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Determination of Biotin with *Lactobacillus arabinosus.*

LEMUEL D. WRIGHT AND HELEN R. SKEGGS. (Introduced by A. D. Welch.)

From the Nutritional Laboratories, Department of Pharmacology, Medical-Research Division, Sharp and Dohme, Inc., Glenolden, Penn.

Snell and Wright reported that biotin is an essential growth factor for *Lactobacillus arabinosus.*[1] It was stated that the microbiological determination of biotin may be accomplished through the use of the same basal medium employed in the microbiological determination of nicotinic acid, provided that biotin is excluded and nicotinic acid is added. Since then several procedures have appeared for the microbiological determination of biotin with *Lactobacillus casei.*[2,3,4]

The growth factor requirements of *L. arabinosus* are less complex than those of *L. casei,* since luxuriant and rapid growth of *L. arabinosus* may be obtained in a medium composed only of certain purified amino acids, glucose, purines and pyrimidines, inorganic salts, and several synthetic members of the vitamin B complex. Growth stimulation by an eluate factor from tomato juice of unknown composition[5] occurs only under certain conditions where the basal medium and the medium for inoculation are prepared from very nighly purified constituents.[6] On the other hand, *L. casei* is stimulated by a variety of unknown factors and, in addition, requires the presence of "folic acid."[7] Desthiobiotin has an antibiotin activity for *L. casei* but it does not affect the growth of *L. arabinosus.*[8,9] Difficulty has been reported in the preparation of an adequate basal medium for the determination of biotin by one of the methods using *L. casei* but certain improvements in procedure have been described.[4]

Saccharomyces cerevisiæ has been used widely as a test organism in the determination of biotin.[10] It has been shown, however, that yeast responds to peroxide-treated biotin,[11] as well as to certain forms of biotin which do not combine with avidin (vitamers[12]) and while such yeast methods may be useful in studies of biotin metabolism they may be criticised because of lack of specificity.

Clostridium butylicum[13] and *Staphylococcus aureus*[14] have been recommended as test organisms for the determination of biotin. These bacteria have not, however, been widely used.

Since *L. arabinosus* has been used successfully for the determination of biotin in these laboratories for some time, the details of the procedure are presented here.

Procedures for the use of *L. arabinosus* in the microbiological determination of pantothenic acid, *p*-aminobenzoic acid, nicotinic

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acid, and a variety of amino acids have appeared.

Procedure. The details of the procedure resemble those described for other microbiological methods involving lactic acid bacteria.

L. arabinosus 17-5* is carried in 1% glucose, 1% yeast extract, 1.5% agar stabs. Transfers from previous stock cultures are made monthly. The fresh transfers are incubated at 30°C for 3 days and then are held in the refrigerator.

One day prior to the procedures leading to the determination of biotin an inoculum is prepared by transfer of the organism from stock culture to a medium of 1% glucose and 1% yeast extract. The latter is incubated at 30°C for 18-36 hours before use. After growth the cells are centrifuged and washed once with sterile saline and a faintly visible saline suspension prepared for seeding the unknown and the standard tubes.

The constituents of the basal medium are shown in Table I. It is convenient to prepare, in one solution of double strength, all the ingredients of the medium with the exception of glucose and the synthetic vitamins. This solution may be preserved under benzene without previous heat sterilization. A stock solution is prepared containing in each 100 ml: thiamin chloride, riboflavin, pantothenic acid, nicotinic acid, 4 mg of each; pyridoxine hydrochloride, 8 mg; and para-aminobenzoic acid, 0.4 mg.

TABLE I.
Basal Medium.

Hydrochloric acid-hydrolyzed, norite-treated, vitamin-free casein (16)	0.5	%
Tryptophane	0.01	"
Cystine*	0.01	"
Glucose	2.0	"
Sodium acetate (anhydrous)	0.6	"
K_2HPO_4	0.05	"
KH_2PO_4	0.05	"
NaCl	0.001	"
$MgSO_4 \cdot 7H_2O$	0.02	"
$FeSO_4 \cdot 7H_2O$	0.001	"
$MnSO_4 \cdot 4H_2O$	0.001	"
Adenine sulfate†	5	ppm
Guanine hydrochloride†	5	"
Xanthine†	5	"
Uracil†	5	"
Thiamine	1.0	"
Calcium pantothenate	1.0	"
Pyridoxin hydrochloride	2.0	"
Riboflavin	1.0	"
Nicotinic acid	1.0	"
Para aminobenzoic acid	0.1	"

* Dissolved separately with the aid of a small amount of concentrated hydrochloric acid.

