[CONTRIBUTION FROM THE UNIVERSITY OF TEXAS, BIOCHEMICAL INSTITUTE, AND THE CLAYTON FOUNDATION FOR RESEARCH]

The Question of the Existence and Significance of Avidin-uncombinable Biotin Isotels[1]

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An avidin-uncombinable factor which promotes the growth of yeast in a biotin test was shown by Oppel[3] to be present in human urine. Recently Burk and Winzler[4] indicated that avidin-uncombinable factors are present in other materials. The sources found to be relatively the richest in uncombinable factors were the urines of rats or mice fed avidin, and Squibb urease. These uncombinable factors were called "miotin" and "tiotin." Sources in which a smaller but considerable proportion of the biotin activity was uncombinable included "vitab" (rice bran concentrate) and beer.

Since we are interested in these biotin isotels and their relationship to the biotin assay developed in this Laboratory[5] we have sought to confirm and extend these findings and contribute to the knowledge regarding the chemical constitutions of these substances and their physiological significance.

The biotin assay method[5] was developed so as to be as specific for *biotin* as possible, with the full realization that the yeast used (as well as other yeasts) may respond to many other substances, including unknown ones, when the test conditions are varied.[6] Obviously the existence of avidin-uncombinable growth factors for yeast has been amply demonstrated previously, and in the present study we sought to investigate further only those factors which function in a test which is known to be reasonably specific for biotin and which excludes the effects of other unrelated yeast nutrilites.

Experimental

The tests for biotin and the use of raw egg white or an avidin concentrate to counteract its activity followed the methods previously published.[5,7] In most of the experiments here outlined whole fresh egg white was used as a source of avidin. In some cases an avidin concentrate with an activity of 87 units[7] per gram was used. In every case the amount of avidin used was ten times that calculated to be required for the biotin found to be present, and no substantial difference was observed whether fresh egg white or avidin concentrate was used.

"Vitab."—Six tests of unhydrolyzed vitab using amounts from 0.011 to 0.027 mg. in each test yielded biotin values from 1.10 to 1.33 γ (av. 1.21 γ) per g. No biotin activity was found in any case when avidin was used. Enzyme-hydrolyzed vitab yielded more irregular biotin values, 0.98–2.06 γ (av. 1.62 γ) per g. In none of the seven assays, however, was biotin activity found after treatment with avidin. Six assays of acid-hydrolyzed vitab yielded values 0.92–1.3 γ (av. 1.12 γ) per g. In none of the cases, whether hydrochloric or sulfuric acid was used (three tests each), was any avidin-uncombinable biotin activity found.

Rat Brain.—Seven assays of rat brain were carried out. The samples involved were from 0.063 to 0.253 mg. of brain tissue (hydrolyzed with 6 *N* HCl) in each test and the biotin values were in the range 0.16 to 0.23 γ (av. 0.19 γ) per g. In no case was avidin-uncombinable biotin activity found.

Beer.—Four assays of untreated beer using 0.29 to 14.6 mg. per test were made. The biotin values varied from 0.0055 γ to 0.0068 γ per g. and in no case was avidin-uncombinable biotin activity observed.

Urease, Squibb.—Sixteen assays were run using 1.1 to 8.8 mg. and in no case was a measurable amount of biotin found. According to Miller, Lampen and Peterson[8] and work done in this Laboratory,[8a] biotin is present in the enzyme urease only as an impurity. The sample of commercial urease we purchased was evidently free from this contaminant.

Normal Rat Urine.—The sample used was sterilized by steaming and kept in a refrigerator. Twenty-five assays of normal rat urine using 0.034 to 0.325 mg. of urine per assay tube, yielded highly uniform biotin values all within the range of 0.14 to 0.18 γ per g. No change in the biotin content of the urine was observed on storage through a period of one month. When avidin-uncombinable biotin activity was tested for, the results were unmistakably positive in fifty-two assays, but were highly irregular, varying nearly a hundredfold from 0.0018 to 0.15 γ per g. Most of the values were, however, within a narrower range, 0.03 to 0.15 γ per g.

