Previously described results (7) led to the conclusion that although cycloheximide is translocated less easily in eastern white pine when compared with western white pine, the antibiotic tends to persist longer in the eastern white pine needle tissue, an effect which may have been due to the use of higher concentrations of the antibiotic. Present work supports this conclusion in so far as it confirms the persistence of low concentrations in eastern white pine needles for at least 57 days without detectable loss.

The use of tritium-labeled cycloheximide to follow uptake and distribution of the antibiotic in pine seedlings demonstrates an accurate technique which appears to be widely applicable to studies of the translocation and persistence of cycloheximide.

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Received for review October 19, 1960. Accepted January 4, 1961.

RUMEN DEGRADATION OF FUNGICIDES

Fate of Tetramethylthiuram Disulfide in the Digestive Tract of the Ruminant Animal

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In experiments in which ruminant animals were fed corn treated with tetramethylthiuram disulfide (TMTD), it was found that the rumen microorganisms degraded the TMTD to carbon disulfide and probably hydrogen sulfide and dimethylamine. In a 6-hour period, 78% of the ingested TMTD was degraded as determined by the carbon disulfide recovered. However, the degradative action within the gastrointestinal tract was not complete, as 4.0% of the ingested TMTD appeared in the feces and 1.5% appeared in the urine as calculated from the carbon disulfide recovered. TMTD or its degradation products could not be demonstrated to be present in the blood and tissues of animals fed TMTD because a volatile substance was recovered from the blood and tissues which invalidated the distillation procedure for these studies.

TETRAMETHYLTHIURAM DISULFIDE is widely used in the production of food crops, yet there are few reports on the toxicity of TMTD to animals. Data collected by the Food and Drug Administration, cited by Dupont (*8*), set the LD_{50} of TMTD for rats at 850 mg. per kg. of body weight and for rabbits at 210 mg. per kg. of body weight, while in the cat 230 mg. per kg. of body weight proved to be fatal. In rabbits and puppies, Hanzlick and Irvine (*12*) found 3.0 grams per kg. of body weight to be the minimum fatal dose. In chronic toxicity studies carried out with rats fed diets containing 0, 1000, and 2000 p.p.m. of TMTD, it was found that some of the animals fed 1000 and 2000 p.p.m. died. However, rats showed no difference in growth patterns when fed for 65 weeks on diets containing 0, 250, and 500 p.p.m. of TMTD (*8*). There are no published reports on the toxicity of TNTD to ruminant animals.

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Materials and Methods

The methods used for assessment of TMTD in these studies were modifications of the distillation procedures described by Clarke *et al.* (*3*) and Lowen (*16*) for the determination of dithiocarbamates. The apparatus used in this study was of a different design, but operated on the same principle as the apparatus described by the above workers. Modifications in apparatus and procedure consisted in the addition of three (total of five) absorption tubes to accommodate solutions for the determination of hydrogen sulfide and to enable the use of both acidic and basic solutions necessary to remove interfering substances when assaying animal products.

Distillation. A procedure described by Diemair, Strohecker, and Keller (*6*) for the determination of hydrogen sulfide in tissues by the formation of methylene blue was adapted to the distillation procedure to measure the hydrogen sulfide liberated from decomposition of TMTD. The hydrogen sulfide procedure was carried out by using a 1.0*N* NaOH solution in two gas absorption tubes adjacent to the distillation flask to absorb the liberated hydrogen sulfide. At the end of the assay, 3.0 ml. of Reissners solution (0.1*N* $FeCl_3$ in 5.0% HNO_3 solution) was added to the absorption tubes containing the 1.0*N* NaOH and absorbed hydrogen sulfide. After standing for 30 minutes, the resulting color, methylene blue, was read on an Evelyn colorimeter using the 660-mμ filter. A calibration curve was prepared for hydrogen sulfide using a solution of methylene blue. The curve approximated Beer's law over the range determined, 0 to 45 μg.

Determinations of the ratios of carbon disulfide to hydrogen sulfide resulting from the decomposition of TMTD in an alcoholic solution were made on whole and ground corn. Twenty milliliters of 1.0*N* NaOH was used in absorption tubes 1, 2, and 3 (adjacent to the distillation flask) and 20 ml. of the copper-amine reagent (*22*) was added to absorption tubes 4 and 5. A series of absorption tubes was used for each gas to ensure complete trapping of the liberated carbon disulfide and hydrogen sulfide. The samples in the distillation flask were acidified with 100 ml. of 1.0*N* H_2SO_4 and the distillation procedure was carried out at boiling temperature for 1.5 hours.

