Further Studies on the Insect Metabolism of Octamethylpyrophosphoramide

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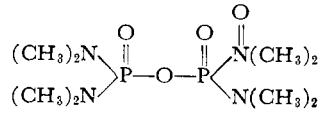
In order to study the metabolism of octamethylpyrophosphoramide (schradan) and establish the reason for its selective toxicity, the identity and nature of the active metabolite produced from it by numerous insect tissues have been studied. The active metabolite, which is a powerful anticholinesterase, is identical with one of the products of permanganate oxidation of schradan. The properties of this substance have been examined. Possible pathways for schradan breakdown which could account for its ineffectiveness in many insects have been studied, but none have been found. The work represents a further small step in understanding of insecticides, which may eventually enable us to produce new insecticides on a rational basis to fulfill many selective requirements.

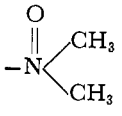
SEVERAL TISSUES FROM VARIOUS INSECTS have the capacity to convert octamethylpyrophosphoramide (schradan) to a powerful anticholinesterase (*27*). Hartley (*14*) first suggested that the corresponding product following liver or plant conversion or permanganate oxidation was



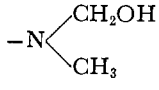
Casida *et al.* (*9*) have published evidence that the plant and mammalian active metabolites are identical, on the basis of partition coefficients and anti-enzyme activity, although one of their results seems rather unlikely. Both metabolites resembled a product of permanganate oxidation of schradan in producing formaldehyde in acid conditions. The identity of the insect metabolite with that of plant and liver was also claimed upon similar evidence, but no quantitative results were given. The chemical and biological properties of schradan have recently been reviewed at some length (*10*, *18*). The present paper is concerned with the identity of the insect metabolite and an investigation of some other possible metabolic pathways.

Materials and Methods

Permanganate oxidation of schradan was accomplished by adding up to 1 gram of schradan [prepared and purified as described in a previous paper (*30*)] to a mixture of 100 ml. of 0.67*M* phosphate buffer, pH 7.4, and 50 ml. of 0.2*M* potassium permanganate. The mixture was allowed to stand until the permanganate was largely decolorized (about 1 hour), then extracted four times with chloroform. The chloroform solution was dried over sodium sulfate and stored at −20° C. This treatment gave two products, one of which had a half life in water at pH 7 and 25° C. of about 10 hours, and another which was much more stable. The less stable component may be the monophosphoramide oxide of schradan, but it could be the methylol isomer of this compound—i.e., with the



grouping isomerized to:



In this paper the term "schradan oxide" refers to this unstable component, whatever its identity.

The insect material was prepared by incubating 25 mid- and hind-guts of roaches (*Periplaneta americana*) in 25 ml. of 0.067*M* phosphate buffer, pH 7.0, containing 0.2 gram of schradan and 2 drops of toluene. Water-saturated air was bubbled through this mixture overnight, then it was filtered and 10 ml. of 5% mercuric chloride were added to precipitate the protein (this method had to be used because of the instability of the product in acid). The solution was centrifuged and the supernatant extracted four times with chloroform to obtain the products. The chloroform solution was dried over anhydrous sodium sulfate and stored at −20° C. When solutions in other solvents were required, the chloroform was taken off under vacuum and the residue dissolved in the appropriate solvent.

Phosphorus analyses were made by the ferrous sulfate–ammonium molybdate method of Rockstein and Herron (*28*) using a Klett-Summerson photometer. Trimethylamine oxide analyses were carried out by a method developed from data given by Ronold and Jakobson (*29*). Five grams of the ground sample were incubated overnight with 50 ml. of water, then centrifuged, and the precipitate was washed twice. Supernatant and washings were combined and made to volume. One half of this solution was made alkaline and steam-distilled into 10 ml. of 0.1*N* hydrochloric acid. About 100 ml. were collected, their volume was reduced on the steam bath and made to 10 ml., and the solution was assayed for trimethylamine by Dyer's colorimetric picrate method (*12*). To the other half were added 2 ml. of 0.1*N* sulfuric acid and 1 ml. of a 20% solution of titanous chloride. After standing for 1 hour, the solution was made alkaline and treated as above. The difference between the two assays gave the trimethylamine oxide content. The method gave 95% recovery of a sample of pure trimethylamine oxide.

