declined as if unfed. Additional growth and longer survival were obtained in fish given nematodes *(Neoaplectana glaseri)* that had been reared free from microörganisms.[13] A diet of autoclaved fish food plus nematodes was not markedly superior to that of nematodes alone. Nematodes supplemented with heat-killed algæ or autoclaved housefly larvæ proved to be the best diets in that life was maintained longer than 4 months, but they were not completely satisfactory. Pond water showed no advantage over tap water, and a change of water did not promote better growth. Probably certain nutritional requirements were not supplied.

TABLE I.
Effect of Various Foods on Size and Survival of Axenic Platyfish.

Food	Effect on fish: Size, cm	Effect on fish: Survival, days
Agar particles	1	14- 21
Commercial fish food	2	60- 90
Commercial fish food + nematodes	2-2.5	75-120
Nematodes	2-2.5	75-109
Nematodes + heat-killed algæ	2-2.5	93-127
Nematodes + autoclaved housefly larvæ	2-2.5	113-127

[13] Glaser, R. W., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 512.

Bacteriological Examination. Whole fish, as well as samples of the water in which they had lived, were tested for contaminating microörganisms under aerobic and anaerobic conditions by culture in infusion broth and sealed piece-meat media incubated at both room temperature and 37°C. Practically all of the fish had remained free of microorganisms. Molds occasionally developed in some containers, and, more rarely, bacterial contamination occurred. Usually it was possible to determine contamination merely by inspection of the aquariums, which showed molds growing or a marked turbidity when bacteria were present.

Summary. An operative procedure on pregnant platyfish, a small viviparous Mexican fish, was devised that regularly yielded young free from contamination. These axenic fish were maintained under conditions free from microörganisms for as long as 4 months and during this period grew appreciably. Under usual aquarium conditions these fish would have reproduced at this age. Several diets were tested for their effect on maintenance and growth and some of them were definitely superior to others—an indication that nutritional factors may be the principal cause of the lack of continued growth and reproduction.

13855

Mechanism of Vitamin B_1 Destruction by a Factor in Raw Smelt.

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The destruction of vitamin B_1 by certain species of fish when incorporated into a diet in the raw form has aroused considerable interest. The work of Green *et al.*[1-4] on carp has treated the nutritional aspects of this problem in some detail. Recent work by Woolley[5] on the causative agent of this destruction in fresh carp indicated either an enzymatic degradation or a tying up of the thiamine molecule in such a manner that it is not available to the living organism. Spitzer *et al.*[6] likewise suggest that the destruction may be due to an enzyme.

We have investigated the vitamin B_1 de-

[1] Green, R. G., Carlson, W. E., and Evans, C. A., *J. Nutrition*, 1941, **21**, 243.

[2] *Ibid.*, 1942, **23**, 165.

[3] Green, R. G., and Evans, C. A., *Science*, 1940, **92**, 154.

[4] Green, R. G., Evans, C. A., Carlson, W. E., and Swale, F. S., *J. Am. Vet. Med. Assn.*, 1942, **100**, 394.

[5] Woolley, D. W., *J. Biol. Chem.*, 1941, **141**, 997.

structive factor in raw fresh water smelt by studies on the correlation of chemical and animal assays for this factor, on the quantitative action of raw smelt on thiamine, on the ability to obtain the factor in an alcoholic extract, on its lability to heat and drying, on its action on the thiamine of living yeast cells, and by attempts to release thiamine from any complexes by acid hydrolysis.

Procedure. A dry non-viable yeast* and a wet viable yeast† were used as the source of vitamin B_1. The dry non-viable yeast was mixed with either raw smelt, heated smelt (100°C for 10-30 min), alcoholic (5%) extracts of raw smelt, the residues of the alcoholic extracts, and air-dried smelt restored to its original moisture content. Non-viable yeast - raw smelt mixtures of varying ratios were prepared to determine the quantitative vitamin B_1 destructive effect of smelt. Moist, viable baker's yeast was suspended in saline and a portion of the suspension was killed by heating at 95°C for 15 min. Both the living and killed suspensions were thoroughly mixed with portions of raw smelt to give a vitamin B_1 - fish ratio analogous to the lowest level of the non-viable yeast-smelt mixtures. These mixtures were air-dried at room temperature under forced ventilation. The moist period of contact of smelt and yeast was approximately 12 hr.

