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Biotin Derivatives in Human Urine. (22227)

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The nature of the biotin derivatives that occur in urine has been the subject of a number of studies since Snell *et al.* (1) first showed that urine is a relatively rich source of the vitamin. Oppel(2) concluded that there exist in urine two forms of biotin, one avidin combinable and one avidin non-combinable. Burk and Winzler(3) postulated the existence in natural material (including urine) of a number of biotin derivatives (miotin, tiotin, and rhiotin) distinguishable by differences in microbiological activity, chemical stability, or avidin combinability. Chu and Williams(4), on the other hand, could not detect the presence in any natural material other than urine of avidin non-combinable forms of biotin. They even seriously questioned the real validity of their own data concerning the existence in urine of small amounts of such a derivative(s).

The above described studies were carried out primarily with *Saccharomyces cerevisiae* as the assay organism. Since yeast growth is notoriously influenced by a variety of stimulatory and inhibitory materials of unknown nature, the specificity of the responses described above with respect to any real relationship to biotin may be questioned. Significant to this discussion also is the fact that none of the compounds whose existence was postulated in the references cited has ever been obtained in the pure or even highly concentrated state. On the contrary, a number of other biotin derivatives have been derived from natural material or products derived from natural material and have been adequately characterized. These derivatives are biocytin (ε-N-biotinyl-L-lysine)(5), biotin d-sulfoxide(6-8), and biotin l-sulfoxide(9-11). A number of other biotin derivatives including biotinamide(12,13), oxybiotin(14), and N-biotinyl derivatives(12,13) of glycine, β-alanine, L-aspartic acid, L-glutamic acid, L-leucine, and p-aminobenzoic acid have been synthesized in the laboratory and studied microbiologically. Differential microbiological assays with a high order of specificity in some instances are now available for studies with these compounds. In combination with the bioautographic technic, such differential microbiological asays can yield considerable information concerning the nature of the biotin derivatives in a given sample. One study(15) employed butanol-acetic acid-water as the developing system and *Neurospora crassa* as the indicating organism. The butanol-acetic acid-water system is remarkably free of complications from salts and other components of natural material. The organism *Neurospora crassa* is extremely versatile as far as response to biotin derivatives is concerned. The following compounds are essentially equally active: Biotin, oxybiotin, biotinamide, desthiobiotin, biotin d-sulfoxide, biotin l-sulfoxide, biocytin, biotinyl β-alanine, biotinyl glycine, biotinyl aspartic acid, and biotinyl leucine(10,12,13,16). In addition, R_F data obtained with *Neurospora crassa* are available for the mixed sulfoxides or the individual d- and l-sulfoxides in some

instances of biocytin, biotinyl β-alanine, biotinyl glycine, biotinyl aspartic acid and biotinyl leucine which are formed when these compounds are paper chromatographed in those solvents which lead to the similar formation of biotin d-sulfoxide and biotin l-sulfoxide from biotin(15,16). Pimelic acid and related dicarboxylic acids are inactive microbiologically. On the other hand, *Lactobacillus arabinosus* is highly specific with respect to its requirement for biotin. Oxybiotin, N-biotinyl glycine, and biotin d-sulfoxide are the only compounds among the biotin derivatives presently available with more than 5% of the activity of biotin in promoting growth of this organism. The difference btween the "biotin" content of a particular sample determined with *Neurospora crassa* and that obtained with *Lactobacillus arabinosus* would appear then to have some significance as a measure of derivatives of biotin. The cyclic urea ring of biotin is essential for avidin combinability. Biotin derivatives containing such a structure combine with avidin with widely varying but characteristic affinities that are always less than that found with biotin itself(17,18). Cognizance of the above considerations has been made in the present studies on the biotin content of human urine.

Materials and methods. "Free" biotin was determined in the urine samples with *Lactobacillus arabinosus* (ATCC 8014) as described previously in detail(19,20). "Total" biotin was determined with *Neurospora crassa*(9,11). The bioautographic procedures also have been adequately described previously(9,11,15). In the present studies 0.2 ml of urine (0.02 ml applied 10 times) or 0.02 γ of biotin or biocytin as biotin (0.02 ml of a 1 γ/ml solution) or 2 mg of urea (0.02 ml of a 20 mg per ml solution applied 5 times) was chromatographed on Whatman No. 1 paper where the solvent phase was obtained from the equilibration of butanol(5), water(4), and acetic acid(1), and the aqueous phase was present in a separate receptacle. After removal of the solvent by air drying in the hood the paper chromatograms intended for bioautography were cut into 21 sections such that each section represented 0.05 of an R_F unit. These sections were individually eluted in Erlenmeyer flasks with 25 ml of basal *Neurospora crassa* medium. After removal of the paper sections following a 1-hour elution period the flasks were plugged, autoclaved, seeded with *Neurospora crassa,* and incubated at 30° for 3-4 days. The extent of growth of the mold was determined by collecting the mycelium on a wire loop, squeezing to incipient dryness, and then drying to constant weight at 80°C (usually 18 hours). When the mycelium weight was plotted as a function of the paper section number a smooth curve connecting the points permitted localization of the R_F values by interpolation to within 0.02 unit. Urea was located on paper chromatograms by spraying with 2% p-dimethylaminobenzaldehyde in 1 N HCl. The avidin combinability of the biotin derivatives in urine was determined with *Neurospora crassa.* A microbiological assay using the usual 8 flasks of biotin standard, 2 series of 4 flasks containing increasing levels of urine, and 2 series of 8 flasks each containing a constant amount of the 2 urine samples studied was prepared. After the usual addition of medium, autoclaving, and seeding, 8 increasing levels of a sterile egg white dilution as a source of avidin were added in duplicate to the series of flasks to which the constant levels of urine had been added previously. After growth of the organism the weight of mycelium in each flask was determined in the usual way.

