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BIOSYNTHESIS OF ERGOTHIONEINE AND HERCYNINE BY MYCOBACTERIA

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ABSTRACT

GENGHOF, DOROTHY S. (Yeshiva University, New York, N.Y.), AND OLGA VAN DAMME. Biosynthesis of ergothioneine and hercynine by mycobacteria. J. Bacteriol. **87**:852-862. 1964.—Ergothioneine and hercynine were found to be synthesized by a wide variety of mycobacteria grown in chemically defined media free from these compounds. The cultures examined included 53 recently isolated and laboratory strains of *Mycobacterium tuberculosis*, 26 "unclassified" mycobacteria (Runyon groups I to IV), and representatives of most other species in the genus. Purification and separation of the betaines was achieved by means of chromatography on two successive alumina columns. Photometric measurement of the diazotized effluents from the second column permitted amounts of each compound to be determined. Measurement of hercynine by this method was made possible for the first time by the development of a standard curve. The pathway of ergothioneine biosynthesis in mycobacteria, as judged by the use of S^{35}-sulfate and L-histidine-2-C^{14} as tracers, appears similar to that found in *Neurospora crassa* and *Claviceps purpurea*, that is, from histidine to ergothioneine via hercynine. None of a small group of bacteria other than mycobacteria was found to produce ergothioneine. Two strains of group A streptococci and one of *Escherichia coli* produced hercyninelike material, as yet unidentified.

Ergothioneine, the betaine of thiolhistidine, was first isolated from ergot by Tanret (1909), who also established its empirical formula. Hercynine, the betaine of histidine, was isolated from mushrooms shortly thereafter by Kutscher (1911), and its chemical relationship to ergothioneine was shown by Barger and Ewins (1911, 1913). In due course, ergothioneine was recognized in pig and human blood (Newton, Benedict, and Dakin, 1927; Eagles and Johnson, 1927). Hercynine, however, was unknown in organisms other than fungi until recently, when it was discovered along with ergothioneine in the tissues of the horseshoe crab, *Limulus polyphemus* (Ackermann and List, 1958), and in cattle erythrocytes and boar's seminal fluid (Ackermann, List, and Menssen, 1959). Despite the presence of both ergothioneine and hercynine in various animal tissues, biosynthesis of these compounds by animals has not been detected in any of the well-controlled radioactive studies reported (Melville, 1959; Stowell, 1961). Until recently, the capacity for ergothioneine synthesis has been known only for various fungi (Melville et al., 1956; Heath and Wildy, 1956; Ban and Stowell, 1956; Ban, 1958); hercynine synthesis has been shown only with *Neurospora crassa* (Askari and Melville, 1962).

Attempts to detect ergothioneine in bacteria were negative (Melville et al., 1956) until a few strains of *Mycobacterium tuberculosis* and of "unclassified" mycobacteria, grown in a synthetic medium, were found to produce the compound (Genghof, 1960). The present work reveals that the biosynthesis of ergothioneine and hercynine extends to essentially all varieties of mycobacteria. In addition, through experiments with *M. tuberculosis* and *M. smegmatis*, grown in the presence of C^{14}-histidine (labeled in the imidazole ring) and S^{35}-sulfate as precursors, the pathway of synthesis has been shown to be similar to that reported for fungi (Melville, 1959; Stowell, 1961; Askari and Melville, 1962). These findings add a new biochemical facet to the well-known close taxonomic relationship between the fungi and the acid-fast bacteria.

MATERIALS AND METHODS

Bacterial strains. The individual mycobacteria used in the present study (Table 1) included 85 strains from human sources (*M. tuberculosis*, "unclassified" mycobacteria, and other varieties) and 16 cultures from nonhuman sources. Stock cultures were maintained on the medium of the American Trudeau Society (Woodruff et al.,