† Dissolved separately by heating with hydrochloric acid.

Scrupulously clean pyrex bacteriological test tubes are used and are supported in a wire rack. Appropriate amounts of a biotin solution are pipetted into a series of tubes to establish a standard curve representing the response of *L. arabinosus* to varying amounts of biotin. Various amounts of the materials to be assayed are pipetted into additional tubes. All tubes are then diluted to 5 ml with distilled water. An amount of medium (double strength) sufficient to supply 5 ml for each assay tube is supplemented with 4 g of glucose and 5 ml of vitamin solution per 100 ml. Five ml of the complete medium is then added to each assay tube. The tubes are plugged with cotton and the test autoclaved at 15 lb pressure for 15 minutes. After cooling to room temperature each tube is seeded aseptically with one drop of the bacterial suspension for inoculation. The test is incubated at 30-33°C for approximately 72 hours. Titration of the lactic acid produced, using bromthymol blue as the indicator, is used as a measure of the response of the organism to biotin. From the response of *L. arabinosus* to pure biotin (Fig. 1), which must be determined with every test, the biotin content of the unknown samples is calculated. We have found it convenient to assay unknown materials at 4 different levels. In a successful assay there should be good agreement between the four levels of unknown material assayed.

Results. Comparable results obtained when a variety of natural materials were microbiologically assayed for biotin by the proposed procedure employing *Lactobacillus arabinosus,* and by the biotin procedure of Landy and Dicken[2] with *Lactobacillus casei* are given in Table II. Both procedures gave similar results when applied to materials autoclaved with acid and to some untreated materials. With certain other water-soluble materials, particu-

* Cultures of this organism may be secured from the American Type Culture Collection, Georgetown University Medical School, Washington, D.C., where it is listed as No. 8014.

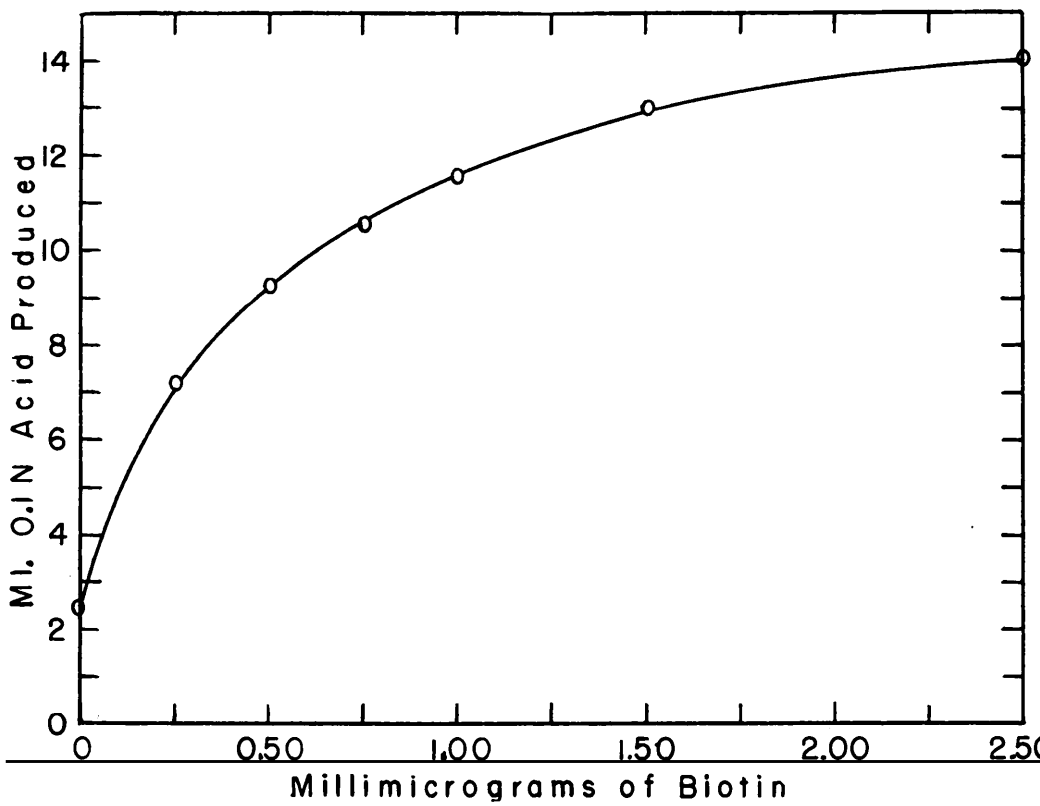

FIG. 1.
The response of *Lactobacillus arabinosus* to added biotin.

