[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY AND CHEMICAL ENGINEERING, THE UNIVERSITY OF TEXAS]

A Quantitative Test for Biotin and Observations Regarding its Occurrence and Properties

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In the course of studies on biotic acid[1] we found that vitamin B_6 serves as a yeast nutrilite[2] and were led to introduce the pure vitamin into our basal culture medium. (The effect of vitamin B_6 on yeast was independently discovered elsewhere at the same time.[3]) In this modified basal medium, yeast became much more sensitive to addenda of small amounts of liver preparations. Shortly after this, through the kindness of Professor Kögl, we were furnished with a sample of biotin, and a few tests demonstrated that in this medium the yeast was responding specifically to biotin.

Some of the observations reported herein were made before we were aware that biotin was the physiologically active substance involved.

Experimental Procedure

The yeast used in this test was isolated in pure culture from a cake (pound goods) of Fleischmann yeast, and was carried thereafter on molasses–agar slants as previously described.[4] Several cultures isolated from this source were found interchangeable for this purpose. The basal medium contains: C. P. sucrose 20 g., $(NH_4)_2SO_4$ 3 g., KH_2PO_4 2 g., $MgSO_4 \cdot 7H_2O$ 0.25 g., $CaCl_2 \cdot 2H_2O$ 0.25 g., H_3BO_3 1 mg., $ZnSO_4$ 1 mg., $MnCl_2$ 1 mg., $TlCl_3$ 1 mg., $FeCl_3$ 0.5 mg., $CuSO_4 \cdot 5H_2O$ 0.1 mg., KI 0.1 mg., *l*-aspartic acid 0.1 g., inositol 5 mg., β-alanine 0.5 mg., thiamin 20 γ and vitamin B_6 20 γ, all added to one liter of distilled water.

Addenda to the basal medium are dissolved in 2 ml. of water in 50-ml. Erlenmeyer flasks and sterilized by steaming for five minutes. After cooling, 10 ml. of the (previously sterilized) basal medium containing 0.02 mg. of suspended yeast is pipetted into each flask which is set unstoppered and without agitation in an incubator at 30° for the growth period of approximately sixteen hours. Very clean glassware is essential for proper results.

At the end of the growth period the yeast suspension is diluted with 10 ml. of saturated aqueous chlor thymol and shaken mechanically for three to five minutes. The amount of yeast present is determined by use of a thermocouple-galvanometer set-up.[5] The same instrument is used to gage the seeding in the basal medium at the start, the concentration being determined indirectly from a heavy suspension from which the proper aliquot is taken. We have found it convenient to use a box type galvanometer with lamp and scale enclosed, and to adjust so that it reads zero when basal medium alone is interposed between the light and the thermocouple, and 100 when an opaque object is interposed. This instrument is superior for this purpose to available photoelectric colorimeters.

Blank cultures (no addenda) and cultures containing known amounts of biotin (0.000025 to 0.00025 γ) are run simultaneously with the unknowns, and the amounts of biotin in the unknown sample are read off the standard curve, in which galvanometer readings are plotted against the known amounts of biotin (either pure or in a standardized extract) added.

Experimental Results

In Fig. 1 are plotted the results of parallel tests of a crude liver preparation and a biotin solution. The two curves are parallel through a wide range. At relatively high concentrations both curves flatten out and do not diverge strikingly. This parallelism means that when the test is applied to such an extract, values obtained at different dosage levels agree, which is true of extracts generally, as is illustrated by data presented below (Table II). This strongly indicates that the test is specific.

