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Effect of Heat Sterilization on Growth-Promoting Activity of Pyridoxine for *Streptococcus lactis* R.

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In a previous publication[1] the existence of a physiologically active metabolite of pyridoxine which possessed much greater activity for *Streptococcus lactis* R than did synthetic pyridoxine itself was demonstrated. For convenience this metabolite (or mixture of metabolites) was called "pseudopyridoxine." It was further shown that the absorption of pyridoxine by this organism from media in which pyridoxine (or a derivative) was in limiting concentration did not occur to a measurable extent. This result is in contrast to those secured with all other known nutrilites which have been investigated. On the basis of this fact, it was postulated that pseudopyridoxine, rather than pyridoxine, was the physiologically essential factor for this organism. The growth-promoting activity of pyridoxine, according to this view, resulted from a transformation of a minute amount of pyridoxine to a more highly active form (pseudopyridoxine), which was then utilized by the organism for growth, while most of the pyridoxine present remained unchanged and hence unavailable for growth of the organism. It was not clear why such a conversion process should cease, and growth of the organism stop, while an excess of unchanged pyridoxine was still present in the medium.

Results cited below show that the conversion results not from activities of the test organism, but rather from interaction of pyridoxine with certain constituents of the medium during heat sterilization. It is shown that the activity of pyridoxine can vary greatly, depending upon how long it is autoclaved with the medium.

TABLE I.
Effect of Autoclaving* with Medium on Growth-Promoting Action of Pyridoxine.

Pyridoxine hydrochloride added, γ per 10 cc	Galvanometer reading†			
	Unheated	Autoclaved 10 min.	Autoclaved 20 min.	Autoclaved 40 min.
0	10	10	10	10
0.2		18	37	43
0.4		26	46	54
0.7	12	35	54	54
1.0	15	42	54	
2.0	15	53		
3.0	21	55		
5.0	29			
10	34			
30	43			
Ratio of activities	1.0	13	41	65

*The culture tubes were autoclaved at 15 lb steam pressure.
†This is a measure of culture turbidity: a reading of zero indicates 100% transmission; a reading of 100 is complete opacity.

Experimental. The medium and technic used were exactly similar to those previously described in detail.[1]

Results. The comparative activity of given amounts of pyridoxine in promoting growth of *Streptococcus lactis* after various periods of autoclaving with the complete medium is shown in Table I. The most

[1] Snell, E. E., Guirard, B. M., and Williams, R. J., *J. Biol. Chem.*, 1942, **143**, 519.

turbid culture obtained gave a galvanometer reading of 55; to achieve this degree of growth required more than 30γ of pyridoxine in the unheated test, whereas only 2, 0.7 and 0.42γ were required to produce a like amount of growth after autoclaving for 10, 20, and 40 min, respectively. The ratio of growth-promoting activity after a given period of autoclaving to that of unheated pyridoxine is given in the last line of the table. These values were calculated from the standard curve obtained after 10 min of autoclaving[1] and demonstrate the profound effect of autoclaving with the medium on the growth-promoting power of pyridoxine for this organism.

Tests were next made to determine which constituents of the medium were responsible for this effect. It was found that autoclaving pyridoxine at pH 7.2 with the hydrolyzed casein of the medium, or with the cystine or tryptophane alone, was effective in increasing its activity. Autoclaving under the same conditions with any of the other constituents of the medium had no such effect. Thus the effect appeared due to interaction of some type between pyridoxine and amino acids during autoclaving. Individual amino acids were therefore compared as to their effectiveness in producing this change. 0.1 mg portions of pyridoxine hydrochloride were added to 10 cc of a solution which contained 50 mg of sodium acetate plus 1 to 5 mg of various amino acids. The pH was 7.2. All tubes were autoclaved at 15 lb pressure for 30 min. The contents of each tube were then assayed with *S. lactis* for their apparent pyridoxine content. The entire assay was autoclaved 10 min as previously described.[1] Results are given in Table II. The effect is quite non-specific, since it is given by each of the amino acids tested. Cystine was especially effective; the next most effective amino acid was glycine. The effect of varying the concentrations of cystine and glycine is shown in Table III. Again, cystine is the more effective in producing the activation.

TABLE II.
Effect of Autoclaving Pyridoxine with Individual Amino Acids on Its Growth-Promoting Activity.

Amino acid	Amt, mg per 10 cc	Activity compared with untreated pyridoxine*
None		1.1
l-Tryptophane	1.0	1.8
l-Cystine	1.0	5.2
dl-Alanine	5.0	2.3
Glycine	5.0	3.9
l-Lysine	5.0	1.9
d-Arginine	5.0	2.6
l-Histidine	5.0	1.3
dl-Phenylalanine	5.0	2.6
dl-Leucine	5.0	2.2
Casein Hydrolysate†	5.0	2.1

*These comparisons were made in an assay where all tubes were autoclaved for 10 minutes. This increase in activity therefore represents that which occurred as a result of the above treatments *in addition* to that occurring during 10 minutes of autoclaving with the medium, as shown in Table I.

