HEAT-LABILE, AVIDIN-UNCOMBINABLE, SPECIES-SPECIFIC AND OTHER VITAMERS[1] OF BIOTIN[2]

By Dr. DEAN BURK and Dr. RICHARD J. WINZLER

NATIONAL INSTITUTE OF HEALTH, BETHESDA, MD.

OPPEL[3] has recently reported that normal human urine contains a considerable proportion (some 20–50 per cent.) of its yeast-growth biotin activity (≃ *ca.* 0.05 mg biotin/l) in a form unaffected by addition of avidin or egg white. We have also found normal urines of dog, horse, rat, mouse, cow and sheep to contain as high or higher proportions of avidin-uncombinable active material,[4] and even greater total activities (≃ 0.08, 0.1, 0.3, 0.5, 0.5 and 1 mg biotin/l). In fact, most all tissues and foodstuffs, analyzed after 2 hours' autoclaving in 5 vol.-per cent. H_2SO_4, were found to contain readily measured proportions of yeast-active, avidin-uncombinable biotin vitamer, thus: 0.1 per cent. or less, biotin concentrates (Smaco #200 and I.G.F. liver extract), egg yolk, egg white, avidin concentrates; 0.1–1 per cent., rat muscle, pancreas, kidney, liver, submaxillary gland and whole carcass, and mouse carcass; 1–10 per cent., rat lung, spleen, testis, adrenals, lymph nodes, skin, intestinal tract contents and feces, and polished rice, dried yeast and various chows; 30–50 per cent., Vitab[4a] (rice bran concentrate), and (unhydrolyzed) beer; 90–100 per cent., urine of rats or mice fed avidin, and Squibb urease.

A remarkable feature of the avidin-uncombinable fraction is that its yeast activity is, unlike that of biotin, greatly reduced (60–90 per cent.) by boiling or autoclaving crude preparations (*e.g.*, urine, Vitab) for 15 minutes to 2 hours at physiological pH values. The reduction obtained increases with pH over the range 4–9, and decreases with dilution of preparation employed during autoclaving. The heat-labile, avidin-uncombinable fraction, which is further characterized by inactivity for Rhizobium growth, has been designated as *miotin*. The residual, comparatively heat-stable, avidin-uncombinable fraction, which evidence indicates is almost certainly produced during autoclaving from miotin, has been designated as *tiotin*.

The activity of miotin preparations is, like that of biotin, little if at all affected by 2 hours' autoclaving in 5 per cent. H_2SO_4 (120° C). Subsequent autoclaving at pH 4–9, however, produces much less reduction in activity than occurs without the prior acid autoclaving. Acid autoclaving thus either produces still another biotin vitamer from miotin of about equivalent activity (but of greater stability to neutral autoclaving) or eliminates some extra component in the crude preparation needed to produce the tiotin of lesser activity than miotin. The existence of such an additional component is also indicated by the dilution effect referred to (*i.e.*, second order reaction involved). The component is not a dissociable inhibitor of miotin because neutral-autoclaved miotin preparations did not inhibit the activity of unautoclaved miotin preparations; other experiments show that the component is not simply bicarbonate. Acid autoclaving of tiotin preparations restores most of the original avidin-uncombinable activity, which is now again little affected by further neutral autoclaving. The reconversion of tiotin to miotin during acid autoclaving (with gain in activity), as well as the conversion of miotin to tiotin during the initial neutral autoclaving, are thus strongly indicated, although formation of still other vitamers of biotin is not precluded. In all the foregoing autoclaving operations, no appreciable formation of avidin-combinable biotin vitamer was definitely detected.

A third definite but unidentified biotin vitamer, *rhiotin*, active for Rhizobium but not for yeast, and

[1] Compounds that act to overcome a given vitamin deficiency (in one or another organism, animal or plant) are vitamers (vitameric), according to nomenclature developed with K. Hickman, Gibson Island Vitamin Conference, July 22, 1942; thus, there are various D vitamers, K vitamers, B_1 (or thiamin) vitamers, niacin vitamers, pantothen vitamers, pyridoxin vitamers, para-aminobenzoic acid vitamers, and, as indicated in this paper, various biotin vitamers. Whereas isomers are compounds with a given molecular formula, vitamers are compounds with a given vitamin activity and usually possess different molecular formulas.

