	5,400	4,900
4,500	---	3,800	4,300
2,200	---	---	---

- 1/ Corrected for background and decay to the first day of feeding.  
 2/ Counted as intact individuals which were discarded after counting.

FIG. 1.—Rate of loss of  $P^{32}$  acquired from larvae or adult feeding by excretion and/or oviposition. Data represents the average of both sexes.

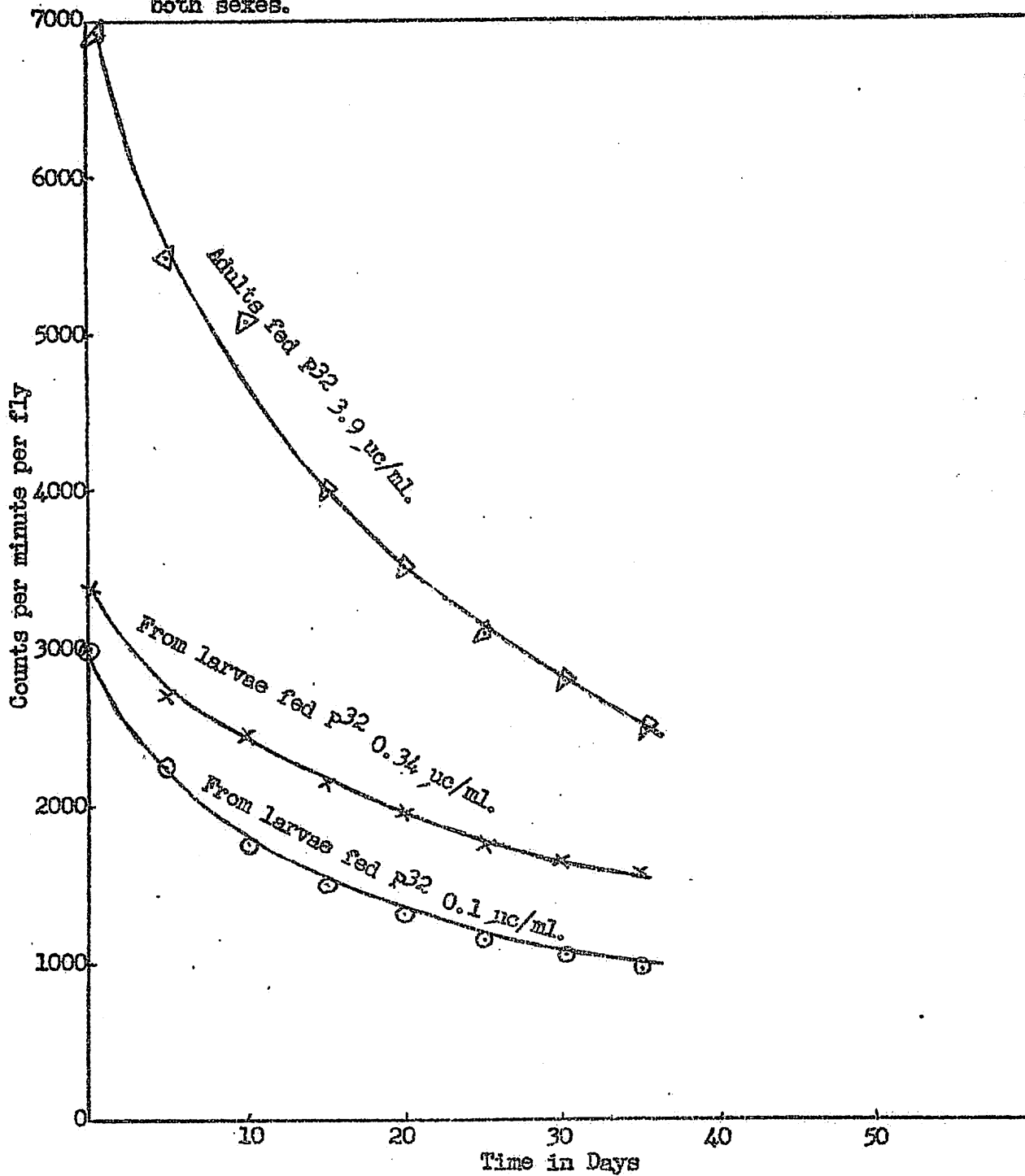
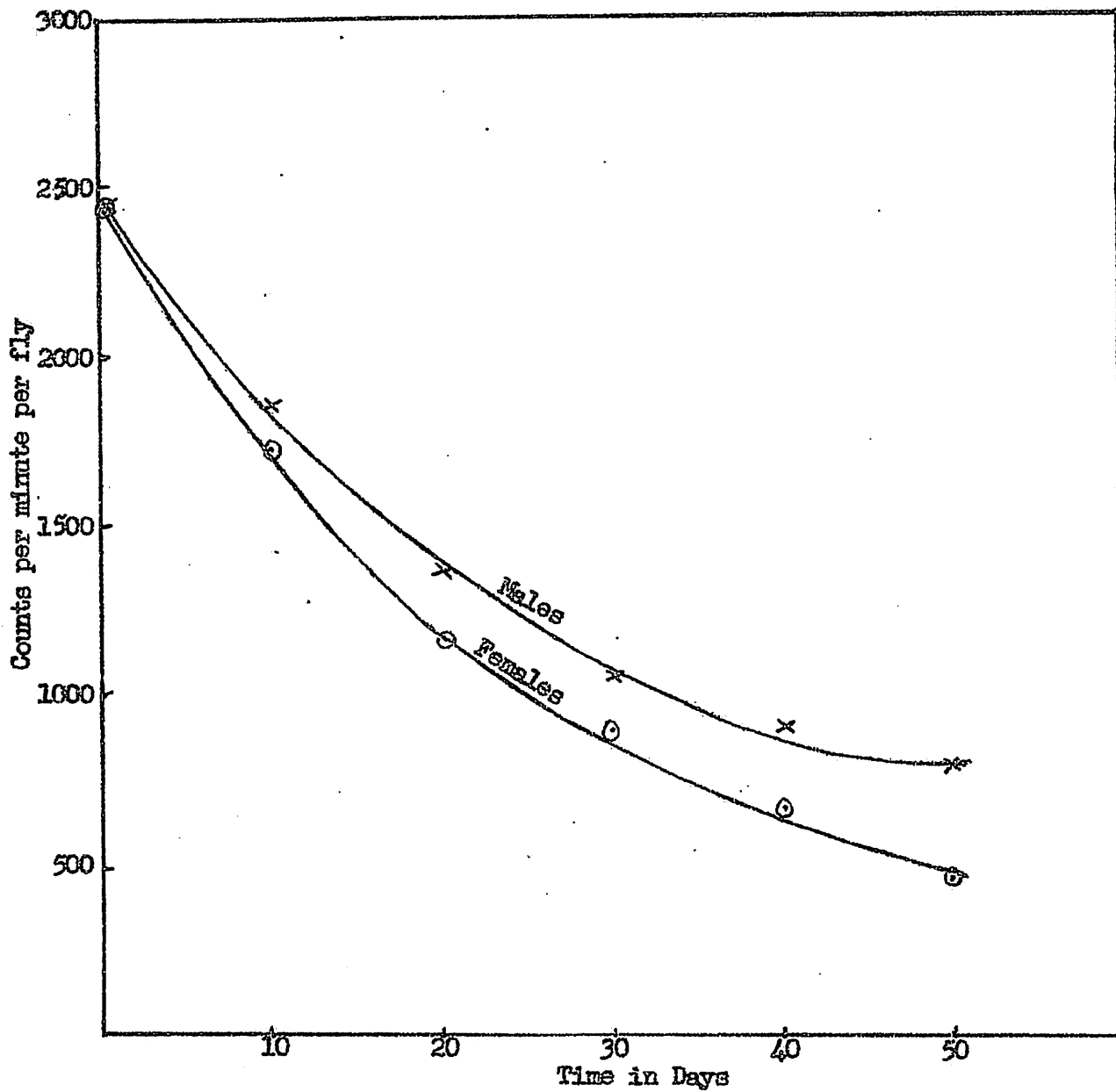