An important factor involved in the variation in the apparent avidin-uncombinable biotin activity was the dosage level at which the urine was tested. Thus thirteen assays involving the use of 0.16 mg. of urine per assay tube yielded an average value of 0.051 γ per g. Eleven assays using 0.33 mg. of urine per assay tube yielded values averaging 0.084 γ per g. Seven assays in which 0.48 mg. of urine was used yielded values averaging 0.115 γ per g. Six assays using 0.65 mg. of urine yielded values which averaged 0.125 γ per g.

Acid-treated samples of the rat urine yielded values ranging from 0.046 γ (0.33 mg. dosage) up to 0.073 γ (1.36 mg. dosage) per g.

Rat urine which had been dialyzed through cellophane[9] was tested in a like manner and gave variable results similar to those obtained with untreated urine. From 5 to 50% of the biotin activity was avidin-uncombinable, depending on whether the dosage level used was low or high.

Rat urine (0.2 cc.) was mixed with 0.3 g. of egg white and dialyzed through cellophane. The dialyzate was then assayed with and without the use of avidin. In three separate experiments the total biotin values remained nearly constant (0.035–0.046 γ per g.) throughout the whole dosage range, whereas the avidin-uncombinable

(1) R. J. Williams, *Science*, **98**, 386 (1943).

(2) Professor of Organic Chemistry on sabbatical leave from National Southwest Associated University, Kunming, Yunnan, China.

(3) T. Oppel, *Am. J. Med. Sci.*, **204**, 856 (1942).

(4) D. Burk and R. Winzler, *Science*, **97**, 57 (1943).

(5) E. E. Snell, R. E. Eakin and R. J. Williams, THIS JOURNAL, **62**, 175 (1940).

(6) R. J. Williams, R. E. Eakin and E. E. Snell, *ibid.*, **62**, 1204 (1940).

(7) R. E. Eakin, E. E. Snell and R. J. Williams, *J. Biol. Chem.*, **140**, 535 (1941).

(8) D. R. Miller, J. O. Lampen and W. H. Peterson, THIS JOURNAL, **65**, 2369 (1943).

(8a) R. J. Williams, F. Schlenk and M. A. Eppright, *ibid.*, **66**, 896 (1944).

(9) The cellophane (viscose sausage casing) was previously boiled and washed with water to remove traces of biotin.

amount varied from practically zero at the lowest dosage level up to 50–60% of the total at high dosage levels.

Normal Human Urine.—Six assays on normal human urine varied from 0.089 to 0.118 γ per g. (av. 0.103 γ) using dosage levels 0.2 to 0.8 mg. of urine. At these levels the urine showed no avidin-uncombinable biotin activity whatever. When tested at higher levels the avidin-uncombinable values were irregular, varying from 2 to 30% of the total found in the original urine.

Human urine (0.2 cc.) was mixed with 0.3 g. of egg white and dialyzed through cellophane. The dialyzate was then tested with and without the addition of avidin. In two of three separate experiments small amounts of biotin activity were found in the dialyzate, equivalent to from 0.5 to 1% of the total in the original urine. In the third experiment no biotin was found. In no case was the dialyzate found to contain biotin activity after subsequent treatment with avidin.

Urine from Egg White-Fed Rat.—A female rat was fed a diet of pure egg white for a week. Samples of the urine were collected and assayed for avidin-combinable and avidin-uncombinable biotin. The results of the forty-eight assays showed an extreme variation due in part to the fact that the biotin activity was low in all samples. The total biotin activity present varied from 5 to 30% of that found in normal rat urine. It appeared to increase considerably by standing (after steaming) for five days in a refrigerator. In one experiment (using 0.2–0.8 mg. of one-day-old urine) there was no avidin-uncombinable biotin found. In another sample (three days old) using tests involving 1–4 mg. of urine, about 85% of the biotin activity appeared to be avidin-uncombinable. Other tests gave values varying from 0 to 50% avidin-uncombinable. In these cases there was not, however, a large drift in values depending on the dosage level used.

Similar experiments were carried out on samples of the urine from the egg white-fed rat which had been mixed with egg white and dialyzed through cellophane. Two experiments showed the presence of no avidin-uncombinable biotin activity in the dialyzate when tested in the presence of avidin; a third showed in a series of ten assays approximately 25% to be avidin-uncombinable with no serious drift in the values with increased dosage level.

Discussion

While it is evident that our immediate results do not agree wholly with those of Burk and Winzler,[4] it should be made clear that we are not questioning their experimental findings. Due to lack of complete information and for other reasons outlined below, we made no attempt to duplicate exactly their experiments.

