[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF WISCONSIN]

Biotin and *p*-Aminobenzoic Acid Content of Some Crystalline Enzymes

BY D. R. MILLER, J. O. LAMPEN AND W. H. PETERSON

Among the members of the vitamin B complex, thiamin, riboflavin, and nicotinic acid have been shown to occur in combination with a protein; *i. e.*, as the prosthetic groups of enzymes involved in respiration. The other members have, however, been assigned no definite role in cellular metabolism. Several of these, notably biotin and *para*-aminobenzoic acid (p. a. b.), occur in tissue in a bound form, and are liberated from natural materials only upon strong hydrolysis.

The possibility that p. a. b. and biotin also may function as the prosthetic groups of enzymes led us to assay a series of crystalline enzymes for their content of these substances. The minimum molecular weight thus obtained in comparison with the molecular weight obtained by other means gives an indication whether or not biotin or p. a. b. is an integral part of the enzyme molecule, and incidentally serves as an index of the purity of the preparation tested.

Sprince and Schoenbach[1] have reported the determination of biotin in purified tobacco mosaic virus. They found, however, that the biotin was free and was separable from the virus by ultracentrifugation. Hence they concluded that the biotin present was a result either of bacterial growth or of the carrying-over of slight impurities from the original tobacco-plant material.

Experimental

Biotin was determined by the microbiological method of Shull, *et al.*[2] The p.a.b. values were obtained by an assay developed in this laboratory[3] with *Clostridium acetobutylicum* S9 as the test organism. For the measurement of "bound" biotin the samples were hydrolyzed with 6 *N* hydrochloric acid for two hours at 15 lb. pressure. Experiments with liver, peptone and yeast[3] have shown large increases in p.a.b. content after strong alkaline hydrolysis; therefore, hydrolyses for "bound" p.a.b. were run for one hour both with 2*N* hydrochloric acid at 15 lb. pressure and with 5*N* sodium hydroxide at 75 lb. pressure.

Table I gives the biotin content of the enzymes studied. The catalase was a crystalline sample furnished by Dr. J. B. Sumner of Cornell University. We also wish to thank Dr. Sumner for the concanavalin A, a crystalline protein from the jackbean, and for the recrystallized jackbean urease. The crystalline rennin[4] was provided by Dr. C. L. Hankinson of the Carnation Company, Milwaukee, Wisconsin. Yeast polypeptidase[5] was furnished by Dr. M. J. Johnson of this laboratory. It was a purified preparation (No. 977) that was not crystalline, but behaved as a homogeneous substance on electrophoresis or ultracentrifugation. The phosphorylase samples and the muscle extracts were gifts of Dr. G. T. Cori of Washington University, St. Louis.

TABLE I

BIOTIN CONTENT OF ENZYMES

Enzyme	γ Biotin per g. Unhydrolyzed	γ Biotin per g. 6 *N* HCl 2 hr. 15 lb.	G. enzyme[b] per mole biotin
Catalase, beef liver	1.50	1.56	160,000,000
Concanavalin A[a]	0.032	0.47	520,000,000
Rennin	.71	1.12	220,000,000
Urease	.05	0.58	420,000,000
Yeast polypeptidase	.033	3.90	63,000,000
Phosphorylase	.84	11.3	22,000,000
Rabbit muscle extract No. 1	.44	0.32	760,000,000
Rabbit muscle extract No. 2	.32	0.32	760,000,000
Phosphorylase (adenylic acid)	..	0.30	810,000,000
Phosphorylase (non-adenylic acid)	..	<0.1	>2,400,000,000

[a] This protein is, of course, not an enzyme but is included because the analyses can serve as a basis of comparison for the enzyme proteins. [b] Calculated from the biotin figures in column 3.