After thorough drying, the smelt-yeast preparations were added to a diet in which the thiamine had been destroyed by prolonged autoclaving. These dry fish-yeast mixtures were incorporated in amounts sufficient to provide, on the basis of the yeast content, an amount of vitamin B_1 greatly in excess of that reported by Elvehjem and Arnold[7] as being necessary for normal chick growth. Day-old chicks were placed on these diets. Two groups of chicks on a diet containing the raw smelt - non-viable yeast mixtures received daily intraperitoneal injections of 3 and 6 γ of thiamine hydrochloride respectively.

These diets were then analyzed for thiamine by the thiochrome method. Samples of the raw smelt-yeast mixtures were heated in 0.1 N H_2SO_4 at 100°C for periods ranging from 10-60 min and then assayed for thiamine by the thiochrome method.

Results. Chemical analyses of the diets revealed that only those containing cooked smelt or raw smelt-viable yeast mixtures retained the approximate amount of added vitamin B_1. Some of the thiamine in the viable yeast-fish mixtures was destroyed since the recovery is less than can be explained as due to variations encountered in the thiochrome procedure.

The destruction of vitamin B_1 was complete in all other fish-yeast mixtures except in the preparation having 43.7 γ of vitamin B_1 per g of raw smelt, in which case a small amount of thiamine was recovered in the diet. The results of the chick assays were in complete agreement with the chemical analyses. Typical polyneuritic symptoms developed in chicks on the ninth day after being placed on the diets shown to be free of vitamin B_1 by chemical analysis. Those chicks on the diet containing a trace of vitamin B_1 (3.4 γ per 100 g of ration) survived approximately one day longer. As little as 3 γ of thiamine hydrochloride injected daily protected the chicks on the vitamin B_1 free diets from polyneuritis although this represented a bare maintenance level as indicated by their growth gains. The results are shown in the table.

Treatment of dried raw smelt - non-viable yeast mixtures with 0.1 N H_2SO_4 for as long as 60 min failed to free any thiamine which could be detected as thiochrome.

Discussion. By chemical analysis of raw fish - non-viable yeast mixtures which have been in contact for a sufficient period of time, it is possible to determine rapidly whether certain species of fish will destroy vitamin B_1. In the experiments herein de-

[6] Spitzer, E. H., Coombes, A. I., Elvehjem, C. A., and Wisnicky, W. R., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 376.

* A dry, killed brewer's yeast assaying 140 γ thiamine per g of yeast.

† A wet, living baker's yeast assaying approximately 205 γ thiamine per g. Furnished through courtesy of Red Star Yeast Co.

[7] Arnold, A., and Elvehjem, C. A., *J. Nutrition*, 1938, **15**, 403.

TABLE I.
Correlation of Chick and Thiochrome Assay for Vitamin B_1 Destruction by Smelt.

Smelt treatment	γ B_1 (as yeast)/g raw smelt (or equivalent)	Calc. γ B_1 (as yeast by addition of smelt-yeast mixt.)/100 g ration	γ B_1/100 g found by chem. assay*	Polyneuritis in chicks‡
Cooked	8.7 (Brewer's)	238	219	—
Raw	8.7 "	238	0	+†
"	21.9 "	458	0	+
"	43.7 "	661	3.4	+
"	9.6 (Baker's)	357	318	—
"	9.6 (Killed baker's)	357	0	+
Air dried	8.7 (Brewer's)	238	222	—
" " + water	8.7 "	238	217	—
5% alc. extr.	8.7 "	238	0	+
Residue from alc. extr.	8.7 "	238	0	+

*Assayed by thiochrome procedure.

†Two groups of 5 chicks on this diet received daily intraperitoneal injections of 3 and 6 of thiamine hydrochloride daily and failed to show any polyneuritic symptoms in 15 days.

‡Eight chicks per group except injection group. Mortality indicated by + was 100%, — no mortality.

scribed the period of contact was determined by the time necessary to dry the fish-yeast mixtures in thin layers under forced ventilation at room temperature. Recent experiments have shown that this period was much longer than necessary to effect marked disappearance of thiamine. No attempt was made, however, to measure the rate of destruction. The greatest amount of destruction that occurred in the mixtures containing 43.7 γ B_1 per g of raw fish does not necessarily represent the highest level of destruction possible. Undoubtedly by using higher levels of vitamin B_1 and longer periods of contact, a greater destruction per unit weight of fish could be obtained.

