Metabolism, in the Rat, of Biotin Injected Intraperitoneally as the Avidin–Biotin Complex[1] (37041)

HAN-MIN LEE, LEMUEL D. WRIGHT,[2] AND DONALD B. MCCORMICK

Graduate School of Nutrition and Section of Biochemistry and Molecular Biology, Cornell University, Ithaca, New York 14850

The vitamin, biotin, can combine with a specific glycoprotein, avidin. The so-formed complex is extremely stable, with a dissociation constant of 10^{-15} *M* (1). This complex is heat resistant (2, 3), stable over a wide pH range (4, 5), and was reported not to be readily dissociated by various proteolytic enzymes or by incubation with liver, kidney, muscle, or blood (6).

Feeding an avidin-containing diet was found to cause a biotin-deficient syndrome (7), though such deficiency was also reported to be cured by parenteral injection of avidin (8). This apparent therapeutic effect of injected avidin was explained by pointing out that there must have been biotin bound in the avidin preparations from which it was liberated *in vivo*. Although seemingly at odds with the reported tissue stability of the complex, this theory was supported by the findings that dialyzable radioactive material, which is still avidin-combinable, is excreted in the urine after parenteral injection into the rats of ureido carbonyl-labeled ^{14}C-biotin complexed with avidin (9, 10). However, the relative, rather than absolute, specificity for binding of biotin left the uncertainty as to whether it is the vitamin and/or metabolites which are excreted following injection of the complex by any route.

Recently, Lee, Wright and McCormick (11) reported on the metabolism of biotin in the rat. Several radioactive metabolites derived from ureido carbonyl-labeled ^{14}C- biotin were isolated from both experiments *in vivo* and *in vitro*. The rates and extents of excretion of the major biotin metabolites in the rat were also studied (12). In the present investigation, the fate of biotin given as the intraperitoneally injected (ip) avidin complex has been studied.

Materials and Methods. Avidin (12 units/mg) was purchased from Worthington Biochemicals Corp. Ureido carbonyl-labeled ^{14}C-biotin (58 mCi/mmole) was purchased from Amersham/Searle Corp. Nonradioactive *d*-biotin was purchased from Hoffmann-La Roche, Inc. The standard biotin catabolites formed by β-oxidation of the valeric acid side chain of ureido carbonyl-labeled ^{14}C-biotin were isolated from culture filtrates of a pseudomonad grown on the ^{14}C-biotin as described by Iwahara *et al.* (13). The *d*- and *l*-sulfoxides were prepared from biotin by oxidizing with hydrogen peroxide in acetic acid according to Melville (14). Radioactive biotin–avidin complex was prepared by mixing avidin (12 units/mg) with the ^{14}C-biotin (325,000 dpm/μg) in 0.9% NaCl, adjusting to a final pH 7.4 with sodium phosphate buffer, and removing excess free biotin by repeated dialysis against the buffered NaCl.

Male, Sprague-Dawley rats (290–320 g), which were obtained from Chordata Corp., were fed for 4 wk a biotin-free, avidin-containing test diet[3] (15). These rats were then divided randomly into two groups of six each. A single ip injection of 2 μg (325,000 dpm/μg)/rat of avidin-bound ^{14}C-biotin was given to rats in one group, while 2 μg

[1] This work was supported in part by Research Grants AM-08721 and AM-12224 from the National Institute of Arthritis and Metabolic Diseases, U.S. Public Health Service, and in part by funds made available through the State University of New York.

[2] Holder of Career Award K6-AM-16,612 from the National Institutes of Health, U.S. Public Health Service.

[3] Purchased from Nutritional Biochemicals Corp., Cleveland, OH; for composition of this diet, see Rubin, Drekter and Moyer (15).

(523,000 dpm/μg)/rat of free ^{14}C-biotin was given to rats of the other group. At the end of 12 days, rats of both groups were given an ip injection of 1 mg unlabeled biotin/animal and switched to a biotin-sufficient diet.[4] Urine, feces, and expired CO_2 from each rat were collected separately for the first day in an all-glass, closed metabolism cage system.[5] Urine and feces of each rat during the following experimental days were collected in an open metabolism cage. Assay of radioactivity in samples was done by scintillation counting according to Yang and and McCormick (16).