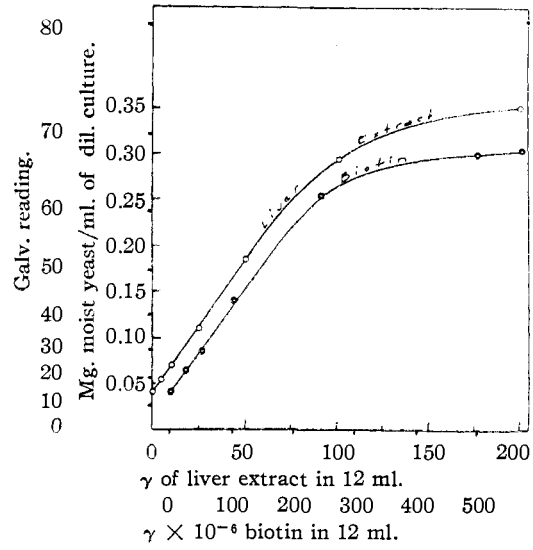

Fig. 1.—Comparative curves, liver extract and biotin.

Under our test conditions biotin gives a response at an exceedingly low dosage. Kögl,[6] speaking of biotin, says "it can be detected in the yeast test even at a dilution of 1 in 4×10^{11}." From Fig. 1 it is seen that in our test a concentra-

(1) Eakin and Williams, *J. Biol. Chem.*, **128**, xxiii (1939).
(2) Eakin and Williams, THIS JOURNAL, **61**, 1932 (1939).
(3) Schultz, Atkin and Frey, *ibid.*, **61**, 1931 (1939).
(4) Williams, Wilson and Von der Ahe, *ibid.*, **49**, 229 (1927).
(5) Williams, McAlister and Roehm, *J. Biol. Chem.*, **83**, 315 (1929).
(6) Kögl, *Proc. Roy. Soc.* (London), **B124**, 1 (1937).

tion of 1 in 5×10^{11} increased the galvanometer reading from 16 to 22. This is considerably greater than the least detectable amount. The advantage of a small dosage in a test of this kind is evident. To get a *measurable* response, less than 0.1 mg. of extracted material (5 γ or more in the case of autolyzed liver extracts) must be added to a 12-ml. culture. The chance of introducing other physiologically active substances which might interfere with the test is thus minimized, and the specificity of the test is ensured.

The active substance concerned in our test has the known properties of biotin. Its activity is not destroyed by heating with normal alkali at 100°, and it withstands refluxing with acidic methanol (*cf.* Table III). The latter procedure brings about esterification but the ester is physiologically active.[7] Biotic acid is destroyed by this treatment. All of the well recognized yeast nutrilites are placed in the basal medium (in this medium β-alanine completely replaces pantothenic acid) and so far as we are aware no known or postulated substance can replace biotin in this test.

Vitamin B_6 must be present in the medium before any significant effect of biotin can be demonstrated. This is illustrated in Table I. The

TABLE I

EFFECT OF BIOTIN IN PRESENCE AND ABSENCE OF VITAMIN B_6

Addition to medium per flask, γ	Galvanometer readings on basal medium: Without vitamin B_6	Containing vitamin B_6
0.0	36.0	30.3
.00003 biotin	38.8	38.8
.00007 biotin	40.0	50.2
.00015 biotin	39.8	58.0
.00300 biotin	38.1	69.1
5.0 liver preparation	39.0	39.2
10.0 liver preparation	38.6	49.4
20.0 liver preparation	36.0	57.0
40.0 liver preparation	31.8	66.0
100.0 liver preparation	25.0	70.0

situation here is somewhat analogous to that with rats, where the presence of vitamin B_6 is necessary before the "filtrate factors" show their full effect.[8,9,10]

The test of Kögl involving a growth response from heavy seeding of "Rasse M" yeast seems not to be as specific as the one we have described. It has been indicated[7] that a crude extract in high dosage gives a 600% increase under his growth conditions, whereas pure biotin causes only a 300% increase. From the published assays[11] it is not clear whether the specificity of the test can be ensured by holding the dosages down to low levels, since assay values at different dosage levels are not given.

In the test investigated by Robbins and Schmidt[12] involving the organism *Ashbya gossypii*, no claim for specificity is made and the authors state that their results are "suggestive only." At different dosage levels the materials did not show the same relative potency, indicating that toxic and/or stimulating principles were influencing the results.