All of the preparations contained some biotin and at least part of this was in a bound form, except in the cases of the catalase and the rabbit muscle extracts. However, the small amounts found yield impossibly large values for the minimum molecular weights as based on one mole of biotin per mole of protein. The molecular weight of catalase is 250,000,[6] that of urease is 480,000,[6] and that of the polypeptidase is 700,000.[5] These data effectively demonstrate that biotin is not an integral part of these enzymes.

The phosphorylase was a crystalline preparation from rabbit muscle containing adenylic acid.[7] It had an activity of 3500 units per mg. of protein.[8] On original assay a value after hydrolysis of 11.3γ of biotin per g. was obtained. Assay of a fresh hydrolyzate about six months later gave 4.1γ of biotin per g., indicating some destruction of biotin on storage. However, even the higher value gives a molecular weight of 22,000,000 which is entirely out of accord with the probable molecular weight of 340,000 to 400,000 as determined by ultracentrifugation and diffusion.[9] The rabbit muscle extracts were impure preparations, No. 1 containing 430 units per mg. of protein, and No. 2, 1705 units per mg. The adenylic acid-containing prosthetic group of the enzyme had been removed from these samples. They contained only 0.32–0.44γ of biotin per g., all of it unbound. This discrepancy between the preparations with and without adenylic acid led us to obtain two additional samples of crystalline phosphorylase. One contained adenylic acid, the other did not. The former assayed 0.3γ of biotin per g., the latter less than 0.1γ per g. This indicates that the high biotin content of the first phosphorylase sample was a chance occurrence, and that biotin is not an integral part of muscle phosphorylase.

(1) Sprince and Schoenbach, *Proc. Soc. Exptl. Biol. Med.*, **49**, 415 (1942).

(2) Shull, Hutchings and Peterson, *J. Biol. Chem.*, **142**, 913 (1942).

(3) Lampen and Peterson, to be published.

(4) Hankinson, *J. Dairy Sci.*, **26**, 53 (1943).

(5) Johnson, *J. Biol. Chem.*, **137**, 575 (1941).

(6) Svedberg and Pederson, "The Ultracentrifuge," Clarendon Press, London, 1940.

(7) Green, Cori and Cori, *J. Biol. Chem.*, **142**, 447 (1942).

(8) We are indebted to Dr. Gerty T. Cori for the figures giving the units of activity possessed by the phosphorylase samples and for information regarding the presence or absence of adenylic acid in them.

(9) Green and Cori, *J. Biol. Chem.*, **151**, 21 (1943).

All of the enzymes contained p.a.b., the greater part of it in a firmly bound form. Maximum yields were obtained by autoclaving in 5 *N* sodium hydroxide at 75 lb. pressure for one hour. Minimum molecular weights calculated from the p.a.b. content again far exceed accepted values, with the exception of the yeast polypeptidase. Here the calculated value of 1,050,000 is not unreasonably far from figure 700,000 given by Johnson.[5] Yeast, however, is an excellent source of p.a.b., with samples containing up to 130γ p.a.b. per g. of dry yeast or about 250–300γ per g. of protein. There has been no increase in p.a.b. content in the purification of the peptidase over the average found in the total protein of the yeast.

The question immediately arises as to whether these enzyme preparations contain foreign proteins. Northrop[10] has pointed out that crystallization is not necessarily an index of purity. All of the preparations did contain "bound" p.a.b. and many of them "bound" biotin in varying amounts. However, nothing is known about the molecular size of these p.a.b.- or biotin-containing impurities. Any foreign material present must be extremely high in p.a.b. if it is to carry all the p.a.b. present. For instance, an impurity amounting to 1% of the urease preparation would have to contain 0.21% p.a.b.

Analysis of materials for their content of various biologically active substances presents itself as a sensitive index of purity. All of the preparations tested here would be regarded as containing some impurity by this criterion.

