Table 1. The *p*H and ethanol concentration of ruminal contents from sheep overfed with cracked wheat.

Time in relation to feeding (hr)	Sheep No. 6*		Sheep No. 7	
	*p*H	Ethanol (μmole/ml)	*p*H	Ethanol (μmole/ml)
−1	6.72	<1	6.60	<1
+6	5.42	2.6	5.55	3.1
14	4.80	3.6	4.65	14.0
22	4.45	13.0	4.88	9.0
28	4.47	18.0	4.90	5.9
30	4.55	19.0	4.85	11.0
37	4.55	33.0	5.25	9.0
46			5.35	6.0
54			5.40	4.1
70			5.80	0

* Sheep died by 46 hours after feeding.

as *n*-propanol, however, have been noted occasionally.

Table 1 gives data on ruminal *p*H and ethanol concentrations after intraruminal dosage of two sheep with 50 g cracked wheat per kilogram of body weight. Sheep No. 6 was a Hampshire cross wether weighing 82 kg and No. 7 was a Cheviot ewe that weighed 54.5 kg. Both sheep were prepared with ruminal fistulas (*5*) and with catheters in the carotid artery (*6*). Both sheep exhibited symptoms typical of ruminants overfed with large amounts of readily available carbohydrate (*2*). Microscopic studies of ruminal contents showed that in both animals, 14 hours after administration of the wheat, ruminal protozoa were no longer motile and there was a decided increase in the proportions of gram positive streptococci and rods. Presumably these were *Streptococcus bovis* and *Lactobacillus sp.* (*7*). Yeast-like cells were not numerous. Sheep No. 6 died 37 to 46 hours after the wheat was administered, while No. 7 recovered, regained its appetite, and had normal rumen motility 70 hours after dosing.

The only blood sample from sheep No. 6 that contained detectable ethanol was the sample collected 37 hours after the wheat was administered. This sample contained less than 1 μmole of ethanol per milliliter of blood. The blood from sheep No. 7 did not contain detectable ethanol during the experiment. The lower limit for detection was about 0.2 μmole/ml. These blood samples were taken from the carotid artery; perhaps ethanol would have been more readily detected in portal blood. Emery *et al.* (*8*) placed relatively large amounts of ethanol (approximately 110 μmole/ml daily) in the rumens of cattle during trials lasting 14 and 21 days. They found a mean concentration of 16 μmole of ethanol per milliliter of blood 2 to 4 hours after ethanol administration but did not report any signs of toxicity. Considering the acute nature of the overfeeding-indigestion syndrome and the absence or low level of ethanol in the blood, it seems likely that ethanol did not contribute significantly to the symptoms of overfeeding observed in our experiments.

Ethanol has been detected in ruminal contents of 12 out of 12 sheep fed cracked wheat. Another sheep dosed with 5.5 kg of crushed apples through a ruminal fistula had a ruminal ethanol concentration of 13 μmole/ml 26 hours after dosage. A 460-kg heifer dosed with 1 kg of glucose had 3 μmole/ml of ethanol in the rumen 8 hours later. Small but detectable concentrations of ethanol have been noted in the ruminal contents of four out of four steers that were on a high concentrate fattening ration (*9*).

Most samples of ruminal material from animals on hay rations contain very small quantities of ethanol or none at all. As part of another study, ruminal samples were obtained from eight lambs that had been maintained on a hay ration. Two of eight samples taken before feeding contained ethanol (1 μmole/ml and less than 0.5 μmole/ml) and one of the eight samples taken 5 hours after feeding hay contained 0.9 μmole/ml. All of these lambs had more than 1 μmole/ml of ethanol in their rumens after they had been fed wheat.

A number of important ruminal bacteria produce ethanol during growth in pure culture (*10*), but the near neutral *p*H and the low concentration of hydrogen in the rumen probably limit its formation (*11*). Perhaps the lowered *p*H after overfeeding inhibits methanogenesis and hydrogen then becomes available for ethanol production. The rate of metabolism of ethanol is slow and it is probably not an important intermediate in the rumen under normal conditions (*12*). However, it may be pertinent that a much greater rate of metabolism was noted when the concentration of ethanol was elevated.