Data on Assays.—For further information regarding the quantitative distribution of biotin, assays on the materials listed below were carried out. Clear water extracts were obtained by autoclaving the material (finely ground if necessary) for ten minutes at 15 lb. (1 atm.) pressure with a large volume of water. If necessary the extract obtained was clarified by filtering with filter-cel. Casein samples were hydrolyzed with sulfuric acid and the $SO_4^{=}$ ion removed with Ba^{++} for assay. Examples are given in Table II showing comparative results at different dosage levels. In Table III single values are given for the sake of brevity. Liberation of biotin by autolysis of liver tissue is

TABLE II

COMPARATIVE RESULTS OF BIOTIN ASSAYS AT DIFFERENT TESTING LEVELS

Material	Fresh material tested (as extract), γ	Dry wt. in extract added, γ	Biotin found, γ	Biotin content found: Fresh substance, γ per g.	Solids in extract, γ per g.
Autolyzed	30	4.5	0.000020	0.67	4.46
Liver I	100	15.0	.000065	.65	4.33
(37°, 24	200	30.0	.000132	.66	4.40
hr., 10% suspension)			Av.	.66	4.4
Unauto-	500	15.5	.000015	.030	0.97
lyzed	1000	31.0	.000032	.032	1.03
Liver I	3000	93.0	.000073	.024	0.78
			Av.	.029	.93
"Liquid	50	1.5	.000025	.50	16.7
Manure"	150	4.5	.000070	.47	15.7
	500	15.0	.000230	.46	15.3
			Av.	.48	15.9
Egg Yolk I	50	1.7	.000015	.30	8.8
	150	5.1	.000045	.30	8.8
	500	17.0	.000137	.27	7.9
			Av.	.29	8.5

(7) Kögl and Tönnis, *Z. physiol. Chem.*, **242**, 43 (1936).
(8) Lepkovsky, Jukes and Krause, *J. Biol. Chem.*, **115**, 557 (1936).
(9) Lepkovsky, *ibid.*, **124**, 125 (1938).
(10) Edgar, El Sadr and Macrae, *Biochem. J.*, **32**, 2200 (1938).
(11) Kögl and Hasselt, *Z. physiol. Chem.*, **243**, 189 (1936).
(12) Robbins and Schmidt, *Bull. Torrey Botan. Club*, **66**, 139 (1939).

TABLE III

BIOTIN CONTENT OF VARIOUS MATERIALS

Material	Biotin content, γ per g.
Cane molasses (Hawaiian Blackstrap)	2.1
Cane molasses (Cuban Blackstrap)	1.7
Beet molasses I	0.06
Beet molasses II	.9
Urine (liquid)	.09
Urine (solids)	2.0
Whey (liquid)	0.12
Whey (solids)	2.5
Egg white (fresh)	0.05
Egg yolk II (fresh)	.37
Egg yolk II (autolyzed 24 hr.)	.35
Yeast (moist, autolyzed)	.13
Beacon Dog Meal	.53
Casein, purified (Eimer & Amend, free of Vitamin "B")	.002
Casein, technical	.125
Potato (fresh)	.010
Autolyzed liver II (solids)	3.89
Autolyzed liver II (refluxed 1 hr. with *N* NaOH)	3.87
Autolyzed liver II (refluxed 1 hr. with 3% HCl in CH_3OH)	3.89

clearly demonstrated and indicates that biotin is bound up with liver proteins or other colloids, an observation apparently not previously made. In the assays of Kögl and Hasselt on animal tissues,[11] livers appeared to be mediocre biotin sources. Had autolysis been carried out, results would doubtless have been different. Whether other tissues yield more biotin on autolysis is not known. Egg yolk extracts are not increased in biotin content by "autolysis."