Serum cholinesterase assays were carried out as described (*30*), except that only 5 minutes elapsed between the addition of water to the inhibitor and the addition of this solution, serially diluted, to the enzyme. This rapidity made it unnecessary to use buffer to dissolve the inhibitor.

Partition coefficients were determined by mechanical shaking of the solvent pair containing the sample for at least 5 minutes, then separation of the phases. The nonaqueous phase was dried with sodium sulfate, and a known volume was evaporated to dryness and dissolved in a known volume of water. Portions were then submitted to anticholinesterase assays against serum cholinesterase. Corrections were made for the time each fraction was exposed to water, using the half life given below.

Paper chromatography was carried out by an adaptation of a method of March, Metcalf, and Fukuto (*20*). Conventional rectangular paper strips

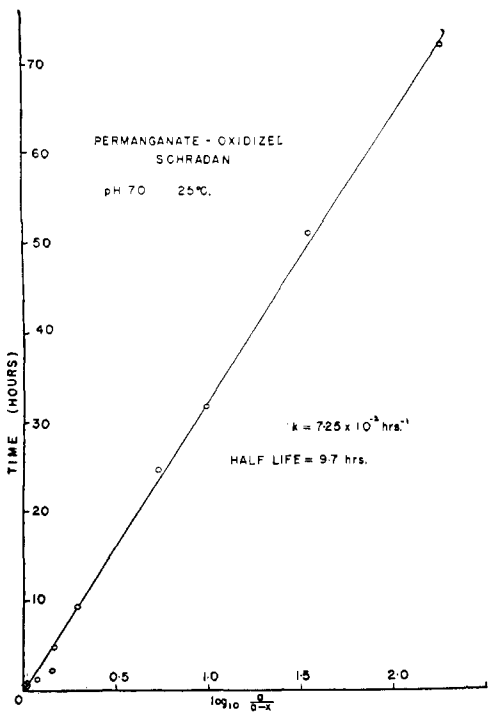
were used, and the glycol impregnation was carried out immediately before use by pouring down the paper a 15% solution of glycol in acetone. The papers were allowed to dry in air for a few minutes and then the compounds in chloroform solution were applied with micropipets.

Identity of Insect Metabolite

Paper chromatography showed that permanganate oxidation of schradan produces two new compounds. One of these is destroyed by equilibrating the chloroform solution of the oxidation products with 10% sodium hydroxide for 3 hours. This less stable component appears to be identical with the compound produced by the conversion of schradan by roach guts. The enzymic assay described below showed the less stable component to be destroyed in 15 minutes by 0.01*N* alkali. This may be due to a lesser sensitivity of the enzymic technique compared with the chromatographic, and to a protective effect of the chloroform in the chromatographic procedure.

The solvent was allowed to travel the full length of the paper (43 cm.) in an attempt to maximize the short travel of the components. The travel of the components was difficult to duplicate in the different runs (the schradan movement varied between 4.8 and 12.0 cm.) and even on a single paper there was sometimes a variation in the travel of identical spots. These problems arose from difficulty in getting reproducible and homogeneous glycol impregnation, even though the acetone method gave

Figure 1. Hydrolysis of permanganate-oxidized schradan



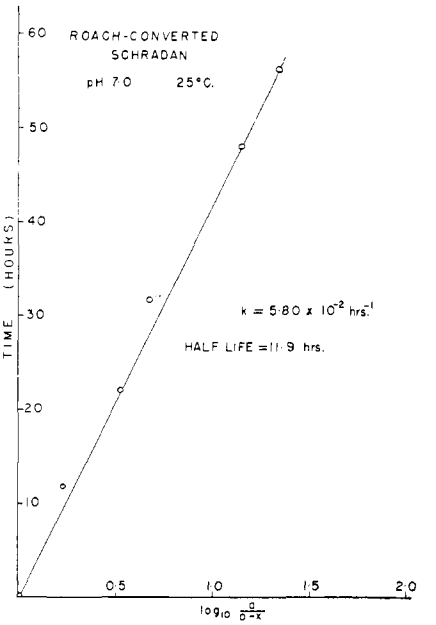


Figure 2. Hydrolysis of roach-converted schradan

much better results than the original ethyl alcohol method (*20*). For these reasons, and because schradan itself was present in all samples, the values calculated were not *Rf*'s, but ratios of schradan travel to component travel. This ratio will be 1 for schradan and >1 for all the others, as none traveled further than schradan.