†Norite-treated: assay of any of the above products alone at dilutions present in the test show an apparent pyridoxine content of zero.

TABLE III.
Effect of Autoclaving with Various Concentrations of Cystine and Glycine on Activity of Pyridoxine.

Amino acid	Amt. mg per 10 cc	Activity compared to untreated pyridoxine*
None		1.1
l-Cystine	0.10	1.5
,,	0.30	2.7
,,	1.00	5.9
,,	3.00	8.2
,,	10.00	10.6
Glycine	0.10	1.3
,,	0.30	1.4
,,	1.00	2.5
,,	3.00	4.2
,,	10.00	4.1

**Cf.* footnote to Table II.

Discussion. These observations indicate that synthetic pyridoxine is almost, if not completely inactive as a growth factor for *S. lactis*. It becomes active due to formation in minute amounts of a substance of unknown structure when pyridoxine is heated with the amino acids of the medium during sterilization. The inactivity of unchanged pyridoxine in promoting growth of this organism explains the previously observed fact that growth ceased in a medium where growth apparently was limited only by the pyridoxine concentration before appreciable amounts of pyridoxine were absorbed. It was shown[1] that a derivative of pyridoxine of similarly high activity for *S. lactis* occurs universally in natural extracts, and that pyridoxine is partially converted to a product

of similarly high activity by animal passage. These conclusions stand unaltered by the present findings. Whether the substances present or formed in these different instances are identical, remains to be demonstrated. Activation of pyridoxine in this manner by heating *in vitro* is not an effect of amino acids alone. Such substances as thioglycollic acid, ammonia, and others also promote the change.

One other strain of *S. lactis* tested behaves the same as that used in this study. Yeast is not dependent upon such a change in the pyridoxine molecule before it is able to use it. Other organisms have not been studied in this regard. Landy and Dicken[2] have recently recommended *Lactobacillus casei* as a test organism for the assay of pyridoxine in natural materials. In our experience, this assay is subject, in a lessened degree, to the same disturbing influences previously outlined[1] and those mentioned above. For example, in one assay carried out according to their procedure, Difco yeast extract contained 2.0, 0.6 or 0.35 mg of pyridoxine per gram as determined after autoclaving the test for 10, 20 or 40 min, respectively. Yeast assay[3] gives 0.015 mg pyridoxine per gram of this material. It is evident that much more investigation into forms of pyridoxine which are physiologically active for animals is required before any organism can be used routinely for the estimation of "vitamin B_6" in natural materials.

Summary. Synthetic pyridoxine is almost inactive as a growth factor for *S. lactis* R in a pyridoxine-free medium if heat sterilization is avoided. Autoclaving media which contain pyridoxine greatly increases its activity for this organism. The same effect was achieved in varying degrees by autoclaving pyridoxine at neutrality with individual amino acids. Cystine and glycine were most effective in producing this change.

2 Landy, M., and Dicken, D. M., *J. Lab. Clin. Med.*, 1942, **27**, 1086.

3 Williams, R. J., Eakin, R. E., and McMahan, J. R., *Univ. Texas Pub.*, 1941, No. 4137, 24.

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Biotin Deficiency in the Rat.

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A number of investigators have reported the occurrence of depigmentation of the pelage of rats maintained on egg-white containing rations. György and co-workers[1,2] noted a brownish discoloration of the black fur, or single gray hairs interspersed in the fur without generalized graying, or both. Sullivan and Nicholls[3] also observed a diffuse, partially decreased pigmentation in some animals receiving egg-white containing rations. Symmetric patterns of grayness such as produced by a deficiency of pantothenic acid were not observed. In the present study the occurrence of a symmetrical achromotrichia is described in black rats maintained on dried, fresh egg-white containing rations after curative therapy had been initiated by the feeding of biotin. The pattern was the reverse of that observed in pantothenic acid deficiency.

A comparison was made of several diets in respect to the production of the biotin deficiency syndrome and the alleviation of symptoms with biotin therapy.

Experimental. Littermate weanling male rats were segregated into each of 6 dietary

1 György, P., in Pfaundler, A., and Schlossmann, M. v., *Handbuch der Kinderheilkunde*, 1935, **10**, 55.

2 György, P., and Poling, C. E., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 773.

3 Sullivan, M., and Nicholls, J., *Arch. Derm. and Syph.*, 1942, **45**, 295.