FIG. 2.—The rates of loss of  $P^{32}$  by males and females produced from larvae feeding one medium containing 0.1  $\mu\text{c}/\text{ml}$ . of  $P^{32}$ .



Line Project I-o-6-1. Substrate and Inhibitor Specificity of the Nerve Cholinesterase of *D. dorsalis*.

The manometric techniques used for determining cholinesterase activity were modifications of those described by Metcalf and March (1950). Except where noted otherwise the flies used were two-day-old female *D. dorsalis* which had been provided with a diet of sugar, water, and soy hydrolysate. In the earlier experiments 2.6 oriental fruit fly heads were used for each flask. It soon became apparent that a rather marked variation in head weight (1.72-1.02 mg./head) was causing considerable variation in the results. All succeeding experiments were carried out using 3.12 mg. of head brei per flask and all previous experiments were corrected for weight variations to this value. Other modifications of technique will be described under the appropriate sections. All values reported were corrected for non-enzymatic hydrolysis.

Substrate Specificity and Effect of Substrate Concentration.—Three potential substrates, acetylcholine (ACh), acetyl-beta methyl-choline (AMeCh) and benzoyl choline (BzCh), were studied. The effects of varying substrate concentrations on their hydrolysis by cholinesterase (ChE) at 37° C. are shown in figure 3. Activity is plotted against varying substrate concentrations. From these curves it is evident that excess ACh or AMeCh inhibits hydrolysis. The optimum substrate concentrations appear to be the order of  $5 \times 10^{-3}$  M for ACh and  $4 \times 10^{-2}$  M for AMeCh. This differs from the findings of Metcalf and March (1950) for the house fly where no optimum was observed for the hydrolysis of AMeCh. The optimum concentration,  $5 \times 10^{-3}$  M was employed for all succeeding experiments with ACh.

Effect of Temperature on the Hydrolysis of ACh by Oriental Fruit Fly ChE: Temperatures ranging from 15° - 45° C. were studied in order to determine the effect of temperature on *in vitro* hydrolysis. The optimum temperature, as determined from figure 4 is of the order of 35° C. From the data presented in previous reports this temperature is near the maximum of the preferred temperature zone. As was observed by Glick (1939) for horse serum ChE the rate of hydrolysis decreases rapidly at temperatures in excess of the optimum, indicating a rapid inactivation of the enzyme at higher temperatures.

Inhibition of ChE by Certain Organic Insecticidal Compounds:—In these experiments the degree of inhibition was determined for a 30-minute period. The inhibitors were placed in the center well at appropriate concentrations in propylene glycol-acetone solutions prior to gassing and equilibration. At the end of the equilibration period the ACh was dumped in from the side arm and after 5 minutes' shaking the stopcocks were closed and measurement of CO<sub>2</sub> evolution commenced. The inhibitors and the ChE were allowed to react for approximately 20 minutes prior to the addition of the substrate. The probit of the percentage inhibition was plotted against log concentration and the concentration for 50% inhibition (IN<sub>50</sub>) determined from the graphs. The results of these experiments are presented in table 3. In comparing the data in table 3 with comparable data for the house fly from Metcalf and March (1950) the IN<sub>50</sub>'s for certain of the compounds are of the same order. Such a comparison is presented in table 4 as well as a comparison of the LD-50 values for the house fly and the oriental fruit fly. These data indicate that as *in vitro* inhibitors the listed compounds are more effective for the oriental fruit fly than the house fly while in general they are less effective as toxicants.

∩ These and the following studies on the mode of action of insecticides are related to line project I-o-3-1. Preliminary Laboratory Testing of Insecticides, in the Chemical Control Section.

FIG. 3. The effect of substrate concentrations on the hydrolysis of ACh, A $\beta$ Ch, and BzCh by oriental fruit fly brain ChE.