Our experiments lead us to conclude that the specificity of the biotin test used is a crucial point in the interpretation of results obtained in the study of "avidin-uncombinable" forms. Avidin-uncombinable yeast nutrilites unquestionably exist, but unless these are known to be related to biotin, they are outside the scope of the present discussion.

One of the most effective measures taken to exclude the effects of other yeast nutrilites, both known and unknown,[6] is to test at very low dosage levels. When materials other than urine are tested at such levels, avidin-uncombinable activity is found to be absent. If they were tested at higher levels; admittedly other nutrilites (avidin-uncombinable) would be effective, but we lack proof that these other nutrilites are related to biotin.

A factor which may be important in making it feasible for us to test routinely at low dosage levels is the use of a thermoelectric turbidimeter[10] for evaluating the yeast growth. This instrument is vastly more sensitive in the lower turbidity range than the photoelectric colorimeters that are often used for this purpose in other laboratories.

When low dosage level testing is adhered to, it is evident that there remains in urine after treatment with avidin, something which functions in the biotin test as we apply it.

A number of observations in this Laboratory as well as elsewhere lead us to suspect that the apparent existence of avidin-uncombinable biotin may be due to the presence, particularly in urine, of substances which interfere with the avidin-biotin combination. Several years ago it was observed that pantothenic acid was not adsorbed from urine under conditions such that it would have been completely removed from a liver extract.[11] Hogg[12] has studied this behavior and found the existence of a group of substances (some of which remain unknown), including hippuric acid, which interfere with this adsorption. Egaña and Meiklejohn[13] have recently found that there are substances in urine which prevent the quantitative adsorption of thiamin by Decalso. Recent studies from this Laboratory on folic acid[14] emphasize that adsorption phenomena involving competitive adsorption may be complicated, relatively little understood and difficult to investigate. While it appears that the avidin-biotin combination is stoichiometric and does not involve adsorption, the equilibrium involved in the combination has not been studied nor has the possible effect of substances which may interfere by being adsorbed.

We do not think that the existence of an avidin-uncombinable substance which can function in the biotin test is ruled out by our experiments. On the contrary we can with reason postulate that a biotin degradation product (present in urine) is responsible for a substantial part of the effect. It is interesting to note in this connection that Burk and Winzler[4] indicate that "tiotin" is almost surely a degradation product of "miotin."

It appears from our investigation that substances which, (1) do not combine with avidin, and (2) promote yeast growth in a test which is designed and made specific for biotin, are of relatively limited occurrence and probably, therefore, of limited significance. Since they appear to occur only in urine, the possibility that they are merely urinary waste products formed from biotin, which escape complete destruction in the body, suggests itself.

(10) R. J. Williams, E. D. McAlister and R. R. Roehm, *J. Biol. Chem.*, **83**, 315 (1929).

(11) Unpublished observation.

(12) J. F. Hogg, "A Study of Adsorption Interference," Master's Thesis, The University of Texas, 1942.

(13) E. Egaña and A. P. Meiklejohn, *J. Biol. Chem.*, **141**, 859 (1941).

(14) E. H. Frieden, H. K. Mitchell and R. J. Williams, THIS JOURNAL, **66**, 269 (1944).

The existence of substances which are isotelic with biotin as far as yeast growth is concerned cannot be doubted. For instance, biotin methyl ester, desthiobiotin,[15] and α-biotin of Kögl and co-workers[16] are known to possess biotin activity. These, however, all combine with avidin and hence are not avidin-uncombinable factors. According to results obtained in this Laboratory,[17] repeated tests of the diaminocarboxylic acid derived from biotin, which does not combine with avidin, have shown it to be inactive in the original yeast assay for biotin.[5] This is in contrast to data obtained by others.[4,18] Again, the discrepancy may be due to variations in testing conditions used.

(15) D. B. Melville, K. Dittmer, G. B. Brown and V. du Vigneaud, *Science*, **98**, 497 (1943).

(16) F. Kögl, J. H. Verbeek, H. Erxleben and W. A. J. Borg, *Z. physiol. Chem.*, **279**, 121 (1943).

(17) E. E. Snell, unpublished observations.