In the experiment *in vitro*, a 20% (w/v) homogenate of normal rat liver was incubated with avidin-bound ^{14}C-biotin (1 μg/g liver) at 37° for 2 hr. The reaction mixture was buffered with a solution of 0.1 *M* potassium phosphate containing 10^{-3} *M* $MgCl_2$ and 10^{-4} *M* $MnCl_2$, final pH 7.4. As control, a heat-inactivated homogenate was incubated in the same way. The detection of $^{14}CO_2$ production during incubation followed the procedure described by Yang and McCormick (16), by which a septum-stoppered flask with appended center well containing base was used. The incubated liver homogenate was dialyzed against 0.2 *M* ammonium carbonate, which was reported to be optimal for maintaining the avidin–biotin complex (5).

The procedure for isolation of radioactive materials from the dialysate of incubated liver homogenate or from urine were as described previously (11) and are similar to those of Yang *et al.* (17). Analytical techniques used for identification of isolated radioactive materials were described in the previous work (11).

Results. No significant amount of radioactivity was excreted as $^{14}CO_2$ for the first day or through feces during the entire experimental period by rats which received either ^{14}C-biotin or the avidin–bound ^{14}C-biotin. Urinary excretion of radioactivity from rats injected with avidin–bound ^{14}C-biotin was much slower than that from rats injected with radioactive biotin (Table I). Administration of milligram levels of nonradioactive biotin at the end of Day 12 increased the urinary excretion of radioactivity in rats of both groups, but clearly more from those which had received the complexed ^{14}C-biotin.

TABLE I. Daily Urinary Excretion of Radioactivity from Rats[a] after IP Injection of ^{14}C-Biotin[b] or ^{14}C-Biotin–Avidin Complex.[c]

Day	% of Administered dose (mean ± SE of 6)	
	^{14}C-Biotin–avidin	^{14}C-Biotin
	Biotin-free diet	
1	3.6 ± 0.5	33.2 ± 3.3
2	2.8 ± 0.4	7.3 ± 0.7
3	1.9 ± 0.6	3.7 ± 0.5
4	0.9 ± 0.2	2.5 ± 0.4
5	0.7 ± 0.1	1.9 ± 0.2
12	0.1 ± 0.0	0.6 ± 0.1
	Biotin-sufficient diet after 1 mg biotin ip	
13	5.0 ± 0.6	2.8 ± 0.4
14	4.8 ± 0.5	2.6 ± 0.3
15	3.1 ± 0.4	2.1 ± 0.3
16	2.3 ± 0.3	1.6 ± 0.3
18	2.2 ± 0.3	1.3 ± 0.3
20	2.3 ± 0.4	1.3 ± 0.3
24	2.5 ± 0.4	—
28	2.4 ± 0.4	1.1 ± 0.1

[a] Male Sprague-Dawley, weighing 290–320 g, fed a biotin-free diet for 4 wk.

[b] 2 μg (1,046,000 dpm)/rat.

[c] 12 units/mg avidin combined with 2 μg ^{14}C-biotin (650,000 dpm)/rat.

Similar major radioactive fractions were isolated from urine collected early (1–5 days) or late (13–17 days) from rats injected with either free radioactive biotin (Fig. 1) or avidin–bound ^{14}C-biotin (Fig. 2). These radioactive fractions were identified as biotin sulfoxides (I), bisnorbiotin (II), and biotin (III) by the criteria of the known positions of elution from the Dowex 1-X2 columns (11, 13), by the mobilities on paper chromatograms developed with various solvent systems, by the color reaction with acidic *p*-dimethylaminocinnamaldehyde sensitive to the 2-ketoimidazole ring, and by the retention of radioactivity. The correspondence in R_f

[4] For composition, see Lee, Wright and McCormick (11).

[5] Purchased from Delmar Scientific Laboratories, Chicago, IL.

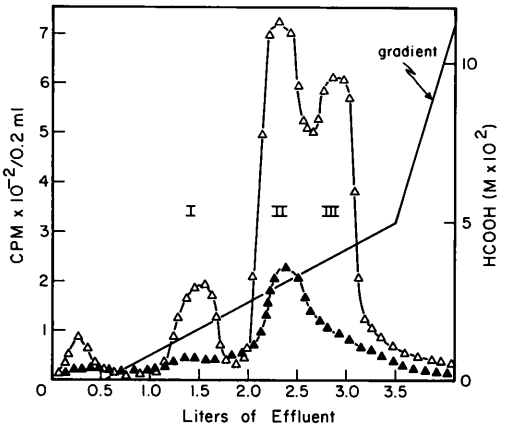

FIG. 1. Anion-exchange chromatographic elution patterns of ^{14}C-biotin and urinary ^{14}C-metabolites from rats that received ^{14}C-biotin. Two batches of rat urine [1–5 days (△); 13–17 days (▲)] were collected after injection of ^{14}C-biotin. The ^{14}C-materials in the urine samples were partially purified by adsorption on and elution from charcoal and by ethanol extraction prior to column chromatography. The column (1.6 × 120 cm) of Dowex 1-X2 (formate, 100–200 mesh) was eluted with a formic acid gradient to obtain compounds identified in Table II.