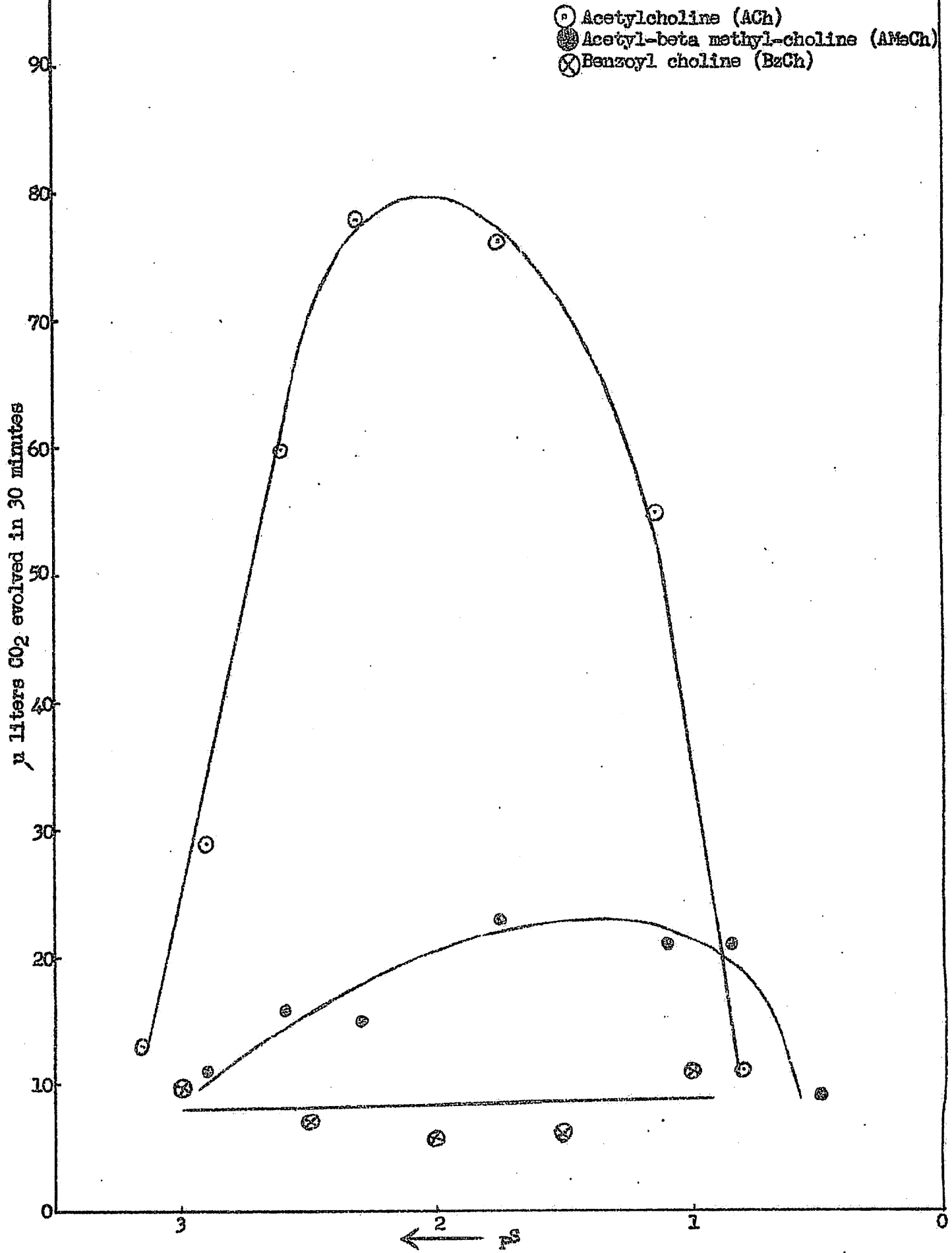


FIG. 4.--The effect of temperature on the hydrolysis of acetylcholine by D. dorsalis brain cholinesterase.

