14269

# Vitamin Requirements of *Torula cremoris.**

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In the course of studies dealing with vitamin deficiencies of microörganisms it was found that a laboratory culture of *Torula cremoris* was unable to grow in a simple medium of inorganic salts, ammonium phosphate and glucose. On substitution of a mixture of 19 amino acids for the ammonium phosphate the torula likewise failed to grow and these negative results were in sharp contrast to the luxuriant growth in dextrose broth or agar.

When a mixture of 16 accessory growth factors and related compounds was added either to the amino acid or the ammonium phosphate basal medium growth of this strain of *Torula cremoris* occurred promptly with marked turbidity in 24 hours.

Upon successive elimination of accessory factors from the mixture it was found that 4 of them were necessary for rapid and luxuriant growth. These were nicotinamide (or nicotinic acid), biotin, pantothenic acid and thiamin. When these were supplied the addition of other factors, which were in the original accessory mixture, did not appreciably increase the rate or luxuriance of growth.

The effect upon growth of various combinations of the above 4 factors was next studied. For these experiments biotin was supplied as the methyl ester in a concentration of 0.002 $\mu$g per ml, calcium pantothenate, thiamin and nicotinamide in concentrations of 0.2 $\mu$g per ml. The ammonium phosphate basal medium was composed of $(NH_4)_2HPO_4$ 0.2%, $KH_2PO_4$ 0.15%, NaCl 0.5%, $MgSO_4$ 0.01% and glucose 0.5%. The amino acid basal medium consisted of 17 amino acids in amounts of 50 to 200 mg each per liter and serine and threonine each 15 mg per liter, $K_2HPO_4$ 0.2%, NaCl 0.5%, $MgSO_4$ 0.01% and glucose 0.5%. The pH of both media was 6.8 to 6.9. It was tubed in 5 ml amounts.

All tubes of each experiment were inoculated with the same amount of a dilution of a 24-hour culture grown in the ammonium phosphate basal medium with the 4 needed vitamins. Plate counts with dextrose agar indicated that the number of yeast cells in the inoculum in the various experiments was usually from 2,200 to 3,000 per ml of medium. Incubation was at 37°C since this yeast grows well at that temperature. The turbidity resulting from growth was measured in a colorimeter with selected 15 mm test tubes containing 5 ml of medium. In Table I a clear tube of the culture medium is shown by zero and increasing turbidities are shown by higher figures.

Omission of thiamin from the 4 vitamins shown in combination 2 of Table I produced only slight delay in the rate of growth in either basal medium. The omission of pantothenic acid resulted in some delay in growth which, peculiarly, was more marked in the amino acid than in the ammonium phosphate medium. This rather striking difference in the rate of growth in the two basal media, in the absence of pantothenic acid, was observed on repeated tests. It emphasizes the importance of the basal medium in relation to the results obtained in studies of accessory growth factor requirements.

The omission of nicotinamide resulted in complete or almost complete cessation of growth. Evidently nicotinamide is essential under the conditions of these experiments.

Omission of biotin gave rise to interesting results. This compound is needed for prompt growth but slow multiplication may be obtained without it when the other three accessory factors are supplied. The rate of growth in the absence of biotin was quite different in the two basal media; in the ammonium phosphate medium usually 5 to 10

* This investigation was aided by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

TABLE I.
Effect of Certain Vitamins upon Growth of *Torula cremoris*.

| Supplement to basal medium | Basal medium: Ammonium phosphate-glucose-salts, Growth by days 1 | 2 | 3 | 7 | Basal medium: Amino acids-glucose-salts, Growth by days 1 | 2 | 3 | 7 |
|---|---|---|---|---|---|---|---|---|
| 1. None (control) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| 2. Nicotinamide, biotin, Ca pantothenate, and thiamin | 22 | 47 | 48 | 56 | 32 | 39 | 42 | 49 |
| 3. (2) minus thiamin | 8 | 44 | 45 | 55 | 21 | 35 | 39 | 47 |
| 4. (2) " Ca pantothenate | 0 | 27 | 40 | 56 | 0 | 0 | 1 | 20 |
| 5. (2) " nicotinamide | 2 | 3 | 3 | 8 | 4 | 6 | 9 | 9 |
| 6. (2) " biotin | 0 | 0 | 0 | 7* | 3 | 19 | 25 | 28 |
| 7. Biotin plus nicotinamide | 0 | 2 | 30 | 53 | 0 | 0 | 0 | 22 |
| 8. " " Ca pantothenate | 0 | 0 | 2 | 2 | 0 | 1 | 1 | 3 |
| 9. Nicotinamide plus thiamin | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 4 |

* Heavier growth later.

days were required for attainment of visible growth, while in the amino acid medium growth of the torula was always quite marked by 48 hours.

This speedier growth in the latter instance was found to be due largely to aspartic acid (100 to 200 $\mu$g per ml of medium). A similar effect was obtained with synthetic dl-aspartic acid.[1] Other amino acids with the possible exception of glutamic acid did not exert this effect. Further work has shown that the effect of aspartic acid is somewhat more pronounced if the tubes are incubated in an atmosphere of 10% $CO_2$ in air, though the growth is not as prompt as that caused by biotin either under ordinary atmospheric conditions or in 10% $CO_2$.