In the assays for TMTD in ingesta, feces, urine, and tissues, the hydrogen sulfide resulting from the decomposition of TMTD could not be measured by the procedure used because of the relatively large amounts of hydrogen sulfide and interfering substances recovered from these materials. The TMTD content of the above materials was estimated from the carbon disulfide recovered. For these assays, absorption tube 1 was charged with 20 ml. of 1.0*N* H_2SO_4, tube 2 with 20 ml. of 1.0*N* NaOH, and tube 3 with 20 ml. of a 10% $Pb(CH_3CO_2)_2$ solution to remove substances which caused turbidity in the copper-amine reagent. Absorption tubes 4 and 5 each contained 10 ml. of the copper-amine reagent which reacted with the carbon disulfide liberated from TMTD.

Animal Investigations. The TMTD balance study was carried out over an 18-day period, using a 7-day collection period, with four male lambs weighing approximately 70 pounds. The animals were fed a maintenance ration, calculated by the formula $TDN(\text{lb.}) = 0.436\ W^{0.73}$ pounds, consisting of 50% ground hay, 31% ground corn, 15% soybean oil meal, and TMTD. The animals were fed twice daily; water was allowed ad libitum. Urine and feces were collected twice daily. One milliliter of toluene was used in the urine containers to inhibit bacterial action. The feces were dried at 90° C. for 24 hours and ground in a Wiley mill. The urine was assayed for TMTD and/or carbon disulfide by acidifying 100-ml. aliquots with 50 ml. of 1.0*N* H_2SO_4 and distilling for 1 hour and 15 minutes. The feces were assayed by weighing out 50-gram samples on a triple-beam balance, acidifying with 500 ml. of 1.0*N* H_2SO_4, and distilling for 1.5 hours.

TMTD Content of Ingesta. Analysis of the ingesta of animals fed TMTD-treated corn was carried out using beef-type animals of mixed Angus and Hereford breeding. Animal No. 1 was a steer weighing 544 pounds; No. 2, a heifer weighing 795 pounds; No. 3, a steer weighing 990 pounds; and No. 4, a steer weighing 1074 pounds. Animals No. 1 and 2 received 12 pounds of TMTD-treated corn daily; animal No. 3 received 16 pounds; and animal No. 4 received 17 pounds of TMTD-treated corn daily. The animals were maintained on the TMTD-treated corn, 389 μg. of TMTD per gram of corn, for several weeks before slaughtering.

The gastrointestinal tract was removed from the animal immediately after slaughter and approximately 0.5-pint samples were taken from the rumen, reticulum, omasum, abomasum, duodenum, ileum, cecum, and rectum. Duplicate aliquots of 24 grams taken from each of the above samples were dried at 90° C. for 24 hours in order to calculate TMTD recovery on a dry weight basis. Duplicate aliquots from each of the above samples were assayed by addition of 100 ml. of 1.0*N* H_2SO_4 and distilling for 1.5 hours.

Degradation of TMTD by Rumen Ingesta. In vitro rumen degradation studies were conducted by incubating, in a vacuum desiccator at 39° to 40° C., rumen ingesta with known amounts of TMTD on treated corn. The rate of liberation of carbon disulfide was measured at 3-hour intervals by aspirating the gases from the desiccator through absorption tubes containing the copper-amine reagent. When the recovery of carbon disulfide dropped to very low levels, the incubation mixture was assayed by distillation. The degradation rate was measured at various pH values.

In vivo degradation action of the rumen microflora on TMTD was investigated using a fistulated animal, estimated weight 600 to 650 pounds. The animal was fed a ration consisting of 6 pounds of TMTD-treated corn (389 μg. of TMTD per gram of corn), 0.5 pound of soybean oil meal, and 8 pounds of low quality alfalfa hay daily. The rumen was emptied and found to contain 95 pounds of ingesta at this level of feed intake. The animal was fed at 8 A.M. and 4 P.M. daily with samples taken from the rumen at 6 hours following the morning feeding to determine the amount of TMTD degraded in the rumen. The solid and liquid portion of the 100-gram rumen samples were separated by manually pressing out the liquid through cheesecloth in order to determine if TMTD was predominantly associated with solids or liquids.