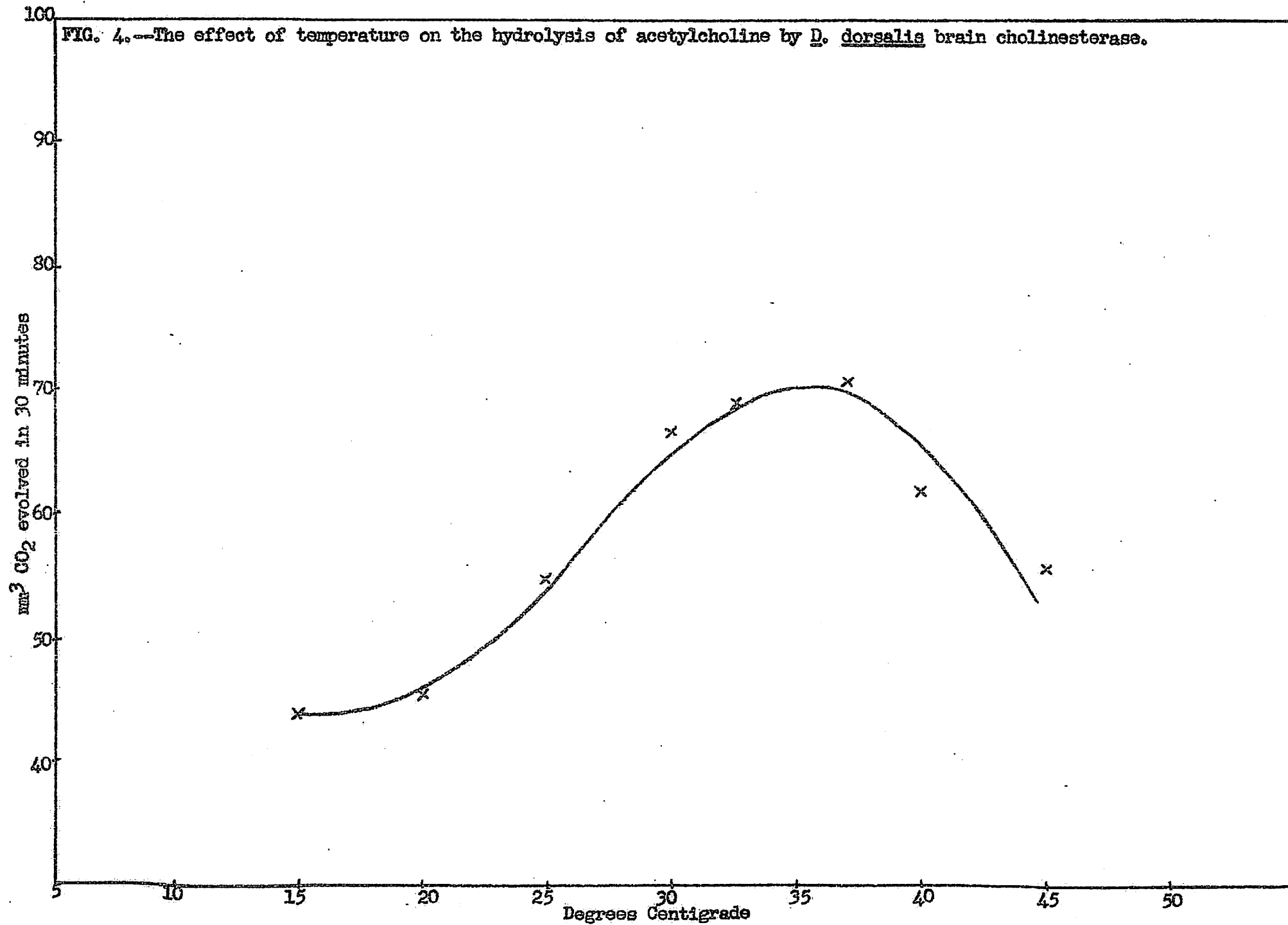


Table 3.—The inhibition of oriental fruit fly brain cholinesterase by certain organic insecticidal compounds.

Compound	Molar concentration for 50 per cent inhibition (1)
1. diethyl p-nitrophenyl phosphate (Para-oxon)	$9.1 \times 10^{-9}$
2. diethyl p-nitrophenyl thiophosphate (Parathion)	$2.3 \times 10^{-7}$
3. di-isopropyl p-nitrophenyl phosphate	$1.5 \times 10^{-8}$
4. di-isopropyl p-nitrophenyl thiophosphate	$3 \times 10^{-6}$
5. ethyl p-nitrophenyl thiobenzene phosphonate (EPN)	$4.0 \times 10^{-6}$
6. tetraethyl pyrophosphate (TEPP)	$7.5 \times 10^{-10}$
7. tetraethyl dithionopyrophosphate	$5.2 \times 10^{-8}$
8. 1-phenyl-3, methyl pyrazol (5) dimethyl carbamate G-22008	$2.0 \times 10^{-8}$

(1) In vitro concentration determined from a 30-minute reaction period.

Table 4.—The comparative effectiveness of certain organic phosphates as in vivo toxicants and in vitro inhibitors of ChE for the oriental fruit fly and the house fly.

Compound	<u>IN<sub>50</sub> House fly</u> (1)	<u>LD-50 House fly</u> (3)
	<u>IN<sub>50</sub> D. dorsalis</u> (2)	<u>LD-50 D. dorsalis</u>
1. diethyl p-nitrophenyl phosphate	2.9	0.55
2. diethyl p-nitrophenyl thiophosphate	2	0.75
4. diisopropyl p-nitrophenyl thiophosphate	6.6	1.2
5. ethyl p-nitrophenyl thiobenzene phosphonate	7.5	0.95

(1) Metcalf and March (1950)

(2) Table 3

(3) Metcalf in Chemical Control Project report Oct.-Dec., 1949.

This evidence further suggests some differences in the reactions of the cholinesterase of the oriental fruit fly and the house fly. The reactions between ChE and these inhibitors, such as suggested by Brauer (1948) or Burgen (1949) may well be effected by other biochemical systems in the organism. In the case of in vivo reactions it is necessary to take into account such factors as rate of penetration, distribution to the site or sites of action and the rate of detoxification, if any.

A further suggestion as to the different reactions of the various species to such toxicants is proposed by Lord and Potter (1950) and (1951) when they present evidence to indicate that these compounds may inhibit other esterases.

These data indicate the necessity for extending these investigations to in vivo conditions and to other biochemical systems. The inhibition exhibited by G-22008, a dimethyl carbamate, is of unusual interest. This material has been found to be a very effective oriental fruit fly insecticide in studies conducted by the Chemical Control project.