In this connection it is interesting to note that casein contains considerable amounts of biotin; purification greatly decreases the amount present.[13]

Manure (and also urine) is a relatively rich source of biotin, but according to our tests it is not as rich as indicated by the tests of Robbins and Schmidt,[12] who judged the extract to be nearly 300 times as rich (on a dry weight basis) as egg yolk.

In view of its properties, occurrence and high physiological activity, the "growth substance" described in 1935[14] may have been biotin. The test organism used in its study became less and less sensitive on subculture and finally was lost. This "growth substance" was removed from casein and other proteins with difficulty.

The absolute values for the biotin content of materials given in Tables II and III are based upon the preparation which Professor Kögl kindly sent us and upon a limited number of samples and should not be accepted as final. If this standard preparation deteriorated during shipment or before use, then values given would be too high. Relative values would not, of course, be affected.

Destruction by Nitrous Acid.—Before we were aware that the active principle in liver extract was biotin, tests revealed that it was destroyed rapidly by nitrous acid.

Following the method of Dunn and Schmidt,[15] we investigated the rate at which the active principle in a liver autolysate was inactivated by standing at 23° in the presence of a given concentration of glacial acetic acid and sodium nitrite. Aliquots were removed and diluted to stop the reaction at time intervals noted in Table IV. Results are compared with those obtained by Dunn and Schmidt[15] for the rate of reaction of α, β and γ amino groups. Only 94% of the activity of the liver could be destroyed by this treatment; the residual activity may arise from the hydroxyl compound which presumably is formed. Assuming this to be true, and recalculating on this basis, it readily is seen that inactivation occurs at a rate most characteristic of α-amino acids. The destructive action of nitrous acid was later confirmed on a small sample of Kögl's biotin. Biotin may therefore be an α-amino acid. Its liberation from tissues by autolysis, the biological activity of its methyl ester and inactivity of its acetyl derivative[7] coupled with its destruction by nitrous acid, all point toward the significance of this amino group in determining its physiological activity.

TABLE IV

EFFECT OF NITROUS ACID ON BIOTIN ACTIVITY OF A LIVER CONCENTRATE AND ON AMINO ACIDS AT 23°

	Biotin		% Amino N liberated from amino acids[a]		
Time, min.	% Destruction	% Destruction (recalcd.)	α-Alanine	β-Alanine	γ-Amino-*n*-valeric acid
0.0	0	0	0	0	0
.5	42	45	..	..	..
1.0	75	80	55	57	20
2.0	88	94	..	..	..
3.0	..	..	99	90	58
4.0	94	100	..	..	..
5.0	..	..	100	97	78
7.0	..	..	..	100	..
9.0	..	..	..	..	95
10.0	94	100	..	..	100
30.0	94	100	..	..	..

[a] Taken from data of Dunn and Schmidt.[15]

(13) The biotin content of hydrolyzed casein has been pointed out before: *cf.* Snell and Williams, THIS JOURNAL, **61**, 3594 (1939).

(14) Williams and Christensen, *Science*, **82**, 178 (1935).

(15) Dunn and Schmidt, *J. Biol. Chem.*, **53**, 401 (1922).

We gladly acknowledge the generous support of this work by The Rockefeller Foundation and Standard Brands, Inc., of New York.

Summary

A sensitive and accurate assay for biotin is described which involves the growth of a strain of *Saccharomyces cerevisiae* from a small seeding. Under the test conditions the response of this organism to biotin is quantitative within a concentration range from 0.00002 to about 0.001 γ of biotin. Assay results on crude extracts agree well at different levels within this range, and the curves obtained are very similar to those obtained with pure biotin. Assay results on a number of natural materials are given.

The effectiveness of biotin under these conditions depends to a large extent on the presence of vitamin B_6 (and of β-alanine) in the medium. Biotin in liver concentrates is destroyed by nitrous acid at a rate which indicates that it may be an α-amino acid.