[2] Reported upon at Gibson Island Chemical Growth Factors Conference, August 21, 1942, and Chemical Society of Washington, October 8, 1942.

[3] T. Oppel, "Studies of Biotin Metabolism in Man," reported at the thirty-fourth annual meeting, Am. Soc. Clin. Invest., Atlantic City, May 4, 1942; *Am. Jour. Med. Sci.* (I, II, III), in press.

[4] The assay and definition of avidin-combinable and -uncombinable biotin vitamers was made by growing organisms (*e.g.*, yeast, Rhizobium) in serial dilutions of unknowns in the presence and absence of avidin (*ca.* 0.001 units/cc), compared with standard dilutions of biotin. The exact quantitative measurement of the avidin-uncombinable fraction requires a definite correction for the fact that avidin not only combines with avidin-combinable biotin vitamers in the growth medium, but, especially at the lower yeast growths, also exerts a considerable toxic effect (increasing up to 50 per cent. over the range 10^{-6} to 10^{-2} avidin units/cc) on the growth of yeast supplied avidin-uncombinable biotin vitamers, and even of yeast internally rich in biotin (no biotin vitamer added to medium). Avidin and avidin-biotin were found by direct measurement to be removed from the medium and highly absorbed by yeast, to the extent of several tenths of a per cent. of the yeast dry weight. Avidin-biotin so absorbed was not available as a source of biotin for growth.

[4a] Kindly supplied by National Oil Products Co., Harrison, N. J.

avidin-combinable and stable to acid or neutral autoclaving, was found in biotin-free concentrates of miotin prepared from human or rat urines (or Vitab hydrolyzates) by treatment with excess avidin followed by precipitation of the biotin-avidin complex with acetone and concentration of the liquid residue at pH 1–2 under reduced pressure at 50–60° C.

Injection tests to date with mice indicate zero or greatly reduced vitamin H activity by either rhiotin or miotin when compared, at yeast-equivalent doses, with curative doses of biotin (0.1 gamma/mouse/day). The vitamin H requirement of the mouse is somewhat higher than that of the rat, and deficiency symptoms are somewhat easier to induce, in agreement with the somewhat higher biotin content of the mouse carcass on a weight basis. The low or zero vitamin H activity of miotin for the rat and mouse is further confirmed by the fact that biotin-deficient animals maintained for weeks or months on diets containing excess avidin continued to excrete normal amounts of avidin-uncombinable (though no avidin-combinable) biotin vitamer in their urine. Furthermore, pure cultures of *E. coli* (and likewise many miscellaneous culture contaminants found in unsterile stock solutions) were found to synthesize and liberate large amounts of extracellular biotin vitamer, over half of which was avidin-uncombinable.

The relations between the foregoing chemically unidentified biotin vitamers and those of known constitution are summarized in Table 1, the data of which must be interpreted with due appreciation of the nature and sensitivities of the usual or implicit techniques employed. We have confirmed the finding[8] that the diaminocarboxylic acid derived from biotin (DAC), a sample of which was initially given us by Professor du Vigneaud, is avidin-uncombinable,[4] and possesses under usual test conditions only about 0.1 the molal equivalent yeast activity of biotin (0.1 BVE for yeast), at half-maximum growth. Much less than this activity will be obtained, especially at the low DAC concentrations, if the yeast inoculum is not quite fresh or is biotin-deficient, or if (as found by du Vigneaud and collaborators also) the CO_2 pressure in the growth medium is inadequate. The CO_2 pressure requirement for growth in DAC is much greater than in biotin, miotin or tiotin, and maximum DAC activity at limiting DAC concentrations requires a CO_2 pressure greater than that in air (> 0.03 per cent. atm.). All three factors indicated will, when inadequate, tend to produce more extended DAC concentration curves, and all probably affect the conversion of DAC to biotin in its utilization by yeast. The conversion of DAC to biotin, even under optimum conditions observed, is very probably the rate-limiting reaction that determines its maximum observed activity in yeast of about 0.1 BVE. We have further found that DAC is inactive for Rhizobium (≦ 0.001 BVE for Rhizobium), and that, like miotin, its activity may be reduced, about as rapidly, by autoclaving, especially in the pH range 4–9. Pimelic acid, which may substitute for biotin in the growth of the diphtheria bacillus,[5] was not observed to substitute for biotin in the growth of Rhizobium or yeast, in either