Competitive Action of Substrate and Inhibitors:--Burgen (1949) reported on the competitive action between acetylcholine and TEPP. He reported that a much greater degree of inhibition was observed when the inhibitor was allowed to react with the enzyme prior to the addition of the substrate. When the inhibitor was added after the substrate the concentration had to be increased by a factor of 100 before comparable degrees of inhibition were observed.

Three methods of addition of the reactants were investigated to determine the degree of competition between the substrate (ACh) and various ChE inhibitors. (1) Various concentrations of the inhibitors were allowed to react with the enzyme preparation for approximately 30 minutes before the addition of the substrate. (2) The inhibitors and the substrate were mixed together and added simultaneously. (3) The substrate was allowed to react with the enzyme 30 minutes before the addition of the inhibitors. In these experiments the inhibitors were made up in propylene glycol-acetone solutions. Due to the problem of temperature equilibration the measurement of gas evolution was started at the time the inhibitor was added. In the case of prior addition of the substrate the curves were corrected for the gas evolved during the 30-minute exposure of enzyme and substrate by values determined from the uninhibited enzymatic hydrolysis.

In preliminary experiments it was observed that the reactions did not reach a maximum until approximately 2 1/2 hours as shown in figure 5 for para-oxon at  $9.2 \times 10^{-8}$  molar concentration. The data in table 5 were determined at 2 1/2 hours from graphs similar to figure 5. These data suggest that within their effective ranges compounds 1, 2, 7, and 8 are the best inhibitors.

In all cases the prior addition of substrate materially reduces the degree of inhibition. The best indication of competitive ability is given by the values for the simultaneous addition of substrate and inhibitor. These values indicate the above series for competitive action. As pointed out by Burgen (1949) the in vivo conditions are similar to those in vitro when the substrate is present prior to the inhibitors. Under these conditions the same compounds, i.e., 1, 2, 7, and 8 appear more effective.

FIG. 5.--The competition between inhibitor and substrate in reacting with the cholinesterase of the oriental fruit fly brain.