The products of roach conversion gave two spots, of ratios 1 and 1.66 ± 0.22. The products of permanganate oxidation gave three spots of ratios 1, 1.48 ± 0.12, and 3.48 ± 0.91. On treatment with alkali as described above, the 1.48 spot disappeared. The ratios of 1.66 and 1.48 are not significantly different at the 5% probability level, using Fisher's *t* test. No spot was found when extracts of control roach homogenates, untreated with schradan, were used.

The half lives of the chemical and roach products were determined by incubating buffered aqueous solutions at 25.0° C. and removing portions at intervals for anticholinesterase assay. The results gave a linear relationship when plotted according to the first-order reaction equation, and are shown in Figures 1 and 2. Each point was obtained from interpolation from a graph showing the anticholinesterase activity of four dilutions of an aliquot. As both plots are linear and pass through the origin, it is apparent that in each case only one effective anticholinesterase is present, and therefore the stable component in the permanganate oxidation product does not interfere in anticholinesterase assays. The *k* values were determined by calculating the regression on the points. They are seen to be similar for the two products.

Brief studies of the stability of the two products under more extreme conditions showed that both were completely destroyed in 15 minutes when in 0.01*N* alkali at 25° C. The half lives in 0.01*N* acid at 25° C. were about 2.9 minutes for the permanganate and 2.6 minutes for the roach product.

Ultraviolet spectra were obtained for the two products, and are given in Figures 3 and 4, along with that of schradan. Both chemical and biological conversion have the effect of enormously increasing the absorption in the 240-mμ region and introducing a new peak at 275 mμ. However, the new peak for the roach product is of different shape from that of the permanganate product, showing an increase in density between 268 and 274 mμ, whereas the permanganate product shows a very slight decrease in density between those wave lengths. The difference in shape was consistently present in several spectral comparisons made at varying concentrations. The conclusion is that although the two products are substantially the same, there is a small but significant difference, probably due to the presence of the stable component in the permanganate-oxidized schradan. Infrared spectra could not be obtained for the low concentration of insect-converted material, so no comparison was possible.

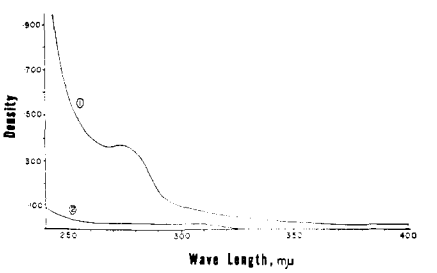


Figure 3. Ultraviolet spectrum of schradan (2) and its roach-conversion product (1)

The pI_{50}'s of the two products against serum cholinesterase were determined in the usual way upon serially diluted samples. The basis for the calculation of the concentration of converted schradan was the chloroform-insoluble phosphorus present when the solution was allowed to stand at pH 7.0 for 3 days, then made alkaline and after 5 minutes extracted four times with chloroform. With this treatment, the schradan oxide should all be hydrolyzed and any schradan or (in the permanganate-oxidized sample) stable component should be completely removed by the chloroform.

The pI_{50}'s found were:

Chemically oxidized schradan	6.36 ± 0.14
Roach-converted schradan	6.45 ± 0.02

These results are not significantly different at the 5% probability level using Fisher's *t* test.

Figure 5 shows a group of curves of

pI against probit of percentage cholinesterase inhibition. The *pI* figures are uncorrected for schradan oxide content (that is why they do not coincide). The lines should be parallel if the two products are identical, and this appears to be so on inspection. However, when the regressions of such lines from 13 samples of each product were determined, and the results pooled using the method of weighted means, the slopes were:

Chemically oxidized schradan	1.50 ± 0.016
Roach-converted schradan	1.39 ± 0.023

This difference is significant at the 1% probability level, when the *t* test is used.

Partition coefficients for the two products were determined, with the results given in Table I. With the method used an error of 3% in the determination of per cent inhibition gives an error of 12% in the estimate of inhibitor concentration at the pI_{50} level, or 18% at the pI_{30} or pI_{70} levels. This is apparent from a scrutiny of Figure 5. However, the accuracy is increased by using several points for the estimate.