MILTON J. ALLISON
R. W. DOUGHERTY
J. A. BUCKLIN, E. E. SNYDER*
National Animal Disease Laboratory, U.S. Department of Agriculture, Ames, Iowa

References and Notes

1. H. M. Cunningham and G. J. Brisson, *Can. J. Agr. Sci.* **35**, 511 (1955).
2. N. Krogh, *Acta Vet. Scand.* **1**, 74 (1959).
3. A gift from Glyco Chemicals, New York, N.Y.
4. Column prepared as suggested by A. D. McGilliard, Dept. of Animal Science, Iowa State University.
5. R. W. Dougherty, *Cornell Vet.* **45**, 331 (1955).
6. W. B. Buck, thesis, Iowa State University (1963), p. 25.
7. R. E. Hungate, R. W. Dougherty, M. P. Bryant, R. M. Cello, *Cornell Vet.* **42**, 423 (1952).
8. R. S. Emery, T. R. Lewis, J. P. Everett Jr., C. A. Lassiter, *J. Dairy Sci.* **42**, 1182 (1959).
9. Steers were made available for sampling through the courtesy of R. Kohlmeier, Dept. of Animal Science, Iowa State University.
10. M. P. Bryant, *Bacteriol. Rev.* **23**, 125 (1959).
11. R. E. Hungate, in *Symbiotic Associations* (Cambridge Univ. Press, London, 1963), p. 282.
12. C. R. Moomaw and R. E. Hungate, *J. Bacteriol.* **85**, 721 (1963).

* Present address: College of Veterinary Medicine, Ohio State University, Columbus.

13 February 1964

Parathion Activation by Livers of Aquatic and Terrestrial Vertebrates

Abstract. *Liver slices from seven terrestrial and six aquatic animals studied "activated" parathion, converting it to paraoxon, a potent anticholinesterase. Although there are similarities between the liver systems which activate parathion and which degrade drugs such as aminopyrine and phenacetin, liver slices of aquatic vertebrates can activate parathion but cannot degrade such drugs.*

The insecticide parathion is converted to paraoxon, a potent anticholinesterase, by liver slices (*1*) or by appropriately fortified liver homogenates (*2*). This activation process, in which the P=S group is converted to a P=O group, can also be accomplished by liver microsomes in the presence of magnesium and a reduced pyridine nucleotide (*3*). The same microsomal preparation can degrade a variety of drugs by such processes as dealkylation and hydroxylation (*4*).

Brodie and Maickel (*5*) showed that terrestrial vertebrates (including birds, mammals, and toads) could degrade such drugs as aminopyrine and phenacetin, but aquatic forms (including fish, turtles, frogs, and salamanders) could not. If the activation of parathion and the degradation of these drugs were carried out by the same enzyme system, one might expect to find that aquatic vertebrates could not activate

Table 1. Metabolism of parathion incubated with liver slices for 30 minutes.

Species	No. of animals and sex*	No. of replicates	Parathion remaining (mμmole) ± S.E.M.	Paraoxon recovered (mμmole) ± S.E.M.	Maximum contamination of paraoxon fraction by ETOH†
		Aquatic animals			
Turtle *(Pseudomys sp.)*	2 M	4	64.8 ± 2.04	0.60 ± 0.12	0.10
Turtle *(Pseudomys sp.)*	2 F	4	54.9 ± 4.32	1.48 ± 0.26	0.14
Brook trout	8 MS	4	78.9 ± 3.14	0.38 ± 0.04	0.07
Brown trout	4 F	4‡	70.0 ± 3.68	0.46 ± 0.12	0.09
Mud puppy	4 F	8	31.6 ± 1.89	0.23 ± 0.04	0.20
Frog *(Rana pipiens)*	4 F	6	52.2 ± 5.34	2.20 ± 0.47	0.16
		Terrestrial animals			
Pigeon	2 MS	4	14.2 ± 2.88	1.52 ± 0.04	0.24
Rabbit (Dutch belted)	2 M	4	36.4 ± 2.15	3.74 ± 0.24	0.18
Guinea pig	2 F	4‡	40.5 ± 0.67	0.18 ± 0.04	0.16
Toad *(Bufo sp.)*	4 F	4	37.6 ± 4.24	2.81 ± 0.45	0.19
Mouse	3 F	5	38.9 ± 1.79	0.46 ± 0.10	0.18
Rat	3 M	6	40.1 ± 2.27	4.59 ± 0.72	0.17
Pig (Yorkshire)	2 MS	4	64.0 ± 1.88	1.34 ± 0.73	0.10