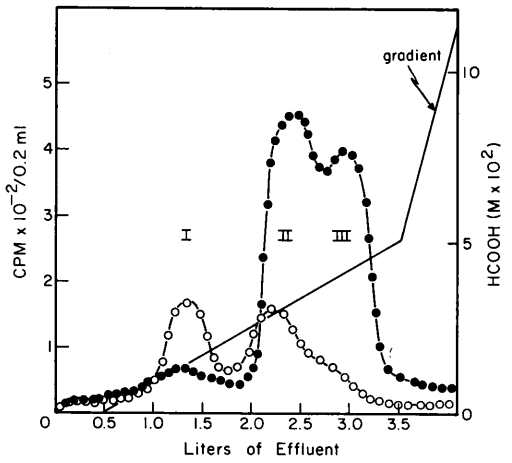

FIG. 2. Anion-exchange chromatographic elution patterns of ^{14}C-biotin and urinary ^{14}C-metabolites from rats that received avidin-bound ^{14}C-biotin. Two batches of rat urine [1–5 days (○); 13–17 days (●)] were collected after injection of avidin-bound ^{14}C-biotin. The ^{14}C-materials in the urine samples were partially purified by adsorption on and elution from charcoal and by ethanol extraction prior to column chromatography as described in Fig. 1.

values of these fractions and those of external and internal standards of biotin and metabolites spotted on paper chromatograms and developed with ascending butanol–acetic acid–water is shown in Table II.

In the study *in vitro*, $^{14}CO_2$ was trapped during incubation of the ^{14}C-biotin–avidin with rat liver homogenate. After incubating for 2 hr, 10.5% of the added radioactivity was dialyzable against 0.2 *M* ammonium carbonate. Most of the dialyzable radioactivity was shown to be biotin sulfoxides. In the control experiment, in which the liver homogenate was heat inactivated, only 1.6% of the added radioactivity was dialyzable after incubation.

TABLE II. Paper Chromatographic Mobilities of ^{14}C-Biotin and Major Urinary ^{14}C-Metabolites Isolated by Anion-Exchange Column Chromatography.

Column fractions[a]	R_f values[b]	Compound identity[c]
I	0.63–0.67	Biotin sulfoxides
II	0.72–0.74	Bisnorbiotin
III	0.83–0.84	Biotin

[a] From chromatography of urine collected from rats that ha dreceived either ^{14}C-biotin or ^{14}C-biotin–avidin.

[b] Determined after ascending development on Whatman No. 1 with *n*-butyl alcohol–acetic acid–water (2:1:1, v/v/v) by localization of radioactive spots with a Nuclear-Chicago Actigraph III radiochromatogram scanner and by specific color reaction of these compounds with acidic *p*-dimethylaminocinnamaldehyde spray reagent (18).

[c] By correspondence to internal and external standards.

Discussion. Similar radioactive biotin metabolites were isolated from rat urine following ip injection of either free or avidin–bound ^{14}C-biotin. This, as well as the general release of ^{14}C, establishes the fact that the animal body can liberate biotin from the injected biotin–avidin complex, even though the complex is reputed to be quite stable in the gastrointestinal tract. The delayed excretion of radioactivity from the avidin-bound ^{14}C-biotin may be due to the slow dissociation of the vitamin from the complex, the dissociated vitamin then being incorporated into the biotin pool of the biotin-