TABLE II

P.A.B. CONTENT OF ENZYME

Enzyme	γ P. A. B. per g. H_2O 1 hr., 15 lb.	2 *N* HCl 1 hr., 15 lb.	5 *N* NaOH 1 hr., 75 lb.	Enzyme[b] per mole P. A. B., g.
Catalase, beef liver	4.3	17	19	7,200,000
Concanavalin A[a]	0.25	9.2	22	6,200,000
Rennin	1.0	7.5	19	7,200,000
Urease	1.9	11.5	21	6,500,000
Yeast polypeptidase	6.6	120	130	1,050,000
Phosphorylase	2	3.6	13	10,500,000
Rabbit muscle extract No. 1	1.7	5.0, 5.4 (6 *N*)	23	5,500,000
Rabbit muscle extract No. 2	1.7	6.0, 5.7 (6 *N*)	25	6,050,000

[a] See footnote to Table I. [b] Calculated from the p.a.b. figures in column 4.

(10) Northrop, "Crystalline Enzymes," Columbia University Press, New York, N. Y., 1939.

The authors are indebted to Miss Florence Fox for assistance in making the biotin determinations.

Summary

Six crystalline and three non-crystalline enzyme preparations and one crystalline protein not an enzyme have been analyzed for their content of biotin and *p*-aminobenzoic acid (p. a. b.). The biotin ranged from 0.32 to 11.3 micrograms per gram and the p. a. b. from 13 to 130 micrograms per gram. Assuming 1 mole of biotin or p. a. b. per mole of enzyme, the minimum molecular weights are far beyond the figures assigned to these enzymes. It is concluded that the biotin and p. a. b. are contained in impurities in the crystalline proteins rather than forming an integral part of the enzyme. Determination of these vitamins in crystalline proteins may be a useful means of detecting impurities.

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[COMMUNICATION NO. 15 FROM THE LABORATORIES OF HOFFMANN-LA ROCHE, INC.]

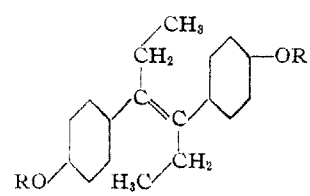
The Synthesis of Estrogenic Indene Derivatives and Remarks on the Configuration of Stilbestrol[1]

BY ULRICH V. SOLMSSEN

The discovery of a great number of estrogenic compounds not chemically related to the natural estrogens, has demonstrated an astonishing unspecificity of estrogenic activity.

Stilbestrol (I), (4,4′-dihydroxy-α,β-diethyl-stilbene) and its hydrogenation product hexestrol (IX), (4,4′-dihydroxy-γ,δ-diphenyl-hexane) first described by Dodds and co-workers,[2,3,4] and since synthesized following various routes, have acquired wide interest as clinical substitutes for the natural estrogens.

Among the many compounds tested by various investigators, the following three indene derivatives reported were found to be inactive: 6- and 7-hydroxy-hydrindene,[5] 4- and 6-hydroxy-α-hydrindone[5] and 5-benzoxy-3-hydrindol.[6] In spite of these negative results, this Laboratory undertook to prepare 2-(*p*-hydroxyphenyl)-3-ethyl-6-hydroxy-indene (V), the structure of which is closely related to that of 4,4′-dihydroxy-α,β-diethyl-stilbene (I) and 4,4′-dihydroxy-α-methyl-β-ethyl-stilbene, if the structure formula for stilbestrol and its derivatives is written according to the British authors.



I R = H
II R = CH_3
III R = $COCH_3$
IV R = $COCH_2CH_3$

(1) Presented at the Meeting of the American Chemical Society, Pittsburgh, Pa., September 6–10, 1943.

(2) Dodds, Goldberg, Lawson and Robinson, *Nature*, **141**, 247 (1938).

(3) Dodds, Goldberg, Lawson and Robinson, *Proc. Roy. Soc.* (London), **127**, 114 (1939).

(4) Campbell, Dodds and Lawson, *Nature*, **142**, 1121 (1938).

(5) Dodds and Lawson, *Proc. Roy. Soc.* (London), **125**, 222 (1938).

(6) Miyasaka, *Pharm. Soc. Japan*, **59**, 407 (1939).