* M, male; F, female; MS, mixed sexes. ‡ In the experiments on the brown trout and guinea pig, three replicates were utilized in calculating the values shown in the parathion column as one replicate was lost. † The values in this column were calculated on the basis of 100 percent of the degradation occurring by de-ethylation. In fact, a figure of 10 percent is probable (*10*).

parathion, and that consequently they would not be poisoned by parathion and related phosphorothionates. However, fish are susceptible to parathion poisoning (*6*) and their cholinesterase is inhibited after exposure to parathion (*7*). In the study reported here we investigated the ability of liver slices from terrestrial and aquatic vertebrates to activate parathion.

Tritiated parathion was synthesized by a procedure proposed by W. C. Dauterman: H^3-ethanol was reacted with P_2S_5, the mixture was chlorinated with SO_2Cl_2 and the resultant diethyl phosphorochloridothionate was reacted with sodium *p*-nitrophenate (yield 2.5 percent, specific activity 7.58 mc/mmole). We chose to use liver slices rather than a microsomal preparation because we wished to avoid establishing cofactor requirements for each of the 12 species tested and because of the variation in distribution of activity between soluble and microsomal fractions (*5*). One-half gram of liver slices was incubated in 10 ml of a Tyrode solution (*8*) with bicarbonate omitted and the *p*H adjusted to 7.1; $10^{-5}M$ H^3-parathion had been added to the preparation. After 30 minutes, the solution was extracted with 15 ml of benzene, of which 5 ml was fractionated on an alumina column (*9*), the residual parathion being eluted with 45 ml of benzene and the residual paraoxon with 50 ml of chloroform. Degradation products, except ethanol, remained in the aqueous phase in the procedure and were not counted. The benzene and chloroform were removed separately under vacuum, and the residues were taken up in 10 ml of toluene. Two-milliliter samples were counted in a toluene scintillator solution with a Packard Tri-Carb scintillation counter. Recovery of parathion was 98.7 percent and of paraoxon was 98.6 percent, when these compounds were added to the liver preparations and promptly extracted.

Some parathion remained on the column and was eluted with the paraoxon by the chloroform eluent. The quantity retained by varying amounts introduced onto the column was determined, and this correction was applied to the amount of apparent paraoxon recovered. Therefore all paraoxon (P=O) values shown in Table 1 are corrected to reflect actual paraoxon levels.

The possibility of H^3-ethanol being released by de-ethylation of parathion and influencing the results was investigated. The partition coefficient of ethanol in water/benzene was 1.69. Tritiated ethanol was chromatographed as above, and it was found that only 0.28 percent was eluted by benzene and 1.14 percent by subsequent addition of chloroform. To show the maximum possible influence of H^3-ethanol on the experimental results, the replicate with the greatest degradation was chosen and, on the assumption of 100 percent degradation by de-ethylation, the percentage of H^3-ethanol that would appear in the chloroform fraction was calculated. The results for each animal are shown in Table 1. De-ethylation is, in fact, a minor route of parathion or paraoxon degradation in the rat liver (*10*).

Attempts were made to improve the paraoxon yield by reducing paraoxon degradation, F^- being used (*11*) with various combinations of Ca^{++} and Mg^{++}, or with Ba^{++} (*12*). No improvements were observed.

