At the present time no hypothesis can be proposed to explain the influence of salts on the production of L forms.

Summary. L type colonies have been isolated from group A streptococci by the combined effect of penicillin and concentrations of various salts considerably higher than usually incorporated in bacteriological media. The appearance and morphology of these cultures correspond to those isolated from the alpha streptococci. There is reason to believe that the technic described will be helpful in the isolation of L forms of other bacterial species.

1. Dienes, L., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 579.
2. ———, *ibid.*, 1939, v42, 636.

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Dietary Conditions Affecting Oxidation of Tyrosine by Rat Liver, with Particular Reference to Thiamine.* (21298)

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The oxidation of tyrosine by mammalian liver enzymes proceeds through several steps before the end-products of the reaction, fumaric and acetoacetic acids, are obtained (1-6). Considerable work has been carried out to determine what cofactors are required for the various steps(5-11). Pyridoxal phosphate, ascorbic acid, and glutathione have been shown to be involved in the oxidation of tyrosine. Williams and Sreenivasan(9-11) found that the dye, 2,6-dichlorophenolindophenol (DCPP) could stimulate this oxidation in addition to the stimulation afforded by glutathione and ascorbic acid. It was considered possible that the DCPP in low concentrations might act as a substitute for a labile cofactor of an unknown nature. Boiled extracts of the active enzyme preparation also produced a stimulation of tyrosine oxidation by dialyzed or aged enzyme preparations in addition to the stimulation produced by glutathione and ascorbic acid.

Upon examination of the possible scheme for tyrosine oxidation, it becomes evident that the oxidation of 2,5-dihydroxyphenylpyruvic acid to homogentisic acid involves an oxidative decarboxylation. In view of the well known participation of thiamine-containing coenzymes in oxidative decarboxylation reactions, it was of interest to investigate the oxidation of tyrosine by enzyme preparations from livers of thiamine deficient rats as compared to those from normal control animals. If thiamine was active in promoting oxidation of tyrosine, its possible identity with the labile cofactor of the DCPP-substituting factor suggested by Williams and Sreenivasan(9-11) might be established.

Experimental. Since thiamine is active in the form of thiamine pyrophosphate (TPP) or, in certain systems, as lipothiamide pyrophosphate (LTPP)†(12), these substances were added to enzyme preparations obtained from normal and thiamine deficient rats. Dialyzed enzyme preparations, both from normal and thiamine deficient animals, have also been employed. A combination of a thiamine deficiency and dialysis of the enzyme preparations would be expected to remove thiamine-containing cofactors more completely than either treatment alone. Male rats of the Sprague-Dawley strain weight 90-110 g were divided into two groups. One group was fed a complete purified ration, and the other the same ration with thiamine omitted. The rations consisted of 20% vitamin-free casein,

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salts IV(13), 5% corn oil, 2% vitamin mix (14) (either complete or without thiamine), and 69% sucrose. The animals received food and water *ad libitum* and given weekly oral supplements of haliver oil fortified with vit E and K. After about 4 weeks on the respective diets, the animals receiving no thiamine had decreased in body weight by 20-30 g, while animals receiving complete ration had gained 150-175 g. Some deaths had occurred in the deficient group by this time. Animals from each group were sacrificed, the livers removed, and the enzyme prepared as previously described(9). Warburg flasks were prepared according to procedures already outlined(9). The various cofactors were added to the main compartment of the flasks, as indicated in the accompanying tables, at the following levels: ascorbic acid, 1000 γ; glutathione, 3120 γ; DCPP, 50 γ; TPP, 50 γ; and LTPP, 10 γ. The flasks were incubated in a Warburg bath at 37°C for 10 minutes before addition of the side arm contents. The stopcocks were then closed, and oxygen uptake recorded for one hour. Nitrogen of the enzyme preparations was determined using a semi-micro Kjeldahl procedure.

Results. The oxygen uptake data for liver enzymes prepared from normal and thiamine-deficient animals are presented in Table I. Contrary to all expectations it can be seen that, in the absence of any added cofactors, the tyrosine oxidase activity was considerably higher in those preparations which were obtained from thiamine deficient rats than from rats fed the complete ration. Addition of ascorbic acid and glutathione stimulated the activity in both normal and deficient preparations, although the degree of stimulation was significantly greater in the latter. Addition of the dye, DCPP, to these two preparations further increased oxidation of the substrate. The slight apparent stimulation by TPP in the presence of ascorbic acid and glutathione is not considered significant in view of the spread of the individual values which were averaged to obtain the figure in the Table. LTPP added in the presence of ascorbic acid and glutathione produced no alteration in effect when compared to that obtained when ascorbic acid and glutathione were added. Therefore, it appears that thiamine, when incorporated either as TPP or LTPP does not produce *in vitro* stimulation of the oxidation of tyrosine by either normal or thiamine-deficient enzyme preparations of rat liver under the conditions employed in these studies. When the activities were calculated on an enzyme nitrogen basis, essentially the same picture was obtained as when the results were expressed in terms of fresh liver.