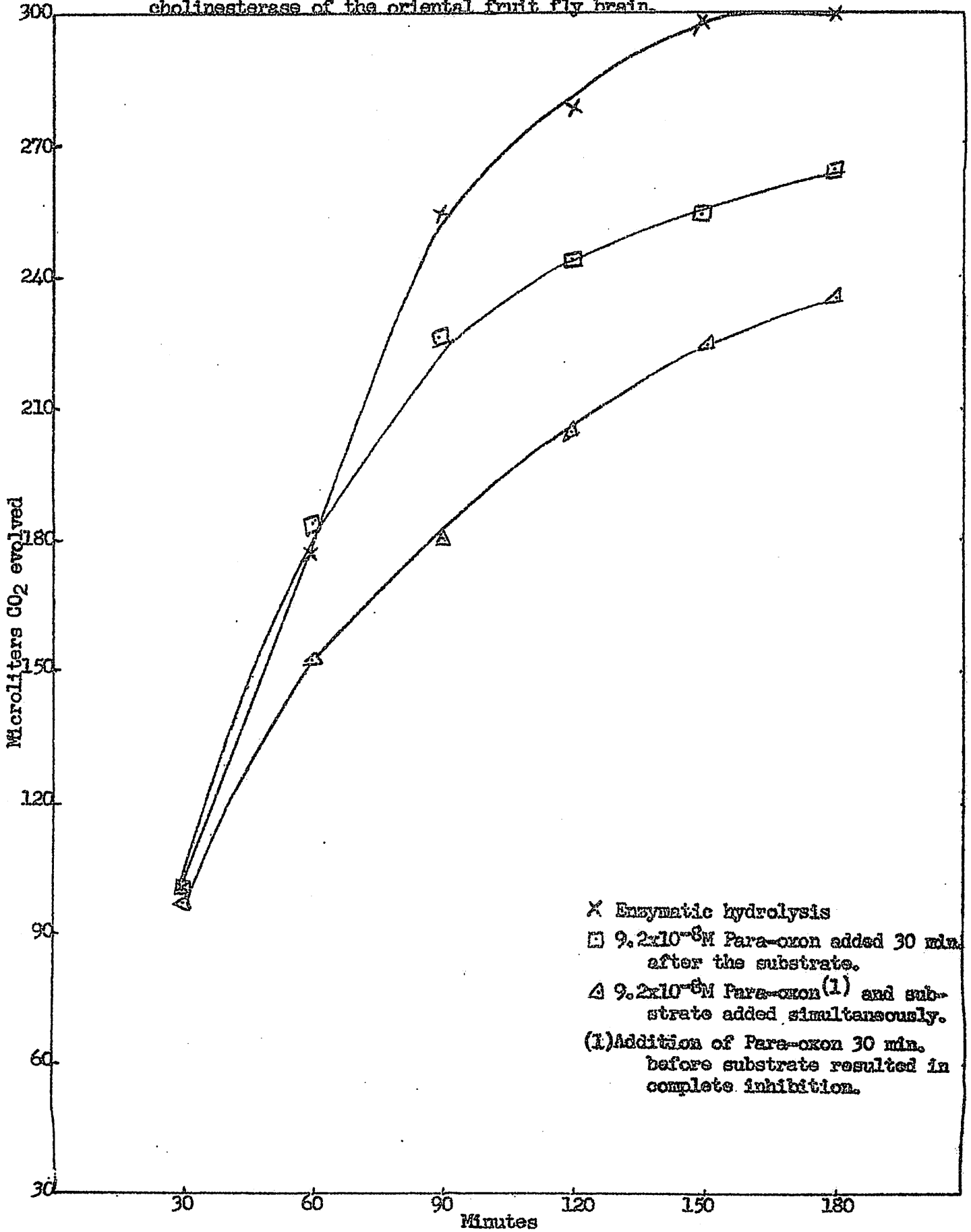


Table 5.--Action of various inhibitors on the cholinesterase of the oriental fruit fly in the presence of acetylcholine as a substrate<sup>(1)</sup>.

		Per Cent Inhibition <sup>(5)</sup>		
		Inh 1st <sup>(2)</sup>	Sub Inh <sup>(3)</sup>	Sub 1st <sup>(4)</sup>
1. diethyl p-nitrophenyl phosphate (Para-oxon)	9.2x10 <sup>-7</sup> M	100	100	53
	9.2x10 <sup>-8</sup> M	100	23	13
	9.2x10 <sup>-9</sup> M	36	0	0
2. diethyl p-nitrophenyl thiophosphate (Parathion)	2.3x10 <sup>-5</sup> M	90	89	62
	2.3x10 <sup>-6</sup> M	90	47	37
	2.3x10 <sup>-7</sup> M	67	16	0
3. diisopropyl p-nitrophenyl phosphate	2.0x10 <sup>-6</sup> M	100	86	67
	2.0x10 <sup>-7</sup> M	95	20	0
	2.0x10 <sup>-8</sup> M	70	2	0
4. diisopropyl p-nitrophenyl thiophosphate	2.0x10 <sup>-4</sup> M	95	73	47
	3.0x10 <sup>-5</sup> M	100	22	2
	4.0x10 <sup>-6</sup> M	58	9	0
5. ethyl p-nitrophenyl thiobenzene phosphonate (EPN)	3.0x10 <sup>-5</sup> M	100	33	17
	6.0x10 <sup>-6</sup> M	66	5	1
	3.0x10 <sup>-6</sup> M	33	0	0
6. tetraethyl pyrophosphate (TEPP)	1.5x10 <sup>-8</sup> M	97	56	33
	1.5x10 <sup>-9</sup> M	58	8	0
	1.5x10 <sup>-10</sup> M	5	4	0
7. tetraethyl dithionopyrophosphate	1x10 <sup>-5</sup>	95	96	56
	1x10 <sup>-6</sup>	68	53	46
	1x10 <sup>-7</sup>	50	7	0
8. 1-phenyl-3 methyl pyrazol <sup>(5)</sup> dimethyl carbamate (G-22008)	2.0x10 <sup>-6</sup>	93	89	64
	2.0x10 <sup>-7</sup>	52	42	17
	2.0x10 <sup>-8</sup>	24	7	0

- (1) In vitro tests at 37° C. with a substrate concentration of 5x10<sup>-3</sup>M.  
(2) Inhibitor allowed to react with enzyme for 20 min. prior to addition of substrate.  
(3) Inhibitor and substrate added to enzyme simultaneously.  
(4) Substrate allowed to react with enzyme for 20 min. prior to the addition of inhibitor.  
(5) Data represent values at the end of a 2-1/2 hour period.

The Effects of Temperature on the Competitive Action of Substrate and Inhibitors.—The effect of temperature on the hydrolysis of ACh by ChE has been described in a previous section. The range of temperatures studied here are below the optimum since higher temperatures may inactivate the enzyme. These experiments were carried out in the same manner as the previous experiments except at temperatures of 15° and 25° C. The data from these experiments are shown in table 6. Regardless of the order of addition of the reactants compounds 1, 7, and 8 show the least effects of lower temperatures. These data suggest that the  $Q_{10}$ 's for the reactions between these compounds and the ChE are higher than for the reaction between ChE and ACh.