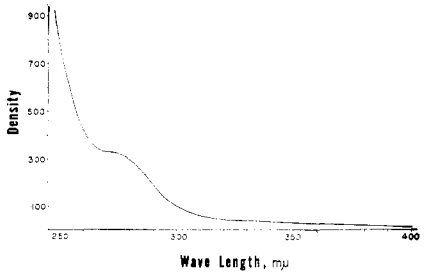


Figure 4. Ultraviolet spectrum of permanganate-oxidized schradan

The agreement for the chloroform-water and benzene-water coefficients is good. There is less agreement between the results for the carbon tetrachloride–water coefficients, and one can only say that they are of the same order. This may arise from the fact that if (as seems probable) schradan partitions much more readily into carbon tetrachloride than does the oxide, the schradan contamination may be serious; in these assays so little oxide is in the organic solvent phase that the serial dilutions prior to addition to cholinesterase only go from 1:1 to 1:10 (ranges of 1:200 to 1:1000 were used for chloroform experiments). Thus, any schradan present may be in sufficient concentrations to affect the enzyme.

The chloroform coefficients agree well with the results given by Casida *et al.* (*8*) for mammalian and plant metabolites (1.58 and 1.68, respectively), but the carbon tetrachloride coefficients are totally different: They give 0.22 for both. This figure appears anomalous, as it suggests that the metabolite is less polar than schradan (whose coefficient they give as 0.004), whereas their other two sets of coefficients (and those of the present authors) show that the metabolite is more polar, as one would expect from the introduction of an electrophilic group. If the figure were correct, the the metabolite could be separated almost completely from schradan by one partitioning from water into carbon tetrachloride. In fact, the separation requires countercurrent distribution (*9*).

Taken individually, few of the results given above offer convincing proof of the identity of schradan oxide with the roach conversion product. Considering, however, the over-all agreement in comparisons of very different properties. there is a high probability that the identity exists, and probably the slight differences noted are due to interference by the great excess of schradan in the roach product or by the stable component in the permanganate product.

Table I. Partition Coefficients for Converted Schradan

	Biological Product	Chemical Product
Chloroform-water	1.72	1.77
Carbon tetrachloride–water	0.0014	0.00092
Benzene-water	0.130	0.124

Effectiveness of Converted Schradan

In a previous paper (*27*) data were given for the relative effectiveness of various tissues in converting schradan, and on this basis a hypothesis was proposed to account for the selective action of the insecticide. The figures were expressed in terms of chlorinated schradan, the only conversion product then available. The results have now been expressed in terms of the schradan oxide and are given in Table II. The general picture remains the same.

The above hypothesis suggested that the only fraction of schradan which was effective in poisoning was that which was converted in the nerve cord, owing to the hydrolyzability or inability to penetrate to the site of action of converted schradan produced in other parts of the body. If this is correct, injected schradan oxide should be nontoxic to insects. This has been confirmed in the case of the roach, no mortalities being observed at doses containing up to 100 γ per gram of schradan oxide. The experiment cannot yet be tried with susceptible insects, because of the contamination of the oxide with schradan, which in their case would produce its own toxic effect. The hypothesis is also in harmony with the findings of Metcalf and March (*23*), who found that the heat isomerization of several thionophosphates gave compounds which were more potent anticholinesterases yet less toxic to the mouse and housefly. They attributed this to the readier susceptibility of the isomers to destructive hydrolysis.

The alternative hypothesis concerning schradan action was given by Casida and Stahmann (*10*), that the conversion product is a poor inhibitor of the cholinesterase of resistant insects. The effectiveness of the chemically produced schradan oxide against the cholinesterase of three resistant insects was tested in vitro. The results were:

Roach (nerve cord brei) pI_{50} = 4.9
Housefly (head brei) pI_{50} = 6.1
Blowfly (head brei) pI_{50} = 4.9

From these results it seems unlikely that ineffective antienzyme activity of the oxide is the cause of the resistance of these insects. The results of March *et al.* (*27*) on the cholinesterase reduction in poisoned insects are also opposed to this hypothesis.

Routes for Schradan Degradation

The observed differential toxicity of schradan could arise from the presence in resistant insects of systems capable of hydrolyzing schradan or its conversion product (which is assumed to be schradan oxide). Such systems might also explain the reported failure to activate schradan of the breis of tissues which are effective when whole or sliced (*13*). Recently systems of widespread occurrence have been demonstrated capable of hydrolyzing diisopropyl fluorophosphate (*22, 25*), tabun [(dimethylamido) ethoxy phosphoryl cyanide] (*4*), diethyl *p*-nitrophenyl phosphate (*1*), and tetraethyl pyrophosphate (*24*).