Table I. Efficiency of Distillation System for Recovery of Carbon Disulfide from Alcoholic Solution and Recovery of Carbon Disulfide and Hydrogen Sulfide from Degraded TMTD in Alcoholic Solution

Standard Alcoholic Solution, μg.	Total Sulfur Present, μg.	Sulfur Recovered, μg. CS_2-S	Sulfur Recovered, μg. H_2S-S	Recovery,[a] %	Ratio, CS_2-S to H_2S-S
		Carbon Disulfide			
200	169	150		89	
400	337	315		94	
600	505	436		86	
800	674	569		84	
1000	842	740		88	
				Av. 88	
		TMTD[b]			
200	107	94		88	
400	213	179		84	
600	320	271		85	
800	427	349		82	
1000	534	438		82	
				Av. 84	
		TMTD[c]			
200	107	79	13	86	6.7:1
400	213	151	33	86	5.1:1
600	320	228	71	93	3.6:1
800	427	294	76	87	4.3:1
1000	534	369	111	90	3.7:1
				Av. 88	

[a] Four replications of each sample.
[b] Boiling 1.0*N* H_2SO_4 added to the sample in the reaction flask.
[c] Sample heated from room temperature to boiling.

Results

Two pathways of degradation have been described for dithiocarbamic acid derivatives, the dithiocarbamates (*3*); one results in formation of two molecules of carbon disulfide from one molecule of dithiocarbamate, while, in the other, one mole each of carbon disulfide and hydrogen sulfide is formed. The former reaction is stated to be quantitative at 100° C. in dilute acid, while the latter reaction is not as well understood, but appears to occur slowly at lower temperatures.

The degradation of TMTD, which may be considered an oxidation prod-

Table II. Assay of TMTD-Treated Corn by Distillation[a] and Recovery of Carbon Disulfide and Hydrogen Sulfide

Corn,[b] Grams	CS_2-S, μg.	H_2S-S, μg.	Recovery TMTD, μg.	Ratio, CS_2-S to H_2S-S
Whole				
0.71	81	27	285	3.3:1
2.13	275	103	332	3.0:1
3.55	603	329	493	3.0:1
4.97	661	259	347	2.9:1
6.39	881	697	463	1.4:1
7.81	752	636	334	1.5:1
8.63	884	852	377	1.2:1
10.65	1634	1080	478	1.7:1
Corn,[c] Grams				
Ground				
0.71	174	53	600	3.7:1
2.13	447	212	579	2.4:1
3.55	689	506	632	2.0:1
4.97	962	536	564	2.0:1
6.39	1153	983	626	1.3:1
7.81	1599	1017	628	1.8:1
8.63	1628	1409	660	1.7:1
10.65	2144	1361	619	1.7:1

[a] Samples heated from room temperature to boiling.
[b] Three replications of each sample.
[c] Six replications of each sample.

Table III. Intake and Excretion Partition of TMTD by Lambs[a]

Lamb No.	Feed, Mg. CS_2	Feces, Mg. CS_2	Urine, Mg. CS_2	Recovery, % Feces + Urine
1	93.9	3.7	2.6	6.7
2	105.7	4.7	0.6	4.6
3	93.9	3.7	1.7	5.7
4	105.7	4.0	1.1	4.8
Total	399.2	16.1	6.0	21.8
Av.	99.8	4.0	1.5	5.5

[a] Seven-day balance study.

Table IV. TMTD Content of Ingesta[a] of Animals Fed TMTD-Treated Corn

Animals	Carbon Disulfide Recovered, μg./Gram of Ingesta			
	No. 1	No. 2	No. 3	No. 4
Ration	120.0	120.0	142.0	142.0
Rumen	7.3	6.1	14.3	25.1
Reticulum	3.8	5.0	12.9	20.1
Omasum	14.3	4.2	7.3	37.1
Abomasum	1.7	...	12.8	14.0
Duodenum	1.3	1.5	1.2	2.6
Ileum	3.4	3.3	7.5	6.2
Cecum	2.9	2.7	4.1	13.4
Rectum	4.2	...	6.9	13.4

[a] Values are per cent of intake.