It is desirable to extend these studies to in vivo conditions and to take into account the effects of other biochemical systems that may be active in other parts of the body.

The inhibition of ChE Following Topical Applications of Para-oxon.—In this experiment six groups of 25 flies each were treated with topical applications of para-oxon in propylene glycol solutions on the dorsal aspect of the thorax. Two concentrations of 0.1/ $\mu$ g and 0.05  $\mu$ g/ fly were used. The volume of solvent was 1mm<sup>3</sup>. At varying time intervals the heads were removed and prepared for manometric assays of ChE activity. Controls for uninhibited ChE were obtained from flies treated with 1mm<sup>3</sup> of propylene glycol.

The results of this experiment are presented in table 7. While the dosages employed are excessive, the data indicate a rapid penetration and inactivation of the brain ChE when the experiments are conducted at room temperature. These tests will be extended to include lower temperatures and other organic insecticides.

The Effects of Diet on ChE Activity:—Flies provided with sugar and water diets were checked against those with a hydrolyzed protein added to the diet. We could observe no differences in the rate of hydrolysis on the degree of inhibition. These tests represent in vitro conditions. Modification of the test conditions to conform with the preceding section may provide a better estimate of the effects of diet on the ChE activity of the oriental fruit fly. In the studies of the Chemical Control project, protein-fed flies have shown a definite resistance to insecticides which act as inhibitors of ChE activity.

Table 6. The effects of temperature on the action of various inhibitors on the cholinesterase of the oriental fruit fly in the presence of acetyl-choline as a substrate.

Compound	PER CENT INHIBITION								
	Inhibitor added first			Substrate & Inhibitor simultaneously			Substrate added first		
	15°C.	25°C.	37°C.	15°C.	25°C.	37°C.	15°C.	25°C.	37°C.
1. diethyl p-nitrophenyl phosphate $1.8 \times 10^{-9}M$	100	100	100	59	69	100	47	40	53
2. diethyl p-nitrophenyl thiophosphate $2.3 \times 10^{-6}M$	84	80	90	18	28	47	8	8	37
3. diisopropyl p-nitrophenyl phosphate $2.0 \times 10^{-6}M$	86	100	100	48	69	86	5	36	62
4. diisopropyl p-nitrophenyl thiophosphate $2.0 \times 10^{-4}M$	100	85	95	27	31	73	9	16	47
5. ethyl p-nitrophenyl thiobenzene phosphonate $3 \times 10^{-5}M$	73	95	100	14	20	33	0	0	17
6. tetraethyl pyrophosphate $1.5 \times 10^{-8}M$	86	85	97	7	31	56	0	0	33
7. tetraethyl dithionopyrophosphate $1 \times 10^{-6}M$	93	90	86	66	70	72	28	34	49
8. 1-phenyl-3 methyl pyrazol (5)-dimethyl carbonate $2 \times 10^{-6}M$	93	93	93	82	89	89	53	49	64

Table 7. The in vivo inhibition of oriental fruit fly brain cholinesterase following topical application of Para-oxon in propylene glycol solution.

Time after treatment	Per cent inhibition <sup>(1)</sup>	
	0.05 $\mu$ g fly	0.1 $\mu$ g fly
Control	0	0
20 minutes	62	90
45 minutes	63	95
90 minutes	100	---

(1) Determined manometrically for a 30-minute period.

#### REFERENCES

- Brauer, R. W. 1948. Inhibition of the cholinesterase activity of human blood plasma and erythrocyte stromate by alkylated phosphorus compounds. *Jour. Pharm. Exptl. Therap.*, 92 (2): 162-172.
- Burgen, A. S. V. 1949. The mechanism of action of anticholinesterase drugs. *Jour. Pharm. Chemotherap.*, 4: 219-228.
- Glick, D. 1939. The effects of temperature on cholinesterase activity. *Proc. Soc. Exptl. Bio. Med.*, 40: 140.
- Lord, K. A., and C. Potter. 1950. Mechanism of action of organo-phosphorus compounds as insecticides. *Nature*, 166: 893.
- Lord, K. A., and C. Potter. 1951. Studies on the mechanism of insecticidal action of organo-phosphorus compounds with particular reference to their antiesterase activity. *Ann. Appl. Biol.*, 38 (2): 485-507.
- Metcalf, R. L. and R. B. March. 1950. Properties of acetylcholine esterases from the bee, the fly, and the mouse and their relation to insecticidal action. *Jour. Econ. Ent.* 43 (5): 670-677.