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The Liberation of Biotin from the Avidin-Biotin Complex (AB).

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In investigations of the relationship of avidin and biotin the means by which biotin bound to avidin may become physiologically active has remained obscure. In the digestive tract the avidin-biotin bond is firm since ingested egg white prevents absorption of biotin. This tight link is surprising in view of the protein-like nature of avidin and the presence of proteolytic enzymes in the stomach and intestine. Parenterally administered, however, avidin-biotin (AB) is apparently broken down since egg white injury may be cured by injection of avidin containing some bound biotin.[1]

AB seems to be more resistant to chemical action than avidin itself. György, Rose, and Tomarelli have shown[2] that while the ability of avidin to combine with biotin is greatly reduced under conditions of temperature and acidity found in the stomach, AB not only is unaffected, but seems in some measure to protect the avidin. Except for steaming, which readily and completely releases biotin, only a few cases of its liberation from avidin have been noted. In experiments where avidin containing AB was irradiated in the presence of riboflavin a considerable portion of the biotin was released.[2] A different type of reaction is observed when biotin sulfone in excess is added to AB.[3] It may be shown by yeast growth tests that biotin is present in free form apparently replaced in the AB complex by biotin sulfone. To enlarge our information on the behavior of AB we have subjected it to various treatments simulating the physiological reactions—enzymatic or oxido-reductive—which might be responsible for liberating it in the body.

Experimental. The methods of biotin and avidin assay are those of Eakin, Snell, and Williams[4] except that *l*-asparagine was substituted for *l*-aspartic acid in the biotin-free medium since the latter acid was not procurable. The avidin used has been either egg white or a purified concentrate. The biotin was crystalline biotin methyl ester. In most of the experiments AB has been prepared with the avidin in slight excess so that small amounts of liberated biotin might be more accurately estimated.

A study of the effect of proteolytic enzymes on AB was made, since, though we had presumed that it was unaffected by the proteolases of the digestive tract, no direct observation of the fact had been made. Solutions of egg white and avidin containing approximately 0.2 μg of biotin per cc were digested at 38°C with 0.2% pepsin at a pH of 1.8 for 24 and 48 hours. After 48 hours there was a trace of free biotin but it was less than 1% of the total amount present. A 24-hour egg white pepsin digest was adjusted to pH 8 and digested for an additional 24 hours with 0.2% trypsin without further liberation of biotin. Similarly a 24-hour avidin pepsin digest was brought to pH 8 and incubated with 0.4% pancreatin. No free biotin was produced. These mixtures were dialyzed to test whether the avidin had been hydrolyzed and the biotin be bound still by some dialyzable fragment of it. The results were negative. Because of the effectiveness of papain as a proteolytic agent an attempt was made to liberate biotin by its means. An egg white solution containing 0.1 μg of biotin with 0.2 mg papain and 0.01 cc of 1.2% HCN per cc as activator was incubated at 70°C for 24, 48, and 96 hours. It was then dialyzed for 24 hours. There was

[1] György, P., and Rose, C. S., *Science*, 1941, **94**, 261.

[2] György, P., Rose, C. S., and Tomarelli, R., *J. Biol. Chem.*, 1942, **144**, 169.

[3] Dittmer, K., du Vigneaud, V., György, P., and Rose, C. S., to be published.

[4] Eakin, R. E., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, 1941, **140**, 535.

no dialyzable biotin and no yeast active biotin in the residue although, after 96 hours, 80% of the egg white protein nitrogen had been converted to non-protein nitrogen. One might have expected that temperature alone would have liberated the biotin in this case.

Release of biotin from avidin by means of tissue enzymes seemed to be a probable explanation of AB activity within the body. Beef liver, kidney, and muscle were used, the same procedure being followed in the 3 cases. Ground tissue (1 g), 1 cc of avidin-biotin containing 1 μg of biotin and 2 cc of an isotonic solution of sodium phosphates (pH 7.4) were mixed, overlaid with benzene for protection against bacterial action and incubated at 38°C for 48 hours. After incubation the solutions were diluted to 10 cc with saline, centrifuged and filtered. The filtrates contained no free biotin.

In blood AB was equally recalcitrant. Both oxygenated and reduced blood were studied since the degree of oxidation might be a factor in the reaction. Mixtures of 2.5 cc of citrated human blood with 1 cc of AB containing 0.6 μg of biotin and 1.5 cc of normal saline were put in tonometers one of which was filled with oxygen, the other with nitrogen, and incubated at 38°C for 24 hours. The mixtures were then dialyzed in the ice box for 24 hours, the reduced sample being protected with oil. No biotin was found in the dialysate nor any free biotin in the residue. A further experiment was made using methylene blue and cysteine to modify the degree of oxidation of the blood. The following systems were studied:

Blood +	AB +	5% cysteine +	5% M.B. +	saline
cc	cc	cc	cc	cc
2.5	1	0	0	1.5
2.5	1	0.5	0	1
2.5	1	0	1	0.5
2.5	1	0.5	1	

In this case defibrinated rabbit's blood was used. Incubation and dialysis were carried out as in the preceding experiment and the results were similarly negative.