The results are shown in Table 1. It is apparent that degradation of parathion occurred in every case, and varied from 20.7 percent in the brook trout to 84.3 in the pigeon. Activation, although less extensive, was always observed. Variation between species was extremely large, with no consistent difference between aquatic and terrestrial forms. Consequently, parathion activation does not parallel the degradation of drugs such as aminopyrine and phenacetin. For instance, the frog is virtually unable to degrade these drugs (*5*) but its liver, as well as that of most terrestrial forms, can activate parathion.

Since paraoxon is readily degraded in mammals (*13*) and amphibia (*13, 14*) the paraoxon levels shown in the table represent not total paraoxon production but the difference between production and destruction. Consequently it would be unsafe to state (for instance) that activation by toad liver is greater than activation by pigeon liver. Our conclusion is simply that activation is observed in both terrestrial and aquatic organisms.

J. L. POTTER
R. D. O'BRIEN
Department of Entomology, Cornell University, Ithaca, New York

References and Notes

1. W. M. Diggle and C. J. Gage, *Biochem. J.* **49**, 491 (1951).
2. A. N. Davison, *Biochem. J.* **61**, 203 (1955).
3. R. D. O'Brien, *Nature* **183**, 121 (1959).
4. B. B. Brodie, J. R. Gillette, B. N. LaDu, *Ann. Rev. Biochem.* **27**, 427 (1958); J. R. Gillette, in *Proc. 1st Intern. Pharmacol. Meeting, Stockholm, 1961* (Pergamon, New York, 1962), vol. 6, p. 13.
5. B. B. Brodie and R. P. Maickel, *Proc. 1st Intern. Pharmacol. Meeting, Stockholm, 1961* (Pergamon, New York, 1962), vol. 6, p. 299.
6. C. Henderson and Q. H. Pickering, *Trans. Am. Fisheries Soc.* **87**, 39 (1957).
7. C. M. Weiss, *Sewage Ind. Wastes* **31**, 580 (1959).
8. R. M. C. Dawson, D. C. Elliott, W. H. Elliott, K. M. Jones, *Data for Biochemical Research* (Oxford Univ. Press, London, 1959), p. 209.
9. H. R. Krueger, R. D. O'Brien, W. C. Dauterman, *J. Econom. Entomol.* **52**, 1063 (1959).
10. T. Shishido and J. Fukami, *Botyu-Kagaku* **28**, 69 (1963).
11. P. A. Dahm, B. E. Kopecky, C. B. Walker, *Toxicol. Appl. Pharmacol.* **4**, 683 (1962).
12. A. R. Main, *Biochem. J.* **74**, 10 (1960).
13. R. D. O'Brien, *ibid.* **79**, 229 (1961).
14. J. L. Potter and R. D. O'Brien, *Entomol. Exptl. Appl.*, in press.

15. This work was supported in part by PHS grant GM07804. We thank B. D. Hilton for preparing the H^3-parathion, A. M. Phillips for providing fish, and F. Matsumura for helpful discussions.

23 December 1963

Insect Chemosterilants with Low Toxicity for Mammals

Abstract. *Hexamethylphosphoramide and hexamethylmelamine are effective as male house fly chemosterilants. Both compounds are structurally similar to the two highly active sterilants tepa and tretamine, but they differ from the aziridinyl compounds in their low toxicity for mammals and in their lack of alkylating properties. This discovery of nonalkylating male sterilants of low toxicity should substantially increase the scope and practicality of the sterile-male control method.*

The most effective male insect chemosterilants so far discovered are biological alkylating agents (*1*). More specifically tepa (*2*), tretamine, and apholate, all derivatives of aziridine (ethylenimine), when applied to a great variety of insects prevent the hatching of eggs laid by females mated to the treated males. Because of the possible mutagenic and carcinogenic properties of aziridinyl compounds (*3*) the practical application of chemosterilants to natural insect populations is restricted. Therefore, the development of nonmutagenic sterilants with low mammalian toxicity will have far-reaching consequences.

Table 1. Male-sterilizing properties of HMPA administered by different methods; at each concentration ten treated males were mated with five virgin females.