TABLE I. Comparison of Liver Tyrosine Oxidase Preparations from Normal and Thiamine-Deficient Rats.

Additions	Normal activity*	Deficient activity*
None	30	48
AA,† GSH	177	229
" " , DCPP	211	505
" " , TPP	187	263
" " , DCPP, TPP	255	489
" " , LTPP	174	260
" " , DCPP, LTPP	254	514

* The data for addition of LTPP or TPP are averages of 3-4 experiments. Otherwise 8-9 determinations made. Values are expressed as $\mu 10_2$/flask for 60 min.

† AA, ascorbic acid; GSH, glutathione; DCPP, 2,6-dichlorophenolindophenol; TPP, thiamine pyrophosphate, LTPP, lipothiamidepyrophosphate.

TABLE II. Comparison of Liver Tyrosine Oxidase Preparations after Dialysis from Normal and Thiamine-Deficient Rats.

Additions	Normal activity*	Deficient activity*
None	0	11
AA, GSH	0	112
" " , DCPP	0	226
" " , TPP	0	162
" " " , DCPP	0	228
" " , LTPP	0	115
" " " , DCPP	0	236

* These data are averages of 6-7 experiments and expressed as $\mu 10_2$/mg nitrogen/hr.

Effect of dialysis of the normal and thiamine deficient preparations. Since it was possible that, even in the deficient preparations, sufficient thiamine might be present to mask the effect of added TPP or LTPP, dialysis of the homogenates was employed to remove cofactors as completely as possible. The enzyme was prepared as previously described(9) from both normal and deficient animals. The crude enzyme was then dialyzed against frequent changes of distilled water at 5° C for 7-9

TABLE III. Effect of *In Vivo* Thiamine Supplements on Tyrosine Oxidase Preparations from Normal and Thiamine-Deficient Animals.

	Additions	Normal activity*	Deficient activity*
A	None	21	80
	AA, GSH	150	278
B	None	28	29
	AA, GSH	167	168

* Part A is avg of 4-5 experiments. These animals were injected with 2 mg of thiamine 24 hr before use. Part B is the avg of 3 experiments. These animals were fed a complete ration 12-13 days before use. The values are expressed as $\mu 10_2$/mg N/hr.

days before enzymatic activity was measured. The results are presented in Table II. When no cofactor was added, oxygen uptake was small for the thiamine deficient group. Addition of the cofactors listed in the Table, however, increased the activity of the enzyme from the thiamine deficient animals 10-20 fold. In no case was any activity obtained under similar conditions for the enzyme preparations from normal control animals. The slight apparent stimulation by TPP in the presence of ascorbic acid and glutathione for the thiamine deficient group is not considered significant because of overlapping of values from this group and the control. Similarly, addition of LTPP to flasks containing ascorbic acid and glutathione produced no stimulation of oxidation of the substrate.

Effect of thiamine supplementation to deficient and normal animals. Since the activity of the tyrosine oxidase system was so greatly increased in liver preparations from thiamine deficient animals, the question whether the increase was a direct or an indirect consequence of the vitamin deficiency arose. In an effort to gain information on this, both thiamine deficient and control animals were injected intraperitoneally with 2 mg of thiamine. The day following the injections, the enzyme was prepared from livers and the tyrosine oxidase activity measured in the usual manner. Other thiamine deficient animals were transferred from the deficient to the complete diet, and after 12-13 days the livers were assayed for tyrosine oxidase activity. The results of these experiments are presented in Table III. Part A of the Table contains data for animals which were analyzed one day after injection of thiamine, and Part B contains results for those deficient animals which were resupplemented with a complete diet for 12-13 days. From Part A it can be seen that, when thiamine was injected into deficient animals, the tyrosine oxidase activity was still considerably higher in deficient than in normal animals. However, when a complete ration was fed to a group of thiamine-deficient animals for a period of about two weeks before analysis, the differences between deficient and control groups disappeared.

Effect of inanition on tyrosine oxidase activity. Since one of the more prominent features of a thiamine deficiency is anorexia, it was possible that the increase in tyrosine oxidase during a thiamine deficiency might have been due to a decrease in food intake. To study this possibility, the effects of inanition on liver tyrosine oxidase activity were investigated. Male rats of the Sprague-Dawley strain weighing 110-140 g were used. They were placed in cages with water but received no food for 4-7 days. At this time livers were removed and assayed for tyrosine oxidase activity. The results of these experiments, presented in Table IV, indicate that the system from starved animals could be stimulated by DCPP in the presence of ascorbic acid and glutathione to produce a greater oxidation of tyrosine than the normal preparation. This observation was also true of the system from thiamine-deficient animals. However, the stimulation by ascorbic acid and glutathione was not as marked in the preparations from starved animals as in those from thiamine-deficient animals.