Biotin and nicotinamide together are sufficient to support growth of *Torula cremoris*, though culture development is slower than when thiamin and calcium pantothenate are also supplied. Other combinations of two accessories resulted in either poor growth or no growth. Results with several of the possible combinations are shown in Table I.

Our results with the combination of nicotinamide and thiamin are similar to those of Robbins and Kavanagh[2] who reported that *Torula cremoris* failed to grow in a medium of aspargine, dextrose, ammonium nitrate and inorganic salts when supplemented with either the pyrimidine or thiazole components of thiamin or with thiamin plus nicotinamide.

When added singly each of the 4 accessories failed to support continued growth with but one exception. In the presence of only nicotinamide slow growth was maintained through 10 successive transfers in the amino acid medium but not in the ammonium phosphate medium.

Successive transfers of the torula were also attempted in the ammonium phosphate basal medium with all of the combinations of accessories shown in Table I. Those in which growth could be carried through 10 successive transfers were combinations 2, 3, 4, 6 and 7. Cultures failed to grow either in the first tube or after the first few transplants in numbers 1, 5, 8 and 9.

Experiments were conducted with decreasing amounts of biotin methyl ester in the presence of an excess of nicotinamide, calcium pantothenate and thiamin in the ammonium phosphate basal medium. The results were in harmony with other reports showing the extreme activity of biotin. One millionth $\mu$g per ml of medium supported very light growth, 0.00006 $\mu$g supported about half maximum growth and 0.002 to 0.003 $\mu$g supported approximately maximum development.

*Summary.* Nicotinamide, biotin, calcium pantothenate, and thiamin must be supplied for prompt and abundant growth of *Torula cremoris* in a basal medium of inorganic salts, glucose and ammonium phosphate

[1] Koser, S. A., Wright, M. H., and Dorfman, A., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 204.

[2] Robbins, W. J., and Kavanagh, F., *Plant Physiology*, 1938, **13**, 611.

or amino acids. The combination of nicotinamide and biotin supports slow growth and under certain conditions nicotinamide alone may suffice.

Tests with the 4 vitamins singly and in various combinations show that the response to the vitamins depends to some extent upon the basal medium used in the tests. The results emphasize the importance of the composition of the basal medium in studies of vitamin requirements.

14270

## Studies on Prothrombin: IV. The Prothrombinopenic Effect of Salicylate in Man.

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Following the demonstration[1] of the quantitative degradation of 3,3'methylenebis(4-hydroxycoumarin) to 2 mols of salicylic acid, Link and his students[2] demonstrated that in the rat salicylic acid induces a prothrombinopenia preventable by natural or synthetic vitamin K.

The purpose of this paper is to report that the fundamental observations made on the rat also apply to man.†

*Experimental.* The action of sodium salicylate, of acetyl salicylic acid with and without dicumarol (3,3'methylenebis(4-hydroxycoumarin)), and the effect of synthetic vitamin K were studied on individuals representing types in which salicylate therapy is commonly used. Since there was variation in age, nutritional state and diet, the salient facts can best be illustrated by representative cases and with dosages comparable to those used therapeutically.[3]

Twenty-seven adults varying in ages between 24 and 80 years were studied. Eight were apparently normal and ambulatory; 12 were chronically ill patients; 6 were cases of Laennec's cirrhosis of the liver; 1 was a case of acute rheumatic endocarditis.

The method used to estimate the prothrombin level (or activity) included a measure of the prothrombin time of whole and diluted (12.5%) plasma.‡ The rationale and clinical applications have been set forth in previous communications.[4-7] By estimation of 12.5% plasma prothrombin time the initial prolongation has usually been detected between 8 and 24 hours after adequate dosage of salicylate. It is obvious from Fig. 1 that detection of a change in prothrombin time on whole plasma is restricted to a very slight shift in seconds, but with 12.5% plasma a more practical working range is afforded.

*Action of Sodium Salicylate.* Fig. 2, curve 1 shows the response in a patient who received 8 g of sodium salicylate on 3 consecutive days. Two others receiving the same

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1 Stahmann, M. A., Huebner, C. F., and Link, K. P., *J. Biol. Chem.*, 1941, **138**, 513.

2 Link, K. P., Overman, R. S., Sullivan, W. R., Huebner, C. F., and Scheel, L. D., *J. Biol. Chem.*, 1943, **147**, 463.

† This was investigated at the same time by O. O. Meyer at the Wisconsin General Hospital, Madison.

3 Hanzlik, P. J., *Action and Uses of the Salicylates and Cincophen in Medicine*, The Williams and Wilkins Co., Baltimore, 1927.

‡ All estimations were done in duplicate.

4 Shapiro, S., Sherwin, B., Redish, M., and Campbell, H. A., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 85.

5 Shapiro, S., Redish, M. H., Campbell, H. A., *Am. J. Med. Sc.*, in press.

6 Shapiro, S., Redish, M. H., Campbell, H. A., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 12.

7 Shapiro, S., Sherwin, B., and Gordimer, H., *Ann. Surg.*, 1942, **116**, 175.