Schradan (final concentration 0.003*M*) was incubated with buffered whole roach breis (final concentrations up to 8%) for periods varying from 10 minutes to 29 hours. The protein was then precipitated with 30% trichloroacetic acid and the filtrate assayed for orthophosphate. None was found, except that due to endogenous orthophosphate. Under similar conditions sodium pyrophosphate yielded 100 γ per

Figure 5. Anticholinesterase activity of converted schradan

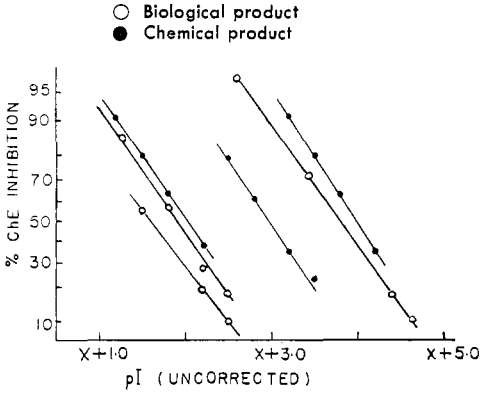


Table II. Relative Effectiveness in Converting Schradan

(Per unit body wt.)

	Resistant										Susceptible							
	Roach Adult				Mealworm Larva				Mourning Cloak Larva				Squash Bug Adult				Milkweed Bug Adult			
	Mean Weight, Mg. ± S.E.M.																			
	737 ± 17				231 ± 7				Variable				93 ± 6				50 ± 3			
	Mean	S.D.	S.E.M.	N	Mean	S.D.	S.E.M.	N	Mean	S.D.	S.E.M.	N	Mean	S.D.	S.E.M.	N	Mean	S.D.	S.E.M.	N
Fore-gut	9.7	5.0	2.1	6																
Mid-gut	65.3	32.8	8.2	16	58.0	20.8	7.4	9	44.8	21.4	7.1	9	181.6	82.6	22.0	14	315.0	108.0	28.9	14
Hind-gut	76.2	18.4	6.5	8																
Fat body	152.9	90.0	21.8	17	495.5	245.2	63.3	15	309.3	75.5	30.8	6	42.9	25.3	6.7	14	97.8	56.7	16.4	12
Nerve cord	3.1	2.0	0.8	6	...	...	...	...	...	...	...	...	2.8	2.2	0.9	6	...	...	...	...
Cuticle	13.2	4.3	1.9	11	0	...	...	6	...	...	...	...	0	...	...	6	0	...	...	6
Muscle	0	...	...	6	...	...	...	...	...	...	...	...	0	...	...	6	0	...	...	6
Residuum	...	...	...	...	...	...	...	...	147.6	37.4	18.7	5	...	...	...	...	...	...	...	...

S.D. Standard deviation *S.E.M.* Standard error of mean *N.* Number of replicates

ml. of orthophosphate after a 5-hour incubation (*26*). It was concluded that a pyrophosphatase existed but that it was not active against schradan. Purified pyrophosphatase from yeast is also inactive against organic pyrophosphate esters (*19*). In a further experiment using a manometric technique it was observed that whole human plasma, which contains all the phosphatases enumerated above, did not hydrolyze schradan.

Schradan oxide had its anticholinesterase activity reduced when various whole insect breis were included with the enzyme-inhibitor system. Table III shows the results.

Table III. Effect of Whole Insect Breis upon Anticholinesterase Activity of Schradan Oxide

(All figures are % cholinesterase inhibition)

Insect Brei	Brei Absent	Brei Present
Roach	31:31	24:24:24
Milkweed bug	35:35	23:24:23
Housefly	64:63	45:54:66

Similarly, the cholinesterase inhibition due to converted schradan produced by a single roach mid-gut was reduced from 90 to 71% by the addition of a brei of one roach mid-gut. These results suggested that either the breis contained a system that hydrolyzed the schradan oxide, or the breis presented alternative phosphorylatable substrates to the oxide, thus competing with cholinesterase for phosphorylation. The effectiveness of breis of milkweed bug (a schradan-susceptible insect) in reducing cholinesterase inhibition showed that such results could not explain the differential toxicity of schradan.