uct of substituted dithiocarbamates, has been investigated under the conditions described above. Data in Table I show the efficiency of the distillation procedure for the recovery of carbon disulfide (reagent grade) from an alcoholic solution and of the recovery of carbon disulfide and hydrogen sulfide resulting from decomposition of TMTD in alcoholic solution. Data further show that TMTD, similarly to dithiocarbamates, releases its sulfur in the form of carbon disulfide and hydrogen sulfide, and the pathway of degradation appears to be influenced by rate of heating. Wider ratios of carbon disulfide to hydrogen sulfide were recovered at the lower concentrations of TMTD with a narrowing of the ratios as the concentration of TMTD increased in the distillation flask. Data show also that low assay recoveries of TMTD, based on the recovery of carbon disulfide alone, result at higher concentrations of TMTD because of the relatively large amount of sulfur released as hydrogen sulfide.

The treated corn used in the animal feeding trials was assayed for TMTD by recovery of both carbon disulfide and hydrogen sulfide. Data in Table II show results of a series of determinations made on whole and ground corn When TMTD was degraded in the reaction flask in the presence of corn, a narrower ratio of carbon disulfide to hydrogen sulfide was obtained—at comparable concentrations—than when TMTD was degraded in an aqueous alcoholic solution. Narrower ratios of carbon disulfide to hydrogen sulfide were obtained as the concentrations of TMTD increased in the reaction flask.

For quantitative results, the preceding data indicated that it was necessary to recover both carbon disulfide and hydrogen sulfide or to establish recovery curves for the type of material under study, and, by means of degradation ratios, to calculate TMTD content on the basis of the carbon disulfide recovered. In feeding experiments with animals and incubation studies, the TMTD of the feed, ingesta, feces, urine, blood, and tissues was calculated from the recovery of carbon disulfide alone, as these substances released hydrogen sulfide and interfering substances in amounts which invalidated the use of the hydrogen sulfide procedure.

The study of the fate of TMTD in the animal was initiated by a balance study, Table III, in which lambs were fed TMTD-treated corn. It was found that 4.0% of the ingested TMTD appeared in the feces and 1.5% appeared in the urine. The excretion data do not account for approximately 95% of the ingested TMTD. The data further indicate that TMTD was either degraded in the gastrointestinal tract or absorbed and metabolized to substances which were not detected by the assay procedure being used.

To determine the sites in the gastrointestinal tract where the concentrations of TMTD decreased, animals fed TMTD-treated corn were slaughtered and the contents of the gastrointestinal tract were assayed for TMTD. The results, Table IV, of the analysis of the ingesta of animals fed TMTD-treated corn show that an approximately 86% decrease in concentration of TMTD occurred in the rumen. Between the rumen and rectum only about an 8% decrease occurred in concentrations of TMTD. Because of variability of data, no specific section of the gastrointestinal tract between the rumen and rectum could be designated as a site where a definite decrease in concentration, due to degradation or absorption, of TMTD occurred.

The data in Table IV indicate that TMTD was either degraded in, or absorbed from, the rumen. To determine if TMTD was degraded, a qualitative assay was made of the rumen gases. Carbon disulfide was found to be present in considerable concentrations, indicating decomposition of TMTD. Trials were made in which it was found that TMTD incubated with rumen in-

gesta was readily degraded. Autoclaved rumen ingesta, 15 pounds for 30 minutes, did not degrade TMTD; reinoculation of the ingesta restored its degradative action on TMTD.

Experiments were conducted in which TMTD was incubated with rumen ingesta. The results, Figure 1, show that approximately 55% of the TMTD was degraded in a 12-hour period. A lower pH of the ingesta at the start of the incubation period increased the rate of degradation of the TMTD and resulted in a higher total recovery. In contrast to the above, it was found that 78% of the ingested TMTD was degraded in the rumen of a fistulated animal in a 6-hour period.

The preceding data show that the degradation of TMTD within the rumen was not complete. This finding was substantiated by the presence of TMTD throughout the gastrointestinal tract and in the feces of the animals fed TMTD-treated corn. Metabolites resulting from the degradative action of the rumen microflora on TMTD, other than carbon disulfide, were not investigated in this study. Despite the high rate of degradation of TMTD in the rumen and the apparent production of carbon disulfide, free carbon disulfide, using vacuum techniques, could not be demonstrated to be present in the blood and tissues of the animals fed TMTD-treated corn. Further work on the metabolism of TMTD in animal tissues could not be pursued using the distillation assay procedure, as a volatile substance was recovered with this procedure from both control and test animals which gave a reaction similar to carbon disulfide with the copper-amine reagent. This substance is under investigation at present.