(18) V. du Vigneaud, K. Dittmer, K. Hofmann and D. Melville, *Proc. Soc. Expll. Biol. Med.*, **50**, 374 (1942).

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Summary

When testing conditions such as have been shown to exclude the effects of unrelated yeast nutrilites are used, avidin-uncombinable forms of biotin appear not to be generally distributed in nature, but to be found in urine samples only. The fact that the material which is physiologically active in the yeast test is partially avidin-uncombinable in urine may be due (1) to the presence in urine of products related to biotin which are effective for yeast but do not combine with avidin or (2) to the presence of substances in urine which interfere with the avidin-biotin combination.

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[CONTRIBUTION FROM THE WM. H. CHANDLER CHEMISTRY LABORATORY, LEHIGH UNIVERSITY]

Experiments in the Synthesis of 3,4-Benzo-9-thiafluorene, a Sulfur Analog of 3,4-Benzophenanthrene

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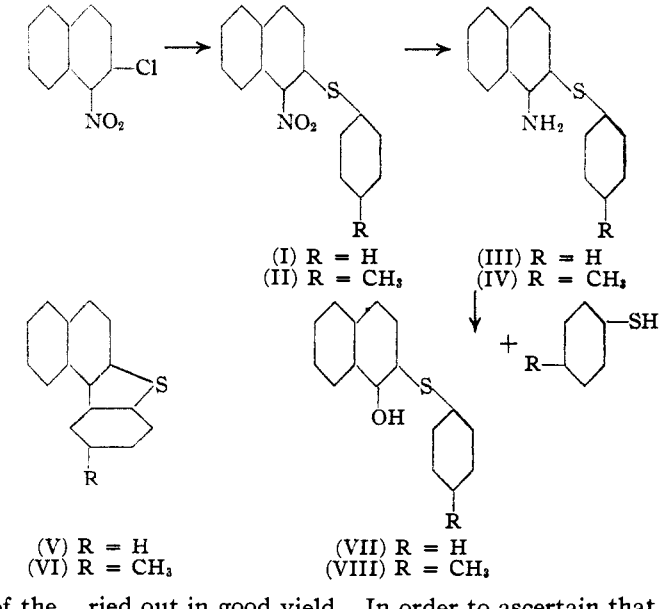
It has been recognized since the time of Victor Meyer's classical experiments on thiophene that a sulfur atom substituted for a vinylene group of an aromatic carbocycle may cause little change in the physical properties of the compounds. Biological effects of corresponding members in the series often are similar also.[2] This present paper reports on a series of experiments designed to produce the 3,4-benzo-9-thiafluorene nucleus and one of its methyl derivatives.

The ring system is angular and is to be related to 3,4-benzophenanthrene. The isomer 2,3-benzo-9-thiafluorene is linear and is one of the sulfur isologs of the angular hydrocarbon 1,2-benzanthracene. While the 2,3-benzo-9-thiafluorene series is well known, a search of the literature reveals the fact that no member of the 3,4-benzo-9-thiafluorene series has yet been prepared. Our interest in this series of compounds, therefore, concerns not only the possible carcinogenic activity of the substances but also the chemistry of their formation and reactions.

This method of synthesis, as outlined by the flow sheet, seemed to us to be especially desirable since it eliminates the possibility of the formation of isomers.

FLOW SHEET



The condensation[3] of 1-nitro-2-chloronaphthalene with thiophenol or with *p*-thiocresol was carried out in good yield. In order to ascertain that no rearrangement took place during condensation,[4] we prepared an authentic sample of *p*-tolyl-4-nitro-1-naphthyl sulfide and, by the method of mixed melting points, found that this compound and the compound prepared from 1-nitro-2-

(1) Original manuscript received July 21, 1943.

(2) One of the recent correlations of this kind was made by Sandin and Fieser, THIS JOURNAL, **62**, 3098 (1940). It was found (Dunlap and Warren, *Cancer Research*, **1**, 953 (1941)) that 4,9-dimethyl-5,6-benzothiophenanthrene exhibited carcinogenic properties similar to one of the corresponding carbocyclic substances, 9,10-dimethyl-1,2-benzanthracene.

(3) Cullinane, Rees and Plummer, *J. Chem. Soc.*, 151 (1939).

(4) *Cf.* Hodgson and Leigh, *ibid.*, 1031 (1938); also 1094 (1939).