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THE ENZYMATIC TRANSFORMATION OF CHOLIC ACID BY THE CULTURE *MYCOBACTERIUM MUCOSUM* 1210

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Abstract—In the enzymatic transformation of cholic acid by the culture *Mycobacterium mucosum* 1210, 7α,12α-dihydroxy-3-oxo-Δ^4-cholenic and 7α,12α-dihydroxy-3-oxo-Δ^4-bisnorcholenic acids have been isolated and identified (in the form of methyl esters). Thus a microbiological α-oxidation of the side chain of cholic acid has been demonstrated.

EARLIER[1] we reported the microbiological transformation of cholic acid (I) by the known *Mycobacterium* Sp. N 1210. In addition to a number of compounds (IIIb, IVb, Vb, VIb, VIIb, VIIIb and IXb) isolated from the methylated ether extract of the cultural medium, two unidentified compounds, m.p. 193–195° (compound F) and m.p. 204–215° (compound E) were isolated.

In the present paper we describe the identification of the compounds F and E, microbiological α-oxidation of the side chain of cholic acid, in addition to the enzymatic transformation of cholic acid by the culture *Mycobacterium mucosum* 1210.

RESULTS AND DISCUSSION

Based on morphological, cultural and physiological indications, the culture 1210 isolated from the soil was assigned to the species *Mycobacterium mucosum*, Krassilnikov 1941. After fermentation and subsequent treatment (see previous communication[1]) the compound F, m.p. 194–196° and the compound E, m.p. 217–219° as major components (in the form of methyl esters) were isolated from the ethyl acetate extract of the culture medium. They were found to be methyl 7α,12α-dihydroxy-3-oxo-Δ^4-cholenate (IIb) and methyl 7α,12α-dihydroxy-3-oxo-Δ^4-bisnorcholenate (Xb), respectively.

The IR and UV spectra of IIb reveals the presence of the Δ^4-3-oxo-grouping, the hydroxy group, the ester bond and the absence of unconjugated oxo-group in 6-membered ring. The mass spectrum of the ester IIb contains the peak of the molecular ion (*m/e* 418), the abundant peak at *m/e* 124 (ion a) which is characteristic of Δ^4-3-oxo-grouping, as well as the peaks at *m/e* 400 and 382 due to the loss of one and two molecules of water from M^+.

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The further fragmentation of the ions $M^+—H_2O$ and $M^+—2H_2O$ is accompanied by elimination of the side chain and results in the formation of the *m/e* 285, 267 (ion b) and 115 ($CH_3\overset{+}{C}HCH_2CH_2COOCH_3$) ions, respectively. The peak of the ion b is a most abundant in the spectrum. We have shown this[1] to be characteristic of methyl 12α-hydroxy-3-oxo-$\Delta^{4,6}$-choladienate.

UV spectrum of Xb reveals the presence of the Δ^4-3-oxo-grouping and the IR spectrum contains absorption bands characteristic of hydroxy and ester groups and the Δ^4-3-oxo-grouping.