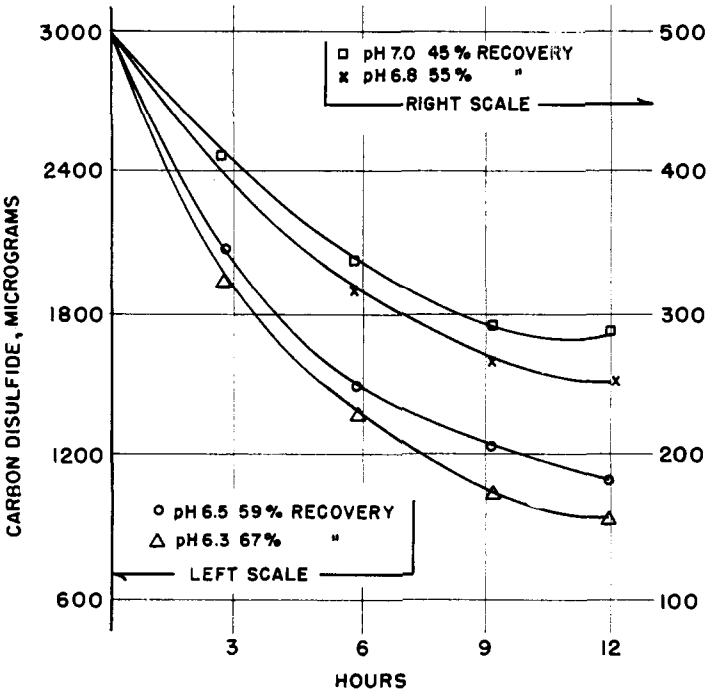

Figure 1. Degradation of TMTD incubated with rumen ingesta

Discussion

The evidence obtained from this investigation shows that when TMTD is consumed by the ruminant animal, the TMTD is rapidly degraded in the rumen with the liberation of carbon disulfide and probably hydrogen sulfide and dimethylamine. The findings that TMTD is degraded in the rumen and that the decomposition is accelerated at lower pH's are consistent with findings that microorganisms degrade dithiocarbamates and that lower pH's accelerate degradation (*23*). The degradative action of the rumen microflora was not complete and the microflora and enzymes of the gastrointestinal tract appeared to possess little or no degradative action against TMTD, as it was found in the feces.

TMTD, or its metabolites, was recovered from the urine. The metabolic fate of TMTD absorbed into the body from the gastrointestinal tract probably depends to some extent on the species of animal. In some species tetraethylthiuram disulfide, TETD, a homolog of TMTD, has been found to be metabolized and in other species excreted as the intact molecule (*11*). Also, animal tissues have been found to degrade TETD to diethyldithiocarbamate which in turn decomposed to carbon disulfide and diethylamine (*19*). Thus, it appears that when ruminant animals ingest TMTD, the toxicity of the intact molecule (*5*) which has been shown to inhibit enzyme systems (*18*) must be considered along with the possible toxic effects of the degradation products: carbon disulfide, hydrogen sulfide, and probably dimethylamine.

The toxic effects of carbon disulfide have been described (*15*), and its specific action partly elucidated as being due to inhibition of the succinic oxidase system (*17*) and stimulation of cholinesterase activity (*9*). The animals studied did not exhibit toxicity symptoms ascribable to carbon disulfide, although the odor of carbon disulfide was apparent when the body cavity of an aminal fed TMTD-treated corn was opened at slaughter. The explanation of this lack of toxicity may be, as has been shown by various workers, that absorbed carbon disulfide can be excreted in the breath and urine as carbon disulfide or metabolized by the tissues to sulfates and excreted in the feces and urine (*21*).

Hydrogen sulfide has been shown to be a highly toxic agent, especially when inhaled (*10*), and its action has been variously ascribed to its irritant properties on the respiratory and nervous systems (*24*), to interference with the oxygen transporting ability of the blood (*20*), and to inhibition of the succinic oxidase system (*2*). The animals investigated exhibited no toxic symptoms as described for hydrogen sulfide. It appears unlikely that toxicity symptoms would arise from the amounts of hydrogen sulfide liberated from the levels of TMTD fed, as this hydrogen sulfide would probably be detoxified by the same mechanism as the hydrogen sulfide normally produced in the gastrointestinal tract (*24*).