TABLE II.
Biotin Content of Various Materials as Determined by *L. arabinosus* and by *L. casei*.

Material	Biotin content	
	L. arabinosus μg/g or ml	*L. casei* μg/g or ml
Curbay powder, untreated	3.7	3.9
Valentine's meat extract, untreated	.038	.038
Bacto peptone, untreated	.15	.16
" " acid autoclaved	.17	.17
Proteose peptone, untreated	.11	.44
" " acid autoclaved	.37	.40
Urine, untreated	.032	.032
Purina chow, acid autoclaved	.19	.24
Yeast extract, untreated	.090	1.6
" " acid autoclaved	1.5	1.8
Malt extract, untreated	.045	.065
Trypsinized vitamin-free casein	.00081	.010
Grass juice powder, untreated	.34	.53
Tryptone, untreated	.16	.22
Klim, untreated	.16	.20
Liver powder 1:20, untreated	.22	.27
" " 1:20, acid autoclaved	.29	.21

larly autolyzed or enzyme-treated substances, higher values were obtained with *L. casei*. Acid hydrolysis (6 N H_2SO_4 at 15 lb pressure for 1 hour) did not affect the amount of biotin found with *L. casei* but increased the observed biotin content when determined with

L. arabinosus to a value equal to that found with *L. casei*. It would appear that there exists in certain enzymatically prepared materials a form of biotin which may be utilized for growth and acid production by *L. casei* but not by *L. arabinosus*. Using yeast extract as an example, it was possible to show that this form of biotin, available to *L. casei* but not to *L. arabinosus*, was avidin-combinable. Acid autoclaving converts this form of biotin to one used equally well by the two organisms. This need not seriously interfere with the use of *L. arabinosus* as an assay organism for biotin since it is well known that there exist in many natural materials combined forms of biotin which are not available to bacteria. Preliminary acid hydrolysis has become an accepted procedure before biotin determination by any method.

The recoveries obtained when biotin was added to several naturally occurring materials are given in Table III. Quantitative recovery of biotin was readily obtained. It is believed that better agreement at varying assay levels for the biotin content of various materials is obtained with the *L. arabinosus* method than is possible with the *L. casei* method of Landy and Dicken.[2]

Since pimelic acid is capable of being utilized in lieu of biotin by the diphtheria bacillus,[15] the ability of *L. arabinosus* to utilize similar dicarboxylic acids was studied. Adipic, pimelic, suberic, azelaic, and sebacic acids were found to possess no biotin-like activity for *L. arabinosus*.

TABLE III.
Recovery of Biotin* Added to Various Materials.

Sample	Biotin content μg/g or ml	Recovery %
Proteose peptone†	0.11	100
Bacto peptone†	0.15	100
Malt extract†	0.050	100
Rat liver‡	0.48	101

* Biotin added to the final solution for assay in an amount which approximately doubled that originally present.

† A solution of these materials assayed without previous treatment.

‡ Autoclaved with 6 N H_2SO_4 at 120°C for 1 hour before determination.

Summary. The microbiological determination of biotin, using *Lactobacillus arabinosus* as the test organism, was studied. The existence of a water-soluble, avidin-combinable form of biotin in certain enzymatically prepared materials which is available to *L. casei*, but not to *L. arabinosus*, was demonstrated. When natural materials are autoclaved with 6 N H_2SO_4, prior to determination of their biotin content, comparable results are obtained with the *L. arabinosus* method and with a method using *L. casei*.

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Methionine Deficiency in Yeast Protein.

A. A. KLOSE AND H. L. FEVOLD.

From the Western Regional Research Laboratory, Albany, California.*

The present shortage of proteins has stimulated interest in the production of yeast for use primarily as a source of food (or feed) protein. Work now in progress in this laboratory[1,2] involves the development of methods to produce torula yeasts grown on molasses and fruit wastes, and the evaluation of these yeasts as a source of feed protein.

* One of four regional research laboratories operated by the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

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