TABLE 1. *Sources and descriptions of strains of Mycobacterium*

Species or group	Description	Received from[a]
Human source		
M. tuberculosis	48 fresh isolates[b]	VE
M. tuberculosis	H37Ra (ATCC 13325)	A
M. tuberculosis	H37Rv	C
M. tuberculosis	H37Rv INH-resistant	WS
M. tuberculosis	3BF80 INH-resistant	WS
M. tuberculosis	3N241 INH-resistant	WS
"Unclassified" mycobacteria		
M. kansasii[c]	A6, A15, A17, 7B, 14B, 17B	AP
M. kansasii	P1, P8, P18, P21, P22, P26	ER
Scotochromogens[d]	P5, P6, P28, 7A	ER, AP
Battey type[e]	P7, P17, P23, P39, P44	ER, AP
M. intracellularis[e]	*Nocardia intracellularis*	AP
Mycobacterium sp.[e]	QT 9, NT 10 (Binford, 1959)	B
M. fortuitum	1031, 1033	RG
Other mycobacteria		
M. ulcerans	ATCC 14188[f]	A
M. balnei (*M. marinum*)	X, XVI (Linell and Norden, 1954)	A
"*M. leprae*"	CUMC 1	C
M. smegmatis	M607, CUMC 2	C
Nonhuman source		
M. avium	Sheard 72	AP
M. bovis	BCG, 2 strains	PI
M. bovis	Ravenel	C
M. bovis	4228-4	WS
M. bovis	4228-4-435 INH-resistant	WS
M. fortuitum	*M. giae* (Darzins, 1952)	A
M. marinum	J. D. Aronson (1926)	A
M. balnei (*M. marinum*)	BIV, V (Linell and Norden, 1954)	A
M. microti	ATCC 11152 (OV 183)[g]	A
M. paratuberculosis	Johne's bacillus	JH
M. phlei	CUMC 3	C
M. piscium	ATCC 9819, (NCTC 2014)	A
M. rhodochrous[h]	P. H. H. Gray	A
M. thamnopheos	J. D. Aronson (1929)	A

[a] A, American Type Culture Collection; AP, Anne Pollak; B, Chapman H. Binford; C, Cornell University Medical College; ER, Ernest H. Runyon; JH, John H. Hanks; PI, Pasteur Institute, Paris, or Phipps Institute, Philadelphia, Pa.; RG, Ruth Gordon; VE, Van Etten Hospital; WS, William Steenken, Jr.

[b] These strains were selected from among the isolates from 92 patients, on the basis of their ability to grow as pellicles on Sauton medium.

[c] These are the "yellow bacilli" of Buhler and Pollak (1953) and the group I photochromogens of Runyon (1959).

[d] Group II scotochromogens of Runyon (1959).

[e] Group III nonphotochromogens of Runyon (1959). *M. intracellularis* (Runyon, 1960) is the *Nocardia intracellularis* of Cuttino and McCabe (1949). *Mycobacterium* sp. (QT 9 and NT 10), classified by Charles Shepard as nonphotochromogens, were isolated by Binford (1959) from the skin of leprous patients by passage in hamsters; QT 9 is from a ninth passage; NT 10 is from a tenth hamster passage.

[f] The Battista strain of Jean C. Tolhurst.

[g] Originally received from A. Q. Wells as *M. muris*.

[h] Received at American Type Culture Collection from P. H. H. Gray as *Proactinomyces globerula;* redesignated *M. rhodochrous* by Gordon and Mihm (1957).

1946), except for *M. fortuitum* which was grown on glucose-yeast-agar.

Determination of erogthioneine. For analysis of ergothioneine and hercynine, the organisms were grown in the 5% glycerol medium of Sauton (1912) adjusted to pH 7.2, or in the same medium modified by addition of 0.05% Tween 80 (Atlas Powder Co., Wilmington, Del.). The ingredients of these synthetic media, analyzed by the method of Melville and Lubschez (1953) and Melville, Horner, and Lubschez (1954), were found to be free from ergothioneine and hercynine. To obtain a sufficient yield of bacterial cells (0.5 to 3 g, dry weight) for analysis, Roux flasks of the Sauton or Sauton-Tween medium were inoculated with organisms grown in the corresponding medium for one or two passages, and incubated at an appropriate temperature. Depending on the strain, the time of incubation ranged from 1 to 23 weeks. Two cultures required exceptional nutrient conditions. *M. paratuberculosis* (Johne's bacillus) was grown on a special semisynthetic agar medium containing mycobactin. (The mycobactin and formula for the medium, no. 8A, were kindly supplied by John H. Hanks.) *M. microti* was cultivated on slants of American Trudeau Society medium without malachite green.