depleted animal. The excretion rate of free biotin is rather fast, even in the biotin-depleted animal (11, 12); thus, the amount of radioactive biotin incorporated into the biotin pool after a single injection of ^{14}C-biotin is limited. Hence, compared to rats injected with free ^{14}C-biotin, those injected with the avidin-bound ^{14}C-biotin excreted larger amounts of radioactivity after a "purging" dose of nonradioactive biotin was given at the end of day 12 (cf. Table I). The different amounts of biotin and metabolites contained in the urine collected from both groups during early (1–5 days) and late (13–17 days) periods may be explained by the following: Oxidation of biotin to the sulfoxides continues as long as there is ample vitamin present, but decreases as the level of the latter falls. As the capacity for sulfoxide formation is limited, however, a considerable quantity of the vitamin is available at earlier times after administration to be excreted unaltered, as well as undergo β-oxidative cleavage of the side chain to yield bisnorbiotin. It should be noted that the ATP-dependent conversion of biotin to its adenylate is prerequisite for carboxyl activation of the vitamin to be covalently incorporated into enzymes (19), as well as to be catabolized in the β-oxidative pathway for fatty acids (20, 21). It is likely that biotin, in excess of that required initially to regenerate needed enzymes, will then be wasted in part through β-oxidation to bisnorbiotin, which continues to be excreted through the urine rather than further broken down.

The findings from the study *in vitro* indicate that rat liver has the ability to dissociate the biotin–avidin complex. Some of the biotin, either before or after dissociation, is oxidized to sulfoxides (11). However, the mechanism for the transportation of biotin–avidin complex out of the parenteral cavity, and the exact role of the liver for the dissociation of this complex *in vitro* is not yet certain. Presumably, some destruction, or at least denaturation, of avidin may occur.

Summary. Radioactive biotin injected intraperitoneally into rats as the avidin complex was found to be excreted much more slowly than the free vitamin. However, the biotin–avidin complex is dissociated *in vivo* and the released biotin both excreted and metabolized to sulfoxides and bisnorbiotin as is free biotin. That liver has the capacity to cause dissociation of the avidin–biotin complex, as well as metabolize the vitamin, was also demonstrated.

1. Green, N. M., Biochem. J. **89**, 585 (1963).
2. Eakin, R. E., Snell, E. E., and Williams, R. J., J. Biol. Chem. **136**, 801 (1940).
3. György, P., Rose, C. S., and Tomarelli, R., J. Biol. Chem. **144**, 169 (1942).
4. Pai, C. H., and Lichstein, H. C., Proc. Soc. Exp. Biol. Med. **116**, 194 (1964).
5. Wei, R. D., and Wright, L. D., Proc. Soc. Exp. Biol. Med. **117**, 341 (1964).
6. György, P., and Rose, C. S., Proc. Soc. Exp. Biol. Med. **53**, 55 (1943).
7. György, P., Rose, C. S., Eakin, R. E., Snell, E. E., and Williams, R. J., Science **93**, 477 (1941).
8. György, P., and Rose, C. S., Science **94**, 261 (1941).
9. Fraenkel-Conrat, J., and Fraenkel-Conrat, H., Biochim. Biophys. Acta **8**, 66 (1952).
10. Wei, R. D., Kou, D. H., and Hoo, S. L., Experientia **27**, 368 (1971).
11. Lee, H. M., Wright, L. D., and McCormick, D. B., J. Nutr. **102**, 1453 (1972).
12. Lee, H. M., McCall, N. E., Wright, L. D., and McCormick, D. B., Proc. Soc. Exp. Biol. Med., in press.
13. Iwahara, S., McCormick, D. B., Wright, L. D., and Li, H. C., J. Biol. Chem. **244**, 1393 (1969).
14. Melville, D. B., J. Biol. Chem. **208**, 495 (1954).
15. Rubin, S. H., Drekter, L., and Moyer, E. H., Proc. Soc. Exp. Biol. Med. **58**, 352 (1945).
16. Yang, C.-S., and McCormick, D. B., J. Nutr. **93**, 445 (1967).
17. Yang, H.-C., Kusumoto, M., Iwahara, S., Tochikura, T., and Ogata, K., Agr. Biol. Chem. **32**, 399 (1968).
18. McCormick, D. B., and Roth, J. A., Anal. Biochem. **34**, 226 (1970).
19. Mistry, S. P., and Dakshinamurit, K., "Vitamins and Hormones" (R. S. Harris, I. G. Wool and J. A. Loraine, eds.), Vol. 22, p. 1. Academic Press, New York (1964).
20. Mahler, H. R., Wakil, S. J., and Bock, R. M., J. Biol. Chem. **204**, 453 (1953).
21. Pullman, M. E., and Schatz, G., Annu. Rev. Biochem. **36**, 652 (1967).

Received Oct. 13, 1972. P.S.E.B.M., 1973, Vol. 142.