TABLE I. Biotin Derivatives in 4 Urine Samples.

"Total" biotin by *Neurospora crassa* assay, γ/ml	"Free" biotin by *Lactobacillus arabinosus* assay, γ/ml	Unknown biotin derivative by difference, γ/ml
.018	.016	.002
.018	.017	.001
.014	.012	.002
.029	.024	.005

Results. As indicated by the microbiological assay data of Table I human urine contains in addition to free biotin, or material with microbiological activity for *Lactobacillus arabinosus,* small amounts of a biotin derivative measurable with *Neurospora crassa.*

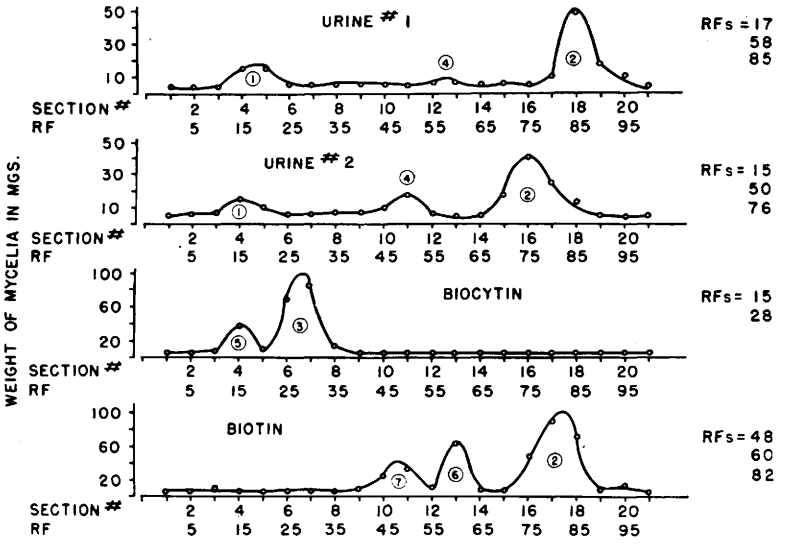

FIG. 1. Summary of bioautographic data. The various peaks are interpreted as follows: (1) unknown biotin derivative, (2) biotin, (3) biocytin, (4) biotin "sulfoxide," (5) biocytin "sulfoxide," (6) biotin d-sulfoxide, (7) biotin l-sulfoxide.

The paper chromatographic data of Fig. 1 confirm the predominant form of biotin in urine measurable with *Lactobacillus arabinosus* as free biotin (R_F ca 0.83). Indicated also by the chromatograms is the presence of biotin d-sulfoxide. This compound together with much smaller amounts of biotin l-sulfoxide (not present in detectable amounts in the present urine chromatograms) is encountered when biotin is paper chromatographed on Whatman No. 1 paper in the butanol-water-acetic acid system. Its presence in the chromatogram when biotin is also present does not necessarily mean that it was originally present in the urine samples studied. The paper chromatograms do indicate the existence in urine of small amounts of a biotin derivative with a low R_F value. The unknown material has an R_F value in the butanol-water-acetic acid system corresponding quite closely to that of the two sulfoxides of biocytin which are not resolved in this solvent or other solvent systems studied. The corresponding sulfoxide of biocytin to which the present biotin derivative conceivably may be related is unknown, and accordingly is referred to hereafter as "biocytin sulfoxide." R_F values for the available N-biotinyl derivatives of glycine, β-alanine, aspartic acid, and leucine as well as the corresponding sulfoxides are all considerably higher(16). Attempts to demonstrate correspondence of R_F values of the unknown component of urine and "biocytin sulfoxide" in a number of other solvent systems have not been conclusive since it became apparent that the R_F values observed in these additional solvents were influenced to some extent by the extraneous components of urine. A certain amount of concentration and purification probably is indicated before paper chromatographic data would be conclusive regarding the identity of the biotin derivative of urine with "biocytin sulfoxide." It seems unlikely that, if the unknown derivative indicated on the chromatograms is "biocytin sulfoxide," it arose from an *in vitro* oxidation of biocytin by the filter paper for the following reasons: (a) it is present in urine prior to any contact with filter paper as indicated by the differential microbiological assays, and (b) biocytin, from which "biocytin sulfoxide" might conceivably have arisen by a certain amount of *in vitro* oxidation analogous to that observed with biotin, was entirely absent as indicated by the chromatograms. Biocytin would not be expected to occur in urine be-

TABLE II. Avidin Combinability of Biotin Derivatives in Urine.