The bacterial pellicles from cultures in Sauton medium or the centrifuged bacterial sediments from cultures in Sauton-Tween medium were collected, washed with distilled water, and exposed to flowing steam for 30 min. The killed cells were next dried in a vacuum oven (80 C, 1 to 6 hr) and stored in a desiccator at room temperature until processed for analysis. (Extraction usually was carried out shortly after drying; however, ergothioneine has proven stable in desiccated cells for more than 1 year, and some bacteria were analyzed after prolonged storage.) In preparation for assay, the dried organisms were weighed and extracted three times with hot (90 to 100 C) water; the combined extracts (15, 10, and 10 ml) were evaporated to dryness in vacuo (ca. 80 C). The residue was redissolved in a minimal volume of water (0.5 to 2.0 ml), and absolute ethanol was added to a concentration of 75% by volume. The clear supernatant fluid obtained by centrifugation was added to a freshly prepared alumina column (80 to 200 mesh, 40 cm by 0.9 cm in diameter) for development with 75% ethanol-1% formic acid (Melville and Lubschez, 1953; Melville et al., 1954); any precipitate was washed twice with the column solvent, and the washings were added to the column. A fraction collector controlled by an automatic drop counter was used to collect 5-ml effluents; 1-ml samples were analyzed for ergothioneine by the modified Hunter diazo test (Melville and Lubschez, 1953), by use of a colorimeter with a green filter (Corning no. 4010, 524-mμ peak). The residual (4 ml) portions of all fractions containing ergothioneine (generally tubes 5 to 10) were combined, dried under nitrogen, and brought into solution in 80% ethanol. This was rechromatographed on a fresh alumina column with 80% ethanol as developer, to obtain ergothioneine and hercynine in separate fractions (generally tubes 10 to 14 for the former and 5 to 9 for the latter).

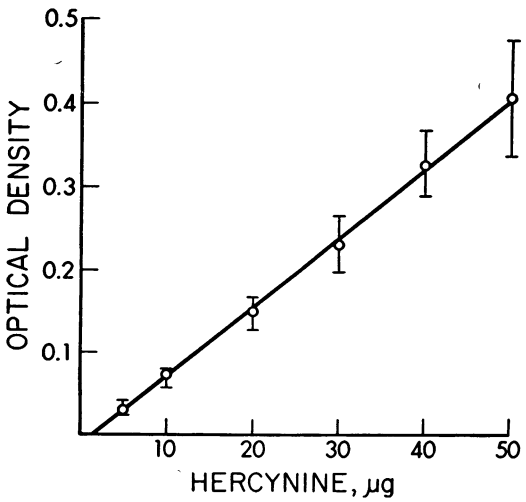

FIG. 1. *Diazo color intensities produced with graded amounts of pure hercynine. Each circle represents the average of five duplicate determinations, with maximal and minimal values indicated.*

Ergothioneine, determined as described above, was expressed as milligrams per 100 grams of dry bacterial cells; a 20-μg sample of authentic ergothioneine (Nutritional Biochemicals Corp., Cleveland, Ohio) was used as a standard with each determination. Hercynine was determined by the following method developed by the authors.

Determination of hercynine. Diazotization of hercynine leads to the formation of material that is colored yellow (dilute solutions) to orange (more concentrated solutions). The relationship of optical density (with a green Corning filter no. 4010, 524-mμ peak) to hercynine concentration was found to approach a straight-line function (Fig. 1). This composite curve was used to esti-

mate the hercynine in effluent fractions derived from the various cultures; the curve represents data from five experiments in each of which graded amounts (5 to 50 μg, in duplicate) of pure hercynine, prepared by D. B. Melville, were diazotized as for ergothioneine and measured. The considerable variation in values about each average point, as shown by differences between the maximal and minimal values, reflect the limited accuracy of the method for hercynine.

Experiments involving radioactive isotopes. Approximately 10 μc of S^{35}-sulfate or 25 μc of L-histidine-*2*-C^{14} were added to 150 ml of Sauton medium prior to inoculation with a pellicle of *M. tuberculosis* strain 5091. After a suitable growth period (4 to 6 weeks), the bacteria were dried, weighed, extracted, and analyzed for ergothioneine and hercynine as described above. Diazotization was carried out on 0.1- to 2.5-ml samples of effluent fractions from the first and second columns; radioactive counts with a windowless gas-flow counter were made on 0.1- to 0.5-ml samples brought to a uniform volume in planchets before drying.

For identification and estimation of S^{35}-labeled ergothioneine, treatment with bromine [which readily oxidizes and liberates the sulfur of ergothioneine (Barger and Ewins, 1911)] was carried out on 0.5 ml of selected 5-ml effluent fractions. Each sample was first dried to remove alcohol, then redissolved in water, and treated for 0.5 hr with a slight excess of bromine water; inorganic sulfate was precipitated, along with carrier sulfate, by barium chloride. The radioactivity of both precipitate and superantant fluid was counted.

To determine the specific activity of the C^{14}-ergothioneine, *M. tuberculosis* 5091 was grown in 150 ml of Sauton medium containing 11.1 μc of L-histidine-*2*-C^{14} (7.5×10^4 disintegrations per min per μmole) plus 50.5 mg of unlabeled histidine. Ergothioneine was recovered in pure form from extracts of the harvested (dried) cells by means of chromatography on three successive alumina columns, the first of which was developed with 75% ethanol-1% formic acid and the latter two with 80% ethanol. To 648 μg of labeled material finally obtained, 64 mg of recrystallized ergothioneine were added; the mixture was dissolved in water and treated with absolute ethanol to 80% by volume to effect crystallization. The crystals were redissolved, and the radioactivity of a sample of the solution was measured by the liquid scintillation counting method. The bulk of the material in solution was dried in vacuo, recrystallized from 80% ethanol, and again analyzed. The process was repeated to provide a third, and final, analysis.