The pharmacodynamics of dimethylamine in animals has received little attention. It has been observed to produce a slight and transient hypotension (*13*) and in rather large doses to increase tonus in isolated rat and guinea pig intestines (*1*). It has also been demonstrated to inhibit competitively certain enzyme systems (*4*) and on continuous subcutaneous injection to stimulate the erythropoietic system (*14*). In this study the effects of dimethylamine on the animal were not investigated. A preliminary trial was conducted in which approximately 3 ml. of dimethylamine in 500 ml. of water, administered to a sheep by stomach tube for 7 days prior to slaughter, was found to impart an odor to the tissues. This odor was different from that of the animals fed TMTD-treated corn, which in this case was ascribed to the presence of carbon disulfide.

Preliminary feeding trials indicated that ruminant animals, as compared to monogastric animals, possessed an apparent immunity to TMTD-treated corn. It appears that the degradative effect of the rumen microorganisms may

Table V. Rate of Degradation of TMTD in the Rumen of a Fistulated Animal

	Water, %	Dry Matter,[a] Rumen	Intake TMTD, 8 A.M., CS_2 μg.	TMTD Rumen, 2 P.M., CS_2 μg.	TMTD,[b] % Degraded in 6 Hours
	85	14.3	50.1	10.5	79
	86	13.3	53.7	9.4	83
	80	19.0	37.6	8.8	77
	86	13.3	53.7	10.3	81
	85	14.3	50.1	10.7	79
	85	14.3	50.1	12.1	76
	85	14.3	50.1	10.0	80
	84	16.2	44.1	9.3	79
	86	13.3	53.7	11.8	78
	85	14.3	50.1	11.8	77
	86	13.3	53.7	11.6	78
Av.	84.8	14.5	49.7	10.6	78.8

[a] Capacity of rumen, 10 gallons; weight of ingesta, 95 pounds.
[b] Calculations based on carbon disulfide/gram of dry matter.

allow the ruminant animal to tolerate considerable amounts of TMTD-treated corn without toxic effects. It seems reasonable to assume that considerable amounts of the volatile gases, carbon disulfide and hydrogen sulfide, could be released from the rumen by belching and aeration of the bolus during rumination, as the ingesta are estimated to remain in the rumen and reticulum for approximately 60 hours and the animal spends approximately 8 hours daily in the act of rumination (*7*). This would appear to reduce the concentration of carbon disulfide and hydrogen sulfide to a level which would not overload the detoxification processes of the body.

The metabolism of TMTD in the animal body was not investigated, as an unknown volatile substance was found to be naturally present in the blood and tissues which gave a reaction similar to carbon disulfide with the copper-amine reagent. These findings invalidated the distillation procedure for the study of TMTD and its degradation products in animal tissues.

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Received for review July 20, 1960. Accepted December 6, 1960. Investigation supported in part by a grant from the Pioneer Hybrid Corn Co. Journal Paper No. J-3922, Iowa Agricultural and Homes Economics Experiment Station; Department of Animal Husbandry and Food Processing Laboratory, Iowa State University, Ames. This work is from a thesis submitted by Ralph C. Robbins to the Iowa State University, Ames, Iowa, in partial fulfillment of the requirements for the M.S. degree.

ISOTOPE-LABELED INSECTICIDES

Ethion-P_2^{32}

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THE PESTICIDE ETHION is used against a number of agriculturally important insects and mites. Feeding experiments with cows were contemplated in which it would be necessary to detect a few parts of ethion per billion. Since the molecule might be metabolized, tagging with tritium, carbon-14, or sulfur-35 could lead to ambiguous results. Tagging with phosphorus-32 seemed preferable because if metabolic decomposition occurred it would be indicated simply by the water-fat partition coefficient.

One difficulty with phosphorus-32 is its short half life (14.3 days) which requires that the synthesis and the subsequent experiments be done promptly. Also, since phosphorus-32 decays with the ejection of energetic beta particles, shielding is necessary.

The $P_2^{32}S_5$ was prepared by a method described by Casida (*3*). Although a radioyield of 80% was obtained in the preliminary experiments with weakly radioactive phosphoric acid, the yield in the principal run was about 45%.