Extensive oxidation of biotin produces compounds inactive or only slightly active in promoting yeast growth, as shown by Brown and du Vigneaud.[5] For our purposes a method used by Nielsen, Shull, and Peterson[6] offered possibilities. These authors found that the mild oxidation of biotin with 0.3% hydrogen peroxide at pH 3 used by Shull, Hutchings, and Peterson[7] produced a biotin derivative still active in promoting yeast growth though inactive for *Lactobacillus casei* and rats. We have used this method for biotin and AB with biotin concentrations of 0.05 to 0.1 μg per cc. Control samples were run, omitting only the H_2O_2 from the procedure. Total biotin of AB was determined by liberation of the biotin by steaming. This steaming was done before the MnO_2 added to decompose the excess H_2O_2 was removed. Otherwise avidin with the biotin bound to it was absorbed on the MnO_2 and lost on filtration. We found a greater loss of biotin activity than did the authors of the method, but our purpose was accomplished. A definitely measurable quantity of a substance with yeast-growth promoting ability was liberated from AB. The experiments were repeated using larger amounts of H_2O_2. A concentration of 0.45% liberated a greater amount of "biotin." A higher concentration (0.6%) offered no further advantage. With the 0.45% level of H_2O_2 amounts of free material up to 20% of the biotin originally present were found.

A sample of peroxide treated AB was dialyzed against 0.9% NaCl. Almost all of the liberated biotin was found in the dialysate. At pH 7 only a small amount of biotin was released from AB by oxidation (2 to 3% of that originally present) nor was there much reduction in the total biotin content. At this pH free avidin is not readily destroyed as it is at pH 3. When additional avidin was added to the oxidized free biotin or to the liberated biotin they were completely rebound. The amount of avidin required was the same as was required for an amount of the original biotin producing the same amount of yeast growth. The decrease in activity of the solutions, therefore, does not seem to be due to conversion of all

5 Brown, G. B., and du Vigneaud, V., *J. Biol. Chem.*, 1941, **141**, 85.

6 Nielsen, E., Shull, G. M., and Peterson, W. H., *J. Nutrition*, 1942, **24**, 523.

7 Shull, G. M., Hutchings, B. L., and Peterson, W. H., *J. Biol. Chem.*, 1942, **142**, 913.

TABLE I.

Material tested	Biotin activity (μg/cc)				
	Original sol.	Sol. treated without H_2O_2	Oxidized solution		
			0.3% H_2O_2	0.45% H_2O_2	0.6% H_2O_2
Biotin	0.089	0.060	0.030	0.022	0.030
AB	0 (0.091)*	0	0.0040	0.019	0.011
AB	0 (0.044)	0 (0.040)		0.0024 (0.037)	

* The figures in parenthesis are total biotin (free + bound).

of the biotin to a less active form but to complete loss of activity of a portion of it.

Discussion. The experiments here described indicate that enzymatic processes are not responsible for liberation of biotin from avidin in the body. The metabolism of AB is more likely linked with oxidation-reduction systems. The oxidation method used in these experiments does not presume to duplicate a physiological process, but the results indicate that oxidation is a means of attack on an otherwise refractory compound. Recent work has shown that biotin is only one of a number of related compounds concerned in metabolic processes. Oppel[8] has described avidin-combining and avidin-non-combining fractions of urine of which the former seemed more closely related to biotin intake. That fraction might be a modified biotin such as that obtained in these experiments which does not differ appreciably from true biotin in its ability to combine with avidin or its yeast growth promoting activity. Burk and Winzler[9] have suggested that biotin and its modifications may act in alternation between avidin-combining and avidin-non-combining form. Further investigations of the various biotin related compounds with respect to their avidin-combining power, their growth promoting effect on various organisms and the methods of conversion from one to the other will be helpful in understanding the function of biotin.

Summary. 1. Under the experimental conditions used biotin could not be liberated from combination with avidin by the proteolytic enzymes—pepsin, trypsin, pancreatin, and papain—nor by incubation with liver, kidney, muscle, or blood. 2. Biotin could be liberated from the avidin-biotin complex by oxidation with 0.45% H_2O_2. Amounts of yeast active material equivalent to 10 to 20% of the biotin originally bound were found.

[8] Oppel, T. W., *Am. J. M. Sc.*, 1942, **204**, 856.

[9] Burk, D., and Winzler, R. J., *Science*, 1943, **97**, 57.