Discussion. Increased oxidation of tyrosine by enzyme preparations from livers of thiamine-deficient rats or from starved rats as compared to the normal controls, is an unex-

TABLE IV. Comparison of Liver Tyrosine Oxidase Preparations from Normal and Starved Animals.*

System additions	Starved	Normal
None	31	35
AA, GSH	131	214
" " , DCPP	351	254

* These data are averages of 6-8 experiments and expressed as $\mu 10_2$/mg N/hr.

pected observation that is very difficult to explain. In many cases of nutritional insufficiencies, decreases in activity of many enzymes have been observed. Thus, inanition lowers the activity of liver xanthine oxidase(15) while feeding of a diet low in protein decreases the activity of xanthine oxidase(16), choline oxidase(17), and succinic oxidase(18). The activity of xanthine oxidase and succinic oxidase has also been decreased by feeding diets lacking in methionine(19). Therefore, the fact that a thiamine-deficiency increases the oxidation of tyrosine by liver preparations and that this effect can be partially duplicated by the effects of simple inanition is difficult to fit with what is known concerning interrelationships of nutritional deficiencies and tissue enzyme activities.

One possibly similar situation is that of the effect of a folic acid deficiency on liver xanthine oxidase activity. Several workers(20-23) have observed that in animals with a folic acid deficiency liver xanthine oxidase is considerably higher than in animals receiving a complete diet.

The failure to demonstrate a requirement for thiamine for the oxidative decarboxylation of tyrosine is quite unusual in view of the fact that similar reactions involving α-ketoglutarate and pyruvate have a definite need for the vitamin(22,23).

Summary. 1. Oxidation of tyrosine by the tyrosine oxidase system from livers of thiamine-deficient animals is markedly higher than that by preparations from normal animals. 2. After dialysis the activity of the normal preparation cannot be restored by the addition of ascorbic acid, glutathione, or 2,6-dichlorophenolindophenol (DCPP). The thiamine-deficient preparations are still very active under these conditions, however. 3. Injection of thiamine into normal and deficient animals one day before assay of the livers for tyrosine oxidase has little effect on the high activity of the thiamine-deficient preparations. Feeding a complete ration for 2 weeks to thiamine-deficient animals, however, decreases the activity to the level present in the normal preparations. 4. Inanition produces an increase in tyrosine oxidase activity by liver enzymes. This may be a partial explanation for the fact that thiamine-deficient rats (which are anorexic) possess a higher activity of this enzyme. The reason for the increase under either of these conditions, however, cannot be explained at present.

1. Lerner, A. B., *J. Biol. Chem.*, 1949, v181, 281.
2. Ravdin, R. G., and Crandall, D. I., *J. Biol. Chem.*, 1951, v189, 137.
3. La Du, B. N., Jr., and Greenberg, D. M., *J. Biol. Chem.*, 1951, v190, 245.
4. Schepartz, B., *J. Biol. Chem.*, 1951, v193, 293.
5. Knox, W. E., and LeMay-Knox, M., *Biochem. J.*, 1951, v49, 686.
6. Knox, W. E., and Edwards, S. E., *Fed. Proc.*, 1954, v13, 242.
7. Painter, H. A., and Zilva, S. S., *Biochem. J.*, 1950, v46, 542.
8. Sealock, R. R., Goodland, R. L., Summerwell, W. N., and Brierly, J. M., *J. Biol. Chem.*, 1952, v196, 761.
9. Williams, J. N., Jr., and Sreenivasan, A., *J. Biol. Chem.*, 1953, v203, 109.
10. ———, *ibid.*, 1953, v203, 605.
11. ———, *ibid.*, 1953, v203, 613.
12. Reed, L. J., and DeBusk, B. G., *J. Am. Chem. Soc.*, 1952, v74, 3457, 3964, 4727.
13. Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1941, v138, 459.
14. Williams, J. N., Jr., Feigelson, P., and Elvehjem, C. A., *ibid.*, 1950, v187, 597.
15. Miller, L. L., *ibid.*, 1948, v172, 113.
16. Williams, J. N., Jr., and Elvehjem, C. A., *J. Biol. Chem.*, 1949, v181, 559.
17. Richert, D. A., and Westerfeld, W. W., *ibid.*, 1952, v199, 829.
18. Wainio, W. W., Eichel, B., Eichel, H. J., Person, P., Estes, L., and Allison, J. B., *J. Nutrition*, 1953, v49, 465.
19. Williams, J. N., Jr., Denton, A. E., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 386.
20. Keith, C. K., Broach, W. J., Warren, D., Day, P. L., and Totter, J. R., *J. Biol. Chem.*, 1948, v176, 1095.
21. Williams, J. N., Jr., Nichol, C. A., and Elvehjem, C. A., *ibid.*, 1949, v180, 689.
22. Stumpf, P. K., Zarudnaya, K., and Green, D. E., *ibid.*, 1947, v167, 817.
23. Lohmann, K., and Schuster, P., *Biochem. Z.*, 1937, v294, 188.

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