Dosage (μg per fly)	Percentage in food	Total No. of eggs	Sterility (%)
Injection method			
0.5		604	1
1.0		300	3
2.5		535	19
5.0		630	38
10.0		352	86
20.0		161	93
40.0		432	100
Topical application			
5.0		119	31
10.0		844	13
25.0		1123	48
50.0		663	91
100.0		617	99.8
200.0		375	100
Oral application			
	0.01	708	3
	0.05	249	86
	0.25	300	100
	0.50	172	100
	1.00	514	99.9

The work in our laboratory (*4*) has centered around the aziridines, and an accurate and highly sensitive method for determining their sterilizing activity in male house flies (*Musca domestica* L.) has recently been reported (*5*). Although it was supposed that the alkylating property of tepa and other aziridine chemosterilants was indispensable for their biological (sterilizing) activity, other phosphoramides which were either related to the decomposition products of tepa or were structurally similar to it were investigated. Outstanding activity was exhibited by hexamethylphosphoramide (HMPA). As can be seen from the structures in Fig. 1, the compounds are sterically and structurally quite similar. Both are soluble in water and in most organic solvents. Aqueous solutions of HMPA were injected into male house flies which were subsequently allowed to mate with virgin females. The extent to which the compound affected the fertility of the flies was determined on the basis of the hatchability of eggs laid by the inseminated females (*5*). The results of the injection experiments are given in Table 1. The results were subjected to probit analysis at the computing laboratory of the Biometrical Services, U.S. Department of Agriculture. The dosage-activity regression equation was found to be $y = 2.7x + 0.32$ and the calculated ED_{50} (*6*) was 5.42 μg for each male fly.

The activity of HMPA was also tested by topical application and by addition to the fly diet. Neither of these methods allows for a quantitative determination of the activity; nevertheless, from a practical standpoint both topical and oral activities are of utmost importance. The results of the experiments are summarized in Table 1.

In the topical application, 0.85 μl of an acetone solution of HMPA was applied to the dorsal side of the fly's thorax. The effects of the treatment were evaluated in the same way as in the injection method (*5*). In the feeding experiments HMPA was added to the fly food, which consisted of nonfat dry milk (50 percent), sugar (1 percent), and water. Flies were allowed to feed on the treated food for 24 hours, and then untreated food was substituted. Evaluation was carried out as in the injection method. Female flies treated with the compound by any of the three methods and mated to untreated males often produced an appreciable number of eggs that failed to hatch. However, the results were erratic and it was concluded that the effects on females were far less pronounced than on males.

Table 2. Sterilizing activity of hexamethylmelamine applied topically to male house flies.

Dosage (μg per fly)	Total No. of eggs	Sterility (%)
10	140	20
20	130	38
40	256	85
60	192	100
80	203	100

One of the important characteristics of an effective chemosterilant is the margin between the sterilizing and lethal doses of the material. Because a sterile male (*7*) which is sexually competitive with normal males is of cardinal importance in the sterile-male control method (*8*) whereas a dead male is useless, any chemosterilant treatment must not cause undue mortality among the treated insects. In practice it is impossible to apply to a natural population just the minimal quantity of a compound required for 100 percent sterilization, and a severalfold overdosage to some individuals is inevitable. In this respect HMPA rates very well because its LD_{50} (as determined by the injection method) is about 95 μg for a fly of either sex. This value corresponds to a safety margin (LD_{50}/ED_{50}) of about 20.

In comparison to HMPA, tepa is

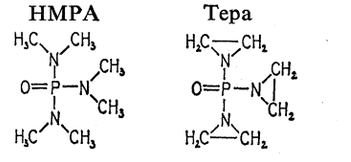

Fig. 1. Comparison of structures of hexamethylphosphoramide and tepa.

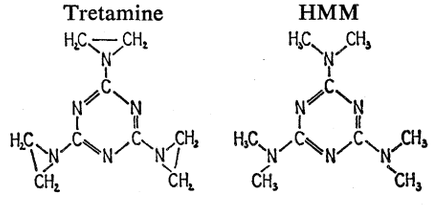

Fig. 2. Comparison of structures of tretamine and hexamethylmelamine.