TABLE 1

SPECIES-SPECIFICITY AND OTHER OBSERVED PROPERTIES OF VARIOUS BIOTIN VITAMERS

Biotin vitamer	Identified					Chemically unidentified		
	Biotin	Methyl ester	Sulf-oxide[7]	Diamino-carboxylic acid (DAC)	Pimelic acid	Miotin	Tiotin	Rhiotin
Species activity								
Yeast (*S. cerevisiae*, Fleischmann strain 139)	+	+	+	+(ca. 0.1 BVE)	−	+	+(≦0.4 BVE)	−
Rhizobium trifolii, str. 209	+	+		−(≦0.001 BVE)		−	−(sl?)	+
Rat	+	+						
Mouse	+					(−)		(−)
C. diphtheriae, Allen str.[5]	+				+			
L. casei[9]	+	−			−			
Thirteen fungi[9]	+				−			
Autoclaving stability (2 hrs., 120°C.)								
pH 4–9	+	−		−		−	+	+
5 vol.–% H_2SO_4	+	−		+		+	−	+
Avidin-combinability[4]	+	+	+	−		−	−	+
Conversion to avidin-combinable biotin vitamer by:								
Yeast				+		+	+	
Phosgene				+		−	−	

[5] V. du Vigneaud, K. Dittmer, E. Hague and B. Long, SCIENCE, 96: 186, 1942.

[6] G. M. Shull, B. L. Hutchings and W. H. Peterson, *Jour. Biol. Chem.*, 142: 913, 1942; L. D. Wright, *Proc. Soc. Exp. Biol. Med.*, 51: 27, 1942.

[7] V. du Vigneaud, Gibson Island Vitamin Conference, July 22, 1942.

[8] V. du Vigneaud, K. Dittmer, K. Hofmann and D. Melville, *Proc. Soc. Exp. Biol. Med.*, 50: 374, 1942.

the presence or absence of added cystine. Robbins and Ma[9] reported that pimelic acid did not substitute for biotin in the growth of some thirteen fungi. Eakin and Eakin[10] inferred a synthesis of biotin by *Aspergillus niger* from either pimelic, suberic or azelaic acid as precursor, with supplementary action by cystine. In further regard to species-specificity of biotin vitamers, it will be recalled that the avidin- combinable, heat-labile methyl ester of biotin is active for yeast and Rhizobium but not for *L. casei*.[6]

The existence of various biotin vitamers, especially their coexistence in natural sources, makes essential their consideration in most phases of biotin investigations, particularly methodology. Thus, total yeast biotin vitamer activity may by no means be a measure of vitamin H activity, with certain preparations and test animals. Rhizobium, employed as a test organism by West and Woglom[11] in their studies of biotin content of tumors and normal tissues, does not measure the miotin or tiotin, but would measure any rhiotin as well as biotin. The use of avidin to determine the biotin requirements of organisms grown in complex media is obviously subject to limitation when other vitamers of biotin are present in the medium. At present it seems that more complete and thorough biotin vitamer analyses would best be carried out by using different organisms, with and without added avidin.[4] In preparing samples for analysis to include bound forms of vitamers (found in most natural materials, with the important exception of urines), it would be best to heat or autoclave wet or mildly oven-dried samples in acid (5 per cent. H_2SO_4) but not neutral solutions.[11a] Heating in neutral solution would tend to decrease the avidin-uncombinable vitamer content; whereas excessive or prolonged oven drying tends to increase the avidin-uncombinable fraction of tissues at the expense of the avidin-combinable fraction, thus, in rat carcass from about 1 to 30 per cent. after many days' oven drying.