Biotin, γ	Urine, ml	Egg white (avidin), ml	Mycelium, mg
0			1.0
.0005			11.6
.0010			19.7
.0015			27.7
.0020			31.9
.0030			42.6
.0050			45.0
	.02 No. 1		13.2
	.04		22.2
	.06		30.0
	.10		42.1
	.02 No. 2		10.0
	.04		17.3
	.06		24.9
	.10		36.8
	.1 No. 1	.002	24.6
	.1	.004	4.0
	.1	.006	1.5
	.1	.01	0
	.1	.02	0
	.1	.04	0
	.1	.06	0
	.1	.10	0
	.1 No. 2	.002	15.4
	.1	.004	3.2
	.1	.006	.7
	.1	.01	0
	.1	.02	0
	.1	.04	0
	.1	.06	0
	.1	.10	0

cause of the rapid hydrolysis of biocytin by an enzyme of blood (and presumably other tissues)(21). If the unknown component of urine is not "biocytin sulfoxide" it is at least a derivative considerably more "hydrophilic" than biocytin. Urea in lieu of biotin was found to be inactive for *Neurospora crassa* and to have an R_F value in the butanol-acetic acid-water system high enough (0.46) to eliminate it from consideration as a non-specific stimulant. As indicated by the data of Table II all the biotin in urine determinable with *Neurospora crassa* is combinable with avidin. These results would indicate that the unknown derivative contains a cyclic urea ring.

The best interpretation of the data described above is that with a biotin auxotroph considerably more versatile with respect to utilization of biotin derivatives than microorganisms previously employed but at the same time considerably more specific with respect to the response being due to material with a structural or functional relationship to biotin, no evidence is available for the existence in human urine of any "unusual" biotin derivatives. Although an unknown form of biotin is present the compound on the basis of paper chromatographic data could very well be "biocytin sulfoxide." No evidence is available for the existence of "avidin uncombinable" forms of biotin.

The isolation and characterization of the derivative according to classical procedures would be a formidable undertaking. Since the factor is present in urine only to the extent of about 2 γ per liter (provided, of course, that its microbiological activity is equal to that of biotin), to obtain the 100 mg that might be required for characterization studies, it would be necessary to work up at least 50,000 liters of urine. These calculations assume 100% recovery of the factor. If, as is more usual in such isolations, the recovery were to run 1 to 10%, the processing of between 500,000 and 5,000,000 liters of urine would be required for successful completion of the problem. When calculated in another way the factor may be described as present to the extent of only about 0.04 γ per g of urinary dry matter. If urine is the only source of the factor a comparison of the occurrence of this factor with the occurrence of other "trace factors" reveals that this compound is probably one of the most sparsely distributed of any biochemical compound presently known.

Summary. Microbiological experiments involving direct assays as well as bioautographic procedures indicate that human urine contains relatively large amounts of free biotin and very small amounts of a "hydrophilic" biotin derivative that may be "biocytin sulfoxide." No evidence was found for the existence of other "unusual" biotin derivatives. All the biotion of urine determinable by an organism more versatile in its response to biotin derivatives than any studied previously is avidin combinable.

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Interaction of Gelatin-Stabilized Radiogold Colloid and Plasma Proteins. (22228)

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It has been demonstrated that the rate of disappearance of gelatin-stabilized radiogold colloid (Aurcoloid) from the circulation is inversely proportional to the amount of gelatin used to stabilize the Aurcoloid(1). Furthermore, the gelatin effect was enhanced by either injection of homologous rat plasma or alpha and beta globulin fractions of human plasma, whereas human serum albumin and gamma globulin had little effect. There was no evidence to suggest that the phagocytic capacity of the reticulo-endothelial system (RES) for the gold particles had been altered by gelatin. The present study was undertaken to investigate the interactions of gelatin with the plasma proteins as a possible explanation for this effect of gelatin.

Materials and methods. The Aurcoloid* was diluted with 50 volumes of distilled, demineralized water and stored in a refrigerator. The P-111 gelatin† solution, similar to that used by the manufacturer to stabilize the Aurcoloid, was handled under sterile conditions and maintained at 37°C in a water bath for at least 15 minutes before use. For the *in vitro* experiments, blood was obtained from adult male rats of the Wistar strain via the abdominal aorta using heparinized syringes. Human blood was obtained by venipuncture using heparinized syringes. After centrifugation, 3 ml volumes of plasma were placed in test tubes to which was added 0.05 ml of the diluted Aurocoloid and 2.5 mg of gelatin. Plasma samples were agitated for 2 minutes, incubated at 37°C for 30 minutes, then electrophoretically separated. In the *in vivo* experiments, adult male rats of Wistar strain weighing between 200-300 g were used. Animals were anaesthetized by intraperitoneal injection of nembutal, 5 mg/100 g of body weight. The external jugular veins were exposed and injections of the Aurcoloid

* Obtained from Abbott Laboratories, Oak Ridge, Tenn.

† Obtained from Charles B. Knox Gelatin Products, Camden, N. J.