The vitamin B_1 destruction by certain species of raw fish is interesting in view of the tying up of another vitamin, biotin, by a protein factor in raw egg white. Unlike biotin, it does not appear that the thiamine inactivation is due to the formation of an analogous complex by a protein factor in the raw fish, since acid hydrolysis does not release any thiamine from the smelt-thiamine mixtures. The destructive factor appears to destroy easily both the free form of thiamine and its pyrophosphate. Crystalline thiamine hydrochloride and the pyrophosphate ester which makes up a large portion of the vitamin B_1 of dried, killed brewer's yeast, is completely destroyed within the stated limits by a factor in the raw smelt.

Woolley[5] states that the mechanism of vitamin B_1 inactivation by carp is not due to a splitting of the thiamine molecule into its pyrimidine and thiazole portions. It is possible, however, that the destructive factor might degrade either the pyrimidine or thiazole portion after a preliminary hydrolysis of the molecule. The failure of thiamine to form thiochrome after treatment with raw smelt and the absence of a hydrolysis labile thiamine-protein complex strongly suggests that the destruction is in the nature of an enzymatic degradation.

The failure of raw smelt to destroy the thiamine in living yeast is due very likely to the inability of the destructive factor to penetrate the living yeast cell. It appears that a portion of the vitamin B_1 in living yeast was destroyed (see table) but this is probably due to the presence of a certain amount of non-viable yeast in the baker's yeast preparation used. Destruction of the living yeast by heating rendered its B_1 content labile. Woolley and Longsworth[8] have shown that the antibiotin factor was ineffective in hindering initiated yeast growth. The antithiamine factor appears to be similar in its inability to penetrate the living cell.

8 Woolley, D. W., and Longsworth, L. G., *J. Biol. Chem.*, 1942, **142**, 285.

A portion of the active destructive agent in raw smelt can be extracted with 5% alcohol. No attempts were made at a quantitative extraction. The residues after alcohol extraction showed high activity. Spitzer *et al.*,[6] on the basis of the chick assay, report that aqueous extracts of carp are inactive while ether extracts showed partial activity. The carp residue after water and ether extraction showed partial activity. Woolley[5] reports that aqueous extracts of carp had 25% of the activity of whole carp suspensions and that 10% NaCl extracts of carp were highly active.

When finely pulverized smelt are thoroughly dried at room temperature, the B_1 destructive factor is lost and subsequent replacement of the original moisture content will not restore this factor. Heating smelt at 100°C for 5 min also causes a complete destruction of the active factor.

The use of certain species of raw fish in the preparation of vitamin B_1 free diets to be used for experimental purposes appears to be worthy of further investigation. Present methods of preparing B_1 free rations by autoclaving for considerable periods of time undoubtedly cause the destruction or alteration of other dietary factors. The use of a fish or a fish extract would circumvent these undesirable effects.

Summary. A viable yeast can protect its thiamine from destruction by a factor present in raw smelt. The antithiamine factor behaves similarly to the antibiotin factor in its inability to penetrate the living cell. The inactivation of thiamine does not appear to be due to the formation of thiamine complexes labile to hydrolysis or to the splitting of the B_1 molecule but rather to an enzymatic degradation of the thiamine molecule. The destructive factor in raw smelt is labile to air drying at room temperatures, heating at 100°C and is extractable in 5% alcohol. Certain raw fish or extracts of these fish may prove valuable in the preparation of vitamin B_1 free diets without the necessity of resorting to prolonged autoclaving.

13856

Estimation of Reduced Degradation Products in Experimental Trinitrotoluene Poisoning.

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Dale[1] presented tentative evidence that administered TNT (trinitrotoluene) in rabbits is partly reduced to dinitro-azoxytoluene and partly to amino compounds. The work of Voegtlin, Hooper and Johnson[2] indicated that in man the portion of the urinary material giving the Webster[3-5] test was the hydroxylamino derivative. It would seem all of these should, under proper conditions, be diazotizable and if coupled with a suitable color reagent, lend themselves to colorimetric estimation. Elvove[6] made a step in this direction, but his method involved reduction of residual nitro groups to nitrite, and application of the Griess reagent. The widely used Webster test suffers from several severe handicaps: (a) the test has

1 Dale, H. H., Medical Research Council of Great Britain, Special Rept. Series No. 58, 1921, 52.

2 Voegtlin, C., Hooper, C. W., and Johnson, J. M., Hygienic Laboratory Bull. No. 126, 1920.

3 Webster, T. A., *Lancet*, 1916, **191**, 1029.

4 Tutin, F., *Lancet*, 1918, **195**, 554.

5 Ingham, J., *Lancet*, 1941, **241**, 554.

6 Elvove, E., *J. Ind. Eng. Chem.*, 1919, **11**, 860.