AUSTIN, TEXAS RECEIVED NOVEMBER 28, 1939

[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF THE UNIVERSAL OIL PRODUCTS COMPANY]

Catalytic Hydration of Acetylene and of Some Alkylacetylenes[1]

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Previously sulfuric acid, sulfuric acid with mercury salts, and the latter composite in the presence of organic solvents have been used as catalysts in the hydration of acetylene and of alkylacetylenes to acetaldehyde and ketones, respectively, as reviewed and described by Thomas, Campbell and Hennion.[2] For the hydration of acetylene several patents claim a similar use of phosphoric acid and mercury salts,[3] and of phosphoric acid on charcoal with[4] and without[5,6] added metals.

In the present work solid phosphoric acid polymerization catalyst[7,8,9] was found to catalyze hydration of butyne-1 rather than its polymerization, the water required for the hydration being removed from the catalyst which was thereby decreased in activity. Continuous hydrations of this and of other alkynes, including acetylene, propyne, pentyne-1, pentyne-2, hexyne-1, and heptyne-1, were effected without rapid decrease in catalyst activity by simultaneous passage of the hydrocarbon and steam through a heated tube containing the granular solid catalyst.

Acetaldehyde formed when steam and acetylene or mixtures of steam with acetylene and ethylene or nitrogen were passed through a tube containing solid phosphoric acid catalyst at 260–300° and atmospheric pressure. Some higher boiling products also formed apparently by condensation of acetaldehyde in the presence of the catalyst.

The different alkylacetylenes tried, both monoalkyl and dialkyl derivatives, underwent hydration to ketones in the presence of solid phosphoric acid catalyst at 204° and atmospheric pressure. Butyne-1 was hydrated also at 150°. Condensation of some of the ketone to oils of relatively high boiling point and refractive index accompanied its formation. By this hydration reaction propyne yielded acetone, butyne-1 formed 2-butanone, pentyne-1 and pentyne-2 each gave 2-pentanone, hexyne-1 produced 2-hexanone and heptyne-1 yielded 4-heptanone. The latter result indicates isomerization possibly involving migration of the triple bond along the carbon chain of heptyne-1 before hydration occurred producing 4-heptanone.

Experimental Part

Sources of Acetylene Hydrocarbons.—Acetylene from a commercial cylinder was purified from acetone vapors by passing through three scrubbers containing 96% sulfuric acid and then through a soda lime tower before use in the hydration experiments. Propyne was prepared following Hennion[10] and butyne-1 was produced according to the

(1) Presented before the Division of Organic Chemistry at the 98th meeting of the American Chemical Society, Boston, Mass., Sept. 12, 1939.

(2) Thomas, Campbell and Hennion, THIS JOURNAL, **60**, 718 (1938).

(3) I. G. Farbenindustrie A.-G., British Patent 460,862 (Feb. 5, 1937).

(4) Walter, U. S. Patent 2,098,842 (Nov. 9, 1937).

(5) Eberhardt, U. S. Patent 2,093,146 (Sept. 14, 1937).

(6) I. G. Farbenindustrie A.-G., French Patent 46,616 (July 11, 1936).

(7) Ipatieff, U. S. Patents 1,993,512-13 (Mar. 5, 1935); 2,018,065-6 (Oct. 22, 1935); 2,020,649 (Nov. 12, 1935); 2,057,433 (Oct. 13, 1936); 2,060,871 (Nov. 17, 1936).

(8) Ipatieff and Schaad, U. S. Patents 2,120,702 (June 14, 1938); 2,157,208 (May 9, 1939).

(9) Universal Oil Products Co., British Patents 437,188 (Oct. 14, 1935); 463,272 (Mar. 25, 1937); 463,864 (Apr. 7, 1937); 464,671-2 (Apr. 19, 1937); French Patent 797,584 (April 29, 1936).

(10) Hennion, Meeting of the Indiana Academy of Science, Manchester, Indiana, November 5, 1937.