Various lines of evidence (biological, kinetic and chemical) indicate that miotin, tiotin and rhiotin are closely related to biotin chemically, and that the mechanism of their substitution for biotin in growth does not rest on some entirely different basis than close chemical relationship. The biochemical conversion of avidin-uncombinable to avidin-combinable biotin vitamers by yeast was tested by growing yeast in preparations containing respectively only biotin, miotin, tiotin, DAC and avidin-biotin,[4] at submaximal concentrations such that the vitamers were completely removed from the growth medium. These variously grown yeast preparations were centrifuged and analyzed for avidin-combinable and avidin-uncombinable forms of yeast-active biotin vitamers. In all cases, 90–98 per cent. combinable form was obtained, indicating extensive conversion of miotin, tiotin and DAC to avidin-combinable vitamer (presumably biotin), and also, to some degree, the conversion of free biotin to avidin-uncombinable form (presumably miotin). The conversion of miotin to avidin-combinable form was quantitative within error of measurement of activity, strongly indicating close chemical relationship with biotin. The conversion of tiotin and DAC by yeast to combinable vitamer involved a three to ten fold increase in total biotin vitamer activity, in line with expectation for the less active DAC, and again indicative that tiotin has a lower molal equivalent activity than biotin or miotin. Miotin added to biotin-deficient yeast in Warburg manometers produced the same rapid kinetic increase in fermentation and respiration rates that was earlier reported for biotin,[12] whereas, at growth-equivalent submaximal concentrations, tiotin and DAC were definitely less effective than biotin or miotin, and required longer periods of time to produce a given effect. Miotin like biotin is dialyzable from urine, and in tissues exists largely in bound form. The miotin/biotin ratio was little affected by ordinary chromatographic procedures with charcoal applied to urine.

The chemical conversion of DAC to biotin has been accomplished with phosgene by Melville, Hofmann and du Vigneaud.[13] We accordingly treated biotin, miotin, tiotin and DAC, respectively, in 2M alkali with 0.2 parts 20 per cent. phosgene in toluene in the cold. A large proportion of the avidin-uncombinable DAC was recovered as more active avidin-combinable biotin vitamer, whereas no effect was noted with the other vitamers so treated. DAC can thus be readily distinguished from miotin, tiotin or rhiotin on the basis of differences in either heat stability, equivalent molal activity, CO_2-growth effect, species-specificity, avidin-combinability or, chemically most decisive, action of phosgene. Nevertheless, demonstration of the possible relationships between these chemically unidentified vitamers with stereo- or other isomers of biotin, carboxy-DAC, dicarboxy-DAC or other derivatives or conjugated forms of biotin must await actual isolation of the unidentified vitamers in the pure state.

In regard to the fundamental function of biotin and biotin vitamers in living matter, it is important to reflect that the existence of a urea ring that

[9] W. J. Robbins and R. Ma, SCIENCE, 96: 406, 1942.
[10] R. E. Eakin and E. A. Eakin, SCIENCE, 96: 187, 1942.
[11] P. M. West and W. H. Woglom, *Cancer Research*, 2: 324, 1942.
[11a] Or, when otherwise desirable, use enzymatic hydrolysis (*cf.* M. A. Pollack, A. Taylor, A. Woods, R. C. Thompson and R. J. Williams, *Cancer Research*, 2: 748, 1942).
[12] D. Burk, R. Winzler and V. du Vigneaud, *Jour. Biol. Chem.*, 140: xxi, 1942.
[13] D. B. Melville, K. Hofmann and V. du Vigneaud, SCIENCE, 94: 308, 1942.

may be opened or closed either chemically[14] or biologically by yeast with respectively loss and gain of CO_2, the increased CO_2 pressure function in DAC as compared with biotin utilization, and other evidence at hand, all suggest the interesting possibility that biotin and its vitamers may act, possibly in alternation between avidin-combinable and -uncombinable forms if these are related to urea ring structure, as a *coenzyme of CO_2 transfer*, either in CO_2 utilization or CO_2 production (just as coenzyme I transfers hydrogen, or adenylic acid transfers phosphate). Such a function could underlie its already established role in heterotrophic fermentation, respiration and growth[12,15,16] as well as a possible role in autotrophic CO_2 assimilation, chemosynthetic or photosynthetic.