The possibility of a schradan oxide–hydrolyzing enzyme was considered first. In these experiments schradan would not interfere, as the previous results had shown. Because the oxide is hydrolyzable even in the absence of breis, and especially in the acid conditions necessary for protein precipitation, its concentration had to be kept low; otherwise the increment of orthophosphate due to an enzymic hydrolysis would be small in comparison to the total orthophosphate production (this contrasts with later experiments upon protein phosphorylation). In order to reduce and control the time of exposure of the oxide to acid conditions, the following method was used: The incubation with brei was concluded by placing in a boiling water bath for 1.5 minutes. Then to each tube was added at zero time 2 ml. of 30% trichloroacetic acid, filtering was started, and a 5-ml. aliquot was taken from the filtrate. Phenolphthalein was added, then at 5 minutes from zero it was titrated to a faint pink with 1*N* sodium hydroxide. At 60 minutes from zero it was extracted four times with chloroform, leaving only orthophosphate. A 5-ml. aliquot was refluxed with 1 ml. of *N* sulfuric acid for 1 hour, then made to 10 ml. and assayed for orthophosphate.

Incubation times of 1 and 15 hours were used, and no soluble orthophosphate was produced above that due to nonenzymic hydrolysis of the oxide and endogenous orthophosphate (oxide alone 10, 11, 10 γ per ml.; brei alone: 15, 15, 15 γ per ml.; brei plus oxide: 22, 25, 25 γ per ml.). It was concluded that no schradan oxide–hydrolyzing system was present.

The possibility that the results of Table III were due to presentation of phosphorylatable substrates by the breis was next examined. This involved incubating the oxide with breis, precipitating the proteins (as the most likely phosphorylatable material), and assaying them for phosphorus. Precipitations were made with trichloroacetic acid, and the precipitate was filtered off, digested with perchloric and nitric acids (*3*), and analyzed for phosphorus. Interferences could arise from (1) the phosphorylation of protein by schradan itself, (2) the adsorption of schradan or the hydrolysis products of the oxide upon the precipitated protein, and (3) the adsorption of the oxide upon the precipitated protein. If possibility (3) occurred, it would be most difficult to differentiate from true protein phosphorylation, except that such an adsorption should not be greater in extent than for (2).

The first experiments used schradan oxide at levels similar to those in the previous work, and failed to give conclusive results. As the suggested competition with cholinesterase for phosphorylation was not strong (Table III), the phosphorylatable sites were either few or difficulty phosphorylatable. Very high concentrations of oxide were therefore used, and at these levels distinct increases in phosphorus in the protein precipitates were found. At these levels the possible interferences outlined above would also be maximal and their extent had to be measured. Interference by schradan was checked by comparing schradan and its oxide at identical concentrations. Adsorption of hydrolysis products was checked by hydrolyzing a sample of schradan in alkali (8 hours at 100° C. in 0.01*N* sodium hydroxide) and using it at the same concentration. Experiment 1 (Table IV) showed that schradan and its hydrolysis products made identical contributions to the protein precipitate. This suggests a nonspecific adsorption of both, with no phosphorylation by the schradan.

Experiments 2 and 3 (Table IV) showed that the oxide contributed more phosphorus than its hydrolysis product. If it is assumed that the hydrolysis products of schradan oxide behave like those of schradan, this contribution may be taken as nonspecific adsorption, and the oxide is additionally effective because it phosphorylates some proteinaceous component.

Therefore, it is concluded that the effects described in Table III are attributable to the presence of phosphorylatable sites in the breis which competed with cholinesterase for the schradan oxide, and thus reduced the effectiveness of enzyme inhibition. A similar conclusion was reached by Hobbiger (*15*) with respect to the low sensitivity of intestinal cholinesterase to tetraethyl pyrophosphate in vitro, but the evidence was only indirect. Jandorf and McNamara (*17*) using radioactive techniques showed that