Paper chromatography. For visualizing ergothioneine and hercynine, 0.025 to 3 ml of effluent were concentrated and subjected to ascending chromatography on Whatman no. 1 paper. Development was with pyridine-water (65:35, v/v) at 4 C for 4 to 5 hr (Askari, 1960), followed by a spray of modified Pauly reagent (Ames and Mitchell, 1952). Comparison with known standards enabled rough estimates to be made of the quantity of each compound.

RESULTS

Biosynthesis of ergothioneine and hercynine by mycobacteria. The recovery of ergothioneine and hercynine from a few laboratory strains and from several freshly isolated strains of tubercle bacilli (Genghof, 1960) prompted the survey of a comprehensive group of mycobacteria (listed in Table 1), including various human pathogens as well as representatives of other species. A major objective was to determine whether the ability to produce ergothioneine and its probable precursor, hercynine, is a general attribute of the *Mycobacterium* genus. Analyses were usually carried out on extracts prepared from 1 to 3 g of dried cells. Chromatography on an alumina column with 75% ethanol-1% formic acid yielded fractions containing both compounds, which were then separated on a second alumina column developed with 80% ethanol. Colorimetric analyses for ergothioneine and hercynine were made by means of the diazo reaction.

From the results obtained in tests of 101 strains of mycobacteria from human and nonhuman sources (Table 2), it is clear that the capacity to synthesize ergothioneine and hercynine is a consistent property of members of the genus *Mycobacterium*. (In addition to the analytical results in Table 2, selected second column fractions from 25 of the strains were subjected to paper chromatography with pyridine-water solvent, and spray of modified Pauly reagent. The presence of material staining and migrating as ergothioneine or hercynine was verified in all but a few instances.) Most freshly isolated strains of *M. tuberculosis* produced 1 to 16 mg of ergothi-

TABLE 2. *Production of ergothioneine and hercynine by mycobacteria cultured on synthetic media*

Source of organism	Mycobacteria	No. of strains	Growth conditions		Dry wt used	Yields after separation on second alumina column (80% ethanol)	
			Medium[a] and temp (c)	Time		Ergothioneine	Hercynine
				weeks	*g*	*mg/100 g*	*mg/100 g*
Human	*M. tuberculosis*						
	Fresh isolates	6	S-37	3-13	2-3	20-41	10-22
	Fresh isolates	21	S-37	5-19	1-3	5-16	5-26
	Fresh isolates	20	S-37	2-16	1-3	1-4.5	5-29
	Fresh isolates	1	S-37	11	2	0[b]	32[b]
	H37Rv, H37Ra	2	S-37	9, 7	2, 3	9, 37	12, 20
	H37Rv, 3BF80[c]	2	S-37	6, 9	2, 1	50, 18	13, 21
	3N241[c]	1	S-37	10	1	0[b]	3[b]
	"Unclassified" group						
	M. kansasii	2	S-37	2, 3	1-3	78, 84	18, 25
	M. kansasii	10	S-37	2-13	0.5-2	5-13[d]	4-68[d]
	Scotochromogens	2	S-37	3, 5	1	1, 13	5, 6
	Scotochromogens	2	ST-37	4	0.1, 0.3	6, 26	15, 18
	Battey type	4	ST-37	6, 7	0.5-3	0-4	3-13
	Battey type	1	ST-37	4	0.2	65	53
	M. intracellularis	1	ST-37	3	0.1	10	48
	M. QT 9, NT 10	2	ST-37	7, 6	1	38, 34	22, 24
	M. fortuitum	2	ST-25	5	3	112, 116	3, 56
	Other mycobacteria						
	M. ulcerans	1	ST-30	7	1	1	11
	M. balnei X, XVI	2	ST-30	4-5	1, 3	0, 1[e]	8, 3
	"M. leprae"	1	S-37	2	3	114	28
	M. smegmatis	2	S-37	1-2	2, 3	12, 100	6, 19
Non-human	*M. avium*	1	S-37	4	0.3	118	30
	M. bovis BCG	2	S-37	3, 4	2	5, 11	25, 10
	M. bovis Ravenel	1	ST-37	3	2	19	10
	M. bovis 4228-4	1	S-37	9	1	75	29
	M. bovis 4228-4-435	1	S-37	23	1	15	9
	M. fortuitum (gia)	1	S-37	3	3	47	30
	M. marinum	1	ST-25	4	5	50	20
	M. balnei BIV, V	2	ST-30	4, 11	0.5, 0.4	0	4, 4
	M. microti	1	AT-37	11	0.5	45	8
	M. paratuberculosis	1	H-37	10	1	44	24
	M. phlei	1	S-37	1-2	2	26	14
	M. piscium	1	ST-23	7	2	25	29
	M. rhodochrous	1	S-25	3	3	1	15
	M. thamnopheos	1	ST-25	2-3	2	3	5