DEAN BURK
RICHARD J. WINZLER
NATIONAL CANCER INSTITUTE,
NATIONAL INSTITUTE OF HEALTH,
U. S. PUBLIC HEALTH SERVICE

OBITUARY

FRANZ BOAS

IN the death of Professor Franz Boas on December 21st at the age of 84 America loses one of its great scientists. To the day of his death he had continued his indefatigible research in ethnology, linguistics and in problems of race and of human growth. The wisdom gained from a long lifetime of scientific research was lodged, during the last years of his life, in a feeble body, but it was not dimmed.

Franz Boas at the time of his death was professor emeritus of anthropology at Columbia University. He was born in Minden, Westphalia, and was educated at the universities of Heidelberg, Bonn and Kiel, where his particular fields of study were physics, geography and mathematics. The subject of his doctoral dissertation presented to the University of Kiel was "The Nature of the Color of Sea Water," and his first act after receiving his degree was typical of the man. He had already arrived at his life-long conviction that for most scientific problems mere examination of the existing data or cunningly devised laboratory experiments are not enough; he saw the necessity of gathering new first-hand material on conditions as they actually exist in human experience. He wanted, in fact, to investigate sea water and ice under winter conditions in the Arctic. There were no scientific funds to send a young man to winter among the Eskimos with his scientific instruments, so he financed himself by arranging with Berlin papers to act as a newspaper correspondent from the Arctic. He set out as a young philosophic materialist accustomed to seek "causes" in the natural environment; as he said much later, he went to the Arctic with "an exaggerated belief in the importance of geographical determinants." He returned with an abiding conviction that if we are ever to understand human behavior we must know as much about the eye that sees as about the object seen. And he had understood once and for all that the eye that sees is not a mere physical organ but a means of perception conditioned by the tradition in which its possessor has been reared.

He turned therefore to the study of culture. After a few years in Germany he returned to America under the auspices of the British Association for the Advancement of Science to study the tribes of the Pacific Coast of Canada. For fifty years he was to continue his intensive work among these tribes, especially among the Kwakiutl. Every detail—linguistic, physical, archeological and cultural—was, it seemed to him, grist for his mill. No student of culture has ever been more tireless. On his first trip he interested himself in the languages, recorded texts in hitherto unwritten tongues, investigated complex forms of social organization and of economics, observed ceremonies and financial exchanges in minute detail. But this seemed to him only a beginning, and in 1897 he interested Morris K. Jesup, then president of the Museum of Natural History, to finance the Jesup North Pacific Expedition in order that archeological, linguistic and cultural investigation might be carried on by a number of investigators both in the New World and in Siberia. Boas directed the work and the resulting publications which he edited are a landmark in the history of the investigation of cultures historically unrelated to Western civilization. Even as late as 1930 he returned to the Kwakiutl for more fieldwork, and in 1937, no longer able to go to them, he brought a Kwakiutl to his home for the winter.

Boas' emphasis on obtaining accurate, detailed knowledge, both intensive and extensive, not only raised the standards of anthropology; it changed its methodology and problems. In phrasing these problems and in insisting that relevant data be used in

[14] Enzymatically, Squibb crude urease added to the growth medium had no effect (except general toxicity at above 100 mg/l) on the yeast growth activity of biotin, tiotin, miotin, DAC or biotin-avidin, each component (vitamer and urease) being varied over a wide range of concentration.

[15] F. E. Allison, S. R. Hoover and D. Burk, SCIENCE, 78: 217, 1933.

[16] P. György, D. B. Melville, D. Burk and V. du Vigneaud, SCIENCE, 91: 243, 1940.