Table IV. Phosphorylation of Breis by Schradan and Products

Contents	Ortho-P in Protein (Digested and in 25 Ml.), γ/Ml.	Conclusions
Experiment 1		
A. Brei, water, buffer	7.2 ± 0.14	B = C
B. Brei, schradan, buffer	8.8 ± 0.62	B and C > A at P = 0.05
C. Brei, hydrolyzed schradan,	8.8 ± 0.55	
Experiment 2		
A. Brei, water, buffer	10.4 ± 0.10	C > A at P = 0.01
B. Brei, schradan oxide, buffer	13.2 ± 0.83	B > C only for P = 0.06
C. Brei, hydrolyzed schradan oxide, buffer	12.1 ± 0.31	
Experiment 3		
Like Experiment 2	4.9 ± 0.29	B > C at P < 0.01
	7.6 ± 0.15	C > A only for P = 0.4
	5.3 ± 0.36	

Breis were of whole roach; final concentration = 5%. Buffer. Veronal, pH 7.0. Incubation. 4 hours, 25° C. Final concentration of schradan (or products) = 0.026*M*. Statistical comparisons by use of *t* test.

diisopropyl fluorophosphate incubated with brain tissue phosphorylated much material other than cholinesterase.

The absence of a schradan-hydrolyzing enzyme in breis is evidence against such a system in intact organisms, but does not preclude it. Nevertheless, known hydrolyzing enzymes do not have the coenzyme or organizational requirements common in oxidases; therefore the evidence for their absence is strong.

Identity of Schradan-Converting System

In a previous paper (*27*) it was suggested that the enzyme there called "trimethylamine oxidase" might be responsible for the conversion of schradan to its oxide. The same suggestion was made independently by Kilby (*18*). The existence of such an enzyme in sterile liver homogenates, effective to a very small extent, has recently been indicated (*2*). If such a system were present in insects, small amounts of trimethylamine oxide might be expected in excreta (as an end product of quaternary nitrogen metabolism) as Chiancone (*11*) found in mammals. The amounts might be increased by injection of choline, by analogy with the findings of Huerga and Popper (*16*) in mammals.

Analyses were therefore carried out on a quantity of silkworm (*Cecropia*) larvae excreta. Further analyses were made on the excreta of two silkworms (weights 14.4 and 12.9 grams) that had been injected previously with 1.5 mg. each of choline chloride. No trimethylamine oxide was found. The method was such that it would readily have detected quantities of 2.5 γ per gram of dried excreta. As a large proportion of the excreta appeared to consist of almost intact leaf fragments, the possibility existed that the quantity of true excreta being assayed was very small. Uric acid was therefore assayed (as it is the usual end product of insect nitrogen metabolism). A modification of Brown's procedure (*7*) was used, involving extraction with petroleum ether, followed by water at 60° C. A Soxhlet extractor was used; for the aqueous extraction the experiment was carried out at reduced pressure. The assays were made by the method of Borsook (*6*). A uric acid content of 1.1% was found.

It was concluded that if trimethylamine oxide is present in this excreta, it is to the extent of less than 1/4000 of the content of uric acid, even in insects injected with choline. This is evidence against the existence of "trimethylamine oxidase" in insects.