[a] S = Sauton medium; ST = Sauton medium containing Tween; H = Hanks medium; AT = American Trudeau Society medium minus malachite green. Numbers represent temperatures.

[b] Value obtained after a single chromatographic separation, with 75% ethanol; the sample was not rechromatographed.

[c] Isoniazid-resistant strains.

[d] Values for two of the ten organisms were obtained after a single chromatographic separation, with 75% ethanol.

[e] For strain X, 3 mg per 100 g were detected in effluents from the first column; none was found on rechromatography.

oneine and 5 to 29 mg of hercynine per 100 g of dry bacterial cells. Of the 48 strains which had grown relatively rapidly, 6 elaborated large amounts of ergothioneine (20 to 40 mg per 100 g). Only one freshly isolated culture failed to form ergothioneine despite its abundant growth and appreciable hercynine content. Laboratory strains of *M. tuberculosis*, including two of three isoniazid-resistant strains, produced considerable amounts of both betaines, although little hercynine and no ergothioneine were found in one isoniazid-resistant strain, no. 3N241.

Several "unclassified" mycobacteria of Runyon groups I to IV produced much larger amounts of ergothioneine than did any of the *M. tuberculosis* cultures. These included 2 strains of *M. fortuitum*, whose ergothioneine content exceeded that of all other human pathogens, 1 of 5 Battey bacilli (strain P23), and 2 of 12 *M. kansasii* (strains A15, P1). Moreover, none of the *M. kansasii* showed the modest yields of ergothioneine (less than 5 mg per 100 g) that characterized about half of the *M. tuberculosis* cultures, though one Battey strain did fail to produce detectable ergothioneine. The range of hercynine synthesis by the "unclassified" mycobacteria was generally similar to that of the freshly isolated tubercle bacilli, but four strains were exceptional in giving the largest yields (48 to 68 mg per 100 g) encountered among any of the mycobacteria. The pronounced capacity of several of the "unclassified" strains (including scotochromogens P6 and P28, and Battey strain P23) to synthesize ergothioneine and hercynine was readily demonstrated with a relatively small mass of cells (0.1 to 0.3 g) derived from diffusely growing cultures.

Of the remaining mycobacteria from human sources, the rapidly growing "*M. leprae*" and *M. smegmatis* produced large amounts of ergothioneine (both strains of the latter species showed yields as high as 100 to 300 mg per 100 g when grown on other synthetic media). At the other extreme, strains of *M. ulcerans* and *M. balnei* (*M. marinum*) from human skin lesions showed low yields of ergothioneine and hercynine, whereas two *M. balnei* isolates from swimming pool water (Table 2) showed no ergothioneine whatsoever. *M. balnei* is considered by some authors (Bojalil, 1959; Bojalil, Cerbón, and Trujillo, 1962) to be the same species as *M. marinum;* however, a strain of *M. marinum* isolated by Aronson (1926) from a saltwater fish produced considerable amounts of ergothioneine and hercynine.

Among mycobacteria isolated from nonhuman sources, *M. avium* was outstanding in synthesizing large amounts of ergothioneine and hercynine. A strain of *M. fortuitum* from the large Brazilian frog, gia, also produced good yields of each compound, but not as much as the strains of human source. *M. paratuberculosis*, grown on slants of Hanks semisynthetic medium because of its fastidious nutritive requirements, produced ergothioneine and hercynine in great excess over the small amount of material resembling hercynine which accompanied the mycobactin component of the medium.