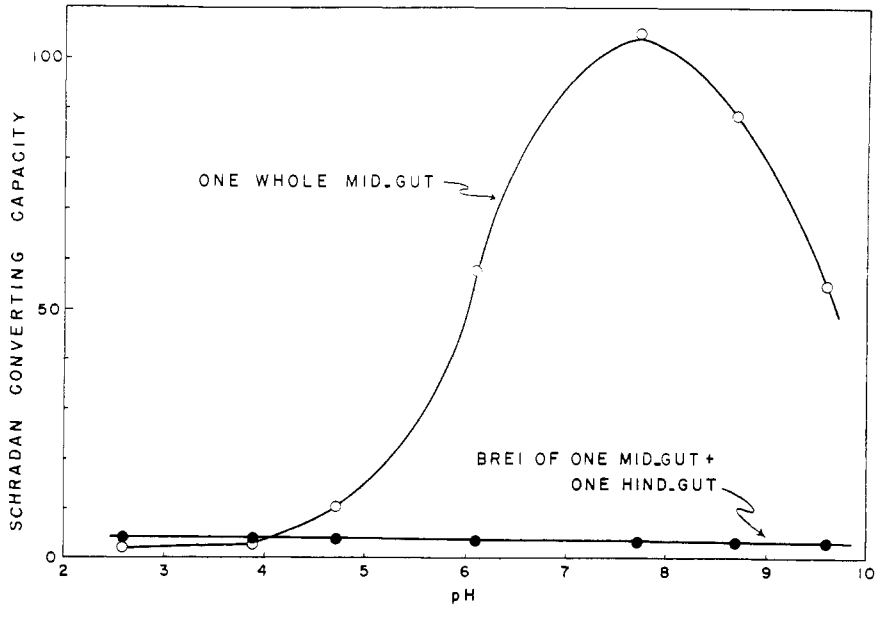
Schradan Conversion by Homogenates

Attempts to prepare an active fortified brei were reported earlier (*27*) and have been continued. So far 16 different combinations of glucose, magnesium, cytochrome C, adenosine triphosphate, nicotinamide, and fluoride have been tried, and also di- and triphosphopyridenenucleotides, ethylenediamenetetraacetic acid (Versene), ferricyanide, and salts of ten common metals. No adequate fortification has resulted. Beck, Stahmann, and Casida (*5*) reported a fortified homogenate of roach caecae, but later Casida *et al.* (*8*) reported ineffective fortification with the same and also with some other components added to liver homogenates.

Nevertheless, it is felt that the problem is one of cofactor dilution rather than, say, enzyme destruction. When the activity of whole mid-gut [calculated as described in (*27*)] was studied as a function of pH, the upper curve in Figure 6 was obtained. When the pH dependence of the small residual activity left after homogenizing was studied, the lower curve was found. The conclusion is that the homogenate activity is pH independent. This appearance would be produced if, after homogenization, the limiting factor were an enzyme cofactor whose effectiveness is pH-independent. (These experiments were made in veronal buffer, and the appropriate corrections for retention were applied.) The possibility also existed that the activity resided in one component of the homogenate—e.g., mitochondria—and that interference with its activity came from another component. Homogenates of roach guts therefore fractionated centrifugally in 0.25*M* sucrose as follows:

A, precipitate at 700 × gravity (probably cell walls, etc.)
B, precipitate at 1500 × gravity (probably nuclei)
C, precipitate at 10,000 × gravity (probably mitochrondria)

Figure 6. Effect of pH upon schradan conversion by roach tissues, whole and homogenized



D, supernatant at 10,000 × gravity (probably soluble and microsomal)

D contained about 50% of the small activity found in the whole homogenate, while A, B, and C shared the other 50% approximately equally. The summation of the net activities of the four components was not greater than the net activity of the whole brei, which indicated that there was no inhibition of one component by another. Fraction D was used in some fortification attempts using nicotinamide, fluoride, magnesium, cytochrome C, and adenosine triphosphate, but it behaved exactly like the whole homogenate.

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RADIATION STERILIZATION

Influence of Gamma Radiation on Proteolytic Enzyme Activity of Beef Muscle

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Approximately 70% of the proteinase activity in beef muscle tissue could be extracted with citrate buffer at pH 9.6. Irradiation with cobalt-60 at dosages of 1.6 × 10^6 rep. reduced the apparent proteinase activity of beef muscle about 50%. This loss occurred largely in the fraction of the enzyme that was extractable at pH 9.6. At lower irradiation dosages (5 × 10^5 rep.) there was little reduction in proteinase activity as measured by liberation of tyrosine from casein substrate. Irradiation reduced the amount of tyrosine extractable from beef, which suggests that the amino acid is changed by irradiation.

Spoilage microorganisms in many foods can be killed by ionizing radiations at dosage levels of 10^5 to 2 × 10^6 rep. (*5, 7, 10, 12*). However, radiation sterilization cannot be applied generally to foods unless enzyme systems that may catalyze undesirable changes during extended storage are also inactivated.

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Studies on purified proteolytic enzyme systems have indicated that these biological catalysts are much more resistant to ionizing radiations than are microorganisms (*3, 10*). However, there have been no investigations on the effect of ionizing radiations on the proteolytic enzyme activity of beef muscle tissue. In fact, very little is known about the proteolytic enzymes in muscle tissue, even though the tenderizing influence of aging meat is usually attributed to the action of these enzymes (*8, 9*). Balls (*2*) reported that the proteinase of beef muscle could be liberated by autolysis of the tissue. He found that the optimal pH for its activity was 4.1 and suggested that it was probably a cathepsin.

The investigations reported here were undertaken to determine the influence of gamma radiation of the over-all proteolytic enzyme activity in beef muscle