In earlier experiments, ergothioneine was not detected in the cells of 12 bacterial strains (representing 11 genera), including *M. smegmatis* ATCC 607 (Melville et al., 1956). Since synthesis has now been demonstrated for most mycobacteria, including the latter culture, the question of ergothioneine production by other microorganisms arose again during the present work. Analysis was made of seven strains of bacteria, including two group A streptococci (type 12, strain 2RP196; and type 3, strain C203S), two *Salmonella schottmuelleri* and individual cultures of *Escherichia coli*, *Corynebacterium diptheriae* type *mitis*, and an unidentified diptheroid. The streptococci [1 to 2 g (dry weight)] from cultures grown in the partially defined medium of Bernheimer et al. (1942) were kindly supplied by R. Rowen. Lesser amounts [0.2 to 0.4 g (dry weight)] were obtained for analysis from the remaining organisms. Three strains of the protozoan *Tetrahymena*, kindly supplied by G. W. Kidder, were also examined. No evidence was obtained for the presence of ergothioneine in any of these. The *E. coli* yielded a modest amount of hercynine or hercynine-like material; extracts of both streptococci, when chromatographed on alumina, yielded material showing diazo colors, also suggestive of hercynine but distributed differently in the effluent fractions. These results suggest the possibility that hercynine or some similar compound, as yet unidentified, may be made by certain bacteria outside the genus *Mycobacterium*.

Evaluation of the technique used for analysis of the betaines. Each sample in Table 2 had been fractionated on two successive alumina columns,

TABLE 3. *Separation of hercynine from ergothioneine by rechromatography of the mycobacterial extracts*

No. of strains tested	First alumina column (75% ethanol)		Second alumina column (80% ethanol)	
	Ergothioneine	Hercynine	Ergothioneine	Hercynine
	mg/100 g	*mg/100 g*	*mg/100 g*	*mg/100 g*
3	123–162	—*	100–118	19–30
4	38–119	15–81	10–116	3–56
21	35–109	—	20–84	7–53
40	9–27	—	4–19	5–68
17	5–11	—	1–5	5–29
2	5, 7	8, 28	Not done	Not done
5	2–3	—	0–4	3–9
2	<0.2	3, 32	Not done	Not done
4	<2	18–40	0.5–1	11–15
3	<2	<5	0	4–12

* Measurement unobtainable because the characteristic yellow-orange color of diazotized hercynine was masked by the stronger pink-magenta color formed by ergothioneine.

with different solvents, because the routine method with a single column does not ordinarily separate ergothioneine from hercynine. The need for separating the compounds prior to measurement is apparent from the results obtained after the first chromatographic fractionation of extracts from all strains (Table 3). Most estimates of ergothioneine made in the presence of hercynine were somewhat high, although they did approach true values due to the dominating magenta color of diazotized ergothioneine. However, estimates of hercynine could not be obtained at all in 86 extracts, as these contained sufficient ergothioneine to cause masking of the weak yellow to orange color of diazotized hercynine. In a few instances, hercynine could be estimated because little or no ergothioneine was produced or because some separation of the compounds had occurred. The use of double chromatography enabled individual estimation of the compounds in every case, and also allowed experiments dealing with their biosynthesis to be made.

Confirmation of the biosynthesis of ergothioneine and hercynine by M. tuberculosis with the aid of radioactive precursors. The betaines produced from defined media by the mycobacteria are known with reasonable assurance to be ergothioneine and hercynine on the basis of their typical positions of elution from alumina columns and their characteristic colors after diazotization. Further evidence, confirming the chemical nature of these substances as well as their de novo synthesis, was obtained with the aid of cultures grown in the presence of S^{35}-sulfate or L-histidine-*2*-C^{14}. The data also provided sufficient definition of the biosynthetic pathway for comparison with that already described for the fungi, e.g., in *N. crassa* (Melville, Eich, and Ludwig, 1957) and *Claviceps purpurea* (Heath and Wildy, 1956; Ban, 1958).

M. tuberculosis (human isolate no. 5091), a niacin-positive and isoniazid-sensitive strain of high pathogenicity for guinea pigs, was grown as a pellicle in flasks of Sauton medium (150 ml) to which either S^{35}-sulfate or L-histidine-*2*-C^{14} was added in trace amounts. After good growth had occurred, the organisms were dried, extracted, and analyzed for ergothioneine and hercynine. Effluents from both the first and second alumina columns were examined for radioactivity and for each of the betaines by diazotization. The results are compared in Fig. 2; the values recorded (counts per min, or micrograms of ergothioneine or hercynine) are from one-tenth (0.5 ml) of each effluent fraction.

In the case of growth with S^{35}-sulfate, the curves of radioactive counts and of readings of the diazo colors for successive fractions from the first alumina column (Fig. 2a) are essentially superimposable. After rechromatography (Fig. 2b), it is evident that the S^{35}-radioactivity is absent from the hercynine peak but is associated with the slower-moving ergothioneine component. That the radioactivity is indeed due to S^{35}-ergothioneine is shown by an additional finding. Each of the radioactive fractions, when treated with bromine water, displayed the ready formation of inorganic sulfate characteristic of er-

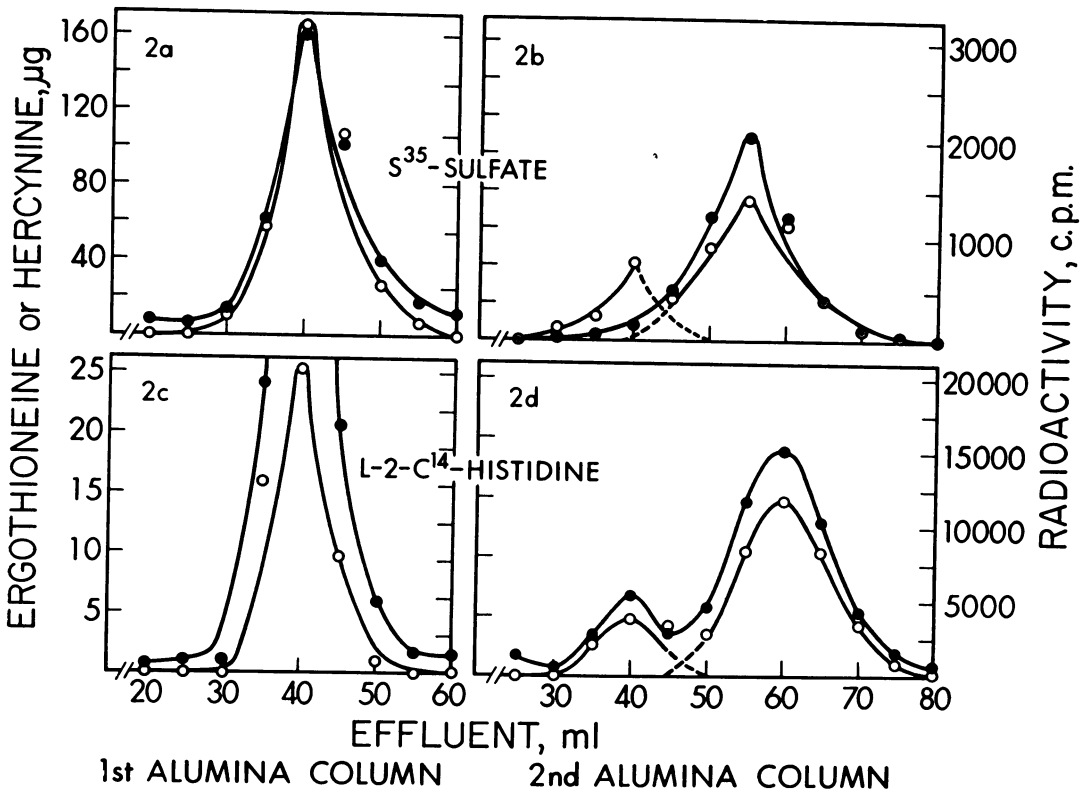

FIG. 2. *Incorporation of S^{35}-sulfate into ergothioneine and of L-histidine-2-C^{14} into hercynine and ergothioneine by Mycobacterium tuberculosis. Distribution of radioactivity (●) and of diazo colors from ergothioneine and hercynine (○) are shown after chromatography in 75% ethanol-1% formic acid (2a, 2c) and rechromatography in 80% ethanol (2b, 2d). In 2b and 2d, the peak of ergothioneine is to the right of that for hercynine; dashed lines are extrapolations based on data obtained by paper chromatography of effluents from the region of incomplete separation of the compounds.*

gothioneine; the sulfate so formed (recovered as $BaSO_4$) accounted for 88% of the total radioactivity of the peak. These findings clearly confirm that *M. tuberculosis* can transform inorganic sulfate to the sulfhydryl of ergothioneine.

In the second experiment, with L-histidine *2-C^{14}* in the medium, the peak of radioactivity in effluents from the first alumina column (Fig. 2c) coincided with that of the diazo colors (from ergothioneine plus hercynine). Rechromatography (Fig. 2d) separated the radioactivity into two peaks, corresponding to the hercynine and ergothioneine regions of the diazotization curve. These data suggest incorporation of the labeled imidazole ring of histidine into each betaine; however, the specific activity calculated for the synthesized ergothioneine was only 2 to 3% of that of the added histidine. Since, according to Melville (1959), suppression of the formation of endogenous histidine is necessary in *N. crassa* to achieve complete incorporation of the labeled imidazole ring into ergothioneine, a further experiment was performed in which the concentration of histidine provided in the medium was greatly increased. The labeled ergothioneine (648 μg) obtained from this culture, after extensive purification by chromatography, was combined with unlabeled ergothioneine (64 mg), and the whole was crystallized from 80% ethanol. As measured by liquid scintillation counting, similar specific activities were obtained for both the added histidine (7.5×10^4 disintegrations per min per μmole) and the ergothioneine successively crystallized to constant specific activity (7.74, 7.12, 7.25×10^4 disintegrations per min per μmole). Melville et al. (1957) already showed the

capacity of *N. crassa* to incorporate the intact imidazole ring of histidine into ergothioneine; more recently, Askari and Melville (1962) described the incorporation of doubly labeled (ring and trimethylamine moiety) hercynine into ergothioneine without preferential loss of isotope. Since the ergothioneine synthesized by *M. tuberculosis* shows the same specific activity as the added histidine, the probability is great that the intact imidazole ring is incorporated by this pathogen also. This knowledge, combined with the results shown in Fig. 2d, strongly suggests that hercynine also incorporates the unchanged imidazole ring.

The data of the experiments of Fig. 2 allow a ready estimate to be made of the extent of loss of ergothioneine and hercynine upon rechromatography. In both experiments, 25 to 30% loss of radioactive counts occurred between the first and second analyses. The cause is not known; Askari (1960) showed that ergothioneine labeled in the methyl groups does not degrade to hercynine during the chromatographic procedure.

The capacity to incorporate the sulfur of sulfate into ergothioneine and the imidazole ring of histidine into hercynine and ergothioneine is not restricted to *M. tuberculosis*. *M. smegmatis*, selected as a representative nonpathogenic mycobacterium, was grown with S^{35}-sulfate and L-histidine-*2*-C^{14} in the manner described for *M. tuberculosis* 5091, and the harvests were similarly analyzed. Although grown for only 8 days, the organisms yielded quantities of labeled ergothioneine and hercynine comparable with those from *M. tuberculosis;* the curves constructed from the data were similar to those shown in Fig. 2.

DISCUSSION

The pathway of ergothioneine biosynthesis in fungi has been fairly extensively studied in both *N. crassa* and *C. purpurea* (Melville, 1959; Stowell, 1961). In these fungi, hercynine (histidine betaine) is first formed from histidine by methylation of the α-amino carbon (Melville et al., 1959; Askari and Melville, 1962); in turn, hercynine is converted to ergothioneine by the addition of sulfur derived most probably from cysteine (Melville et al., 1957). The final step, originally suggested by the latter authors, was described by Ban (1958) as occurring through formation of the thio ether, S-alanyl-ergothioneine, which then splits to form ergothioneine and alanine or serine. Thiolhistidine has been excluded as a precursor (Melville et al., 1957). Parts of the pathway as revealed in the present study with mycobacteria are similar to those described for the fungi. Thus, S^{35}-sulfate is incorporated into ergothioneine as expected, and the intact 2-C^{14}-labeled imidazole ring of histidine appears in both hercynine and ergothioneine. In addition, unpublished experiments have shown that essentially only hercynine synthesis occurs in sulfur-starved cells; upon addition of sulfur as amino acid or as sulfate, ergothioneine is quickly synthesized with concomitant reduction of hercynine levels in the mycobacterium.

Since essentially all mycobacteria appear able to produce ergothioneine, an explanation is necessary to account for the failure reported earlier (Melville et al., 1956) of *M. smegmatis* ATCC 607 to form ergothioneine. The much larger harvest of organisms obtained in the current work probably accounts for the yield of 12 mg per 100 g (Table 2) of ergothioneine observed, for experience has shown that an organism may produce little or no ergothioneine under conditions where growth is slow and the dry weight yield is low, but may elaborate the compound under conditions permitting better growth. In other words, rapid and voluminous growth seems to favor ergothioneine accumulation. In earlier work, the ergothioneine content of *M. tuberculosis* H37Rv was attributed to uptake of the compound from the bovine albumin present in the Dubos medium; present experiments leave no doubt about the synthetic ability of this strain.

In view of the capacity of *M. tuberculosis* to synthesize ergothioneine in vitro, the report of Fraser (1951), that high blood ergothioneine levels occur in tuberculous Canadian Indians and Eskimos (though not in most white patients), assumes considerable interest. We have initiated a study to determine whether tubercle bacilli do, in fact, produce a sufficient amount of the compound during infection to elevate the blood level in the host. So far, tests made upon a small group of Negro and Puerto Rican patients with early pulmonary tuberculosis have failed to show any increase in blood ergothioneine concentration over the normal. It is, of course, possible that these negative findings may reflect the effectiveness of early chemotherapy. Further studies are

planned upon patients with more advanced disease, who either have not been treated or have suffered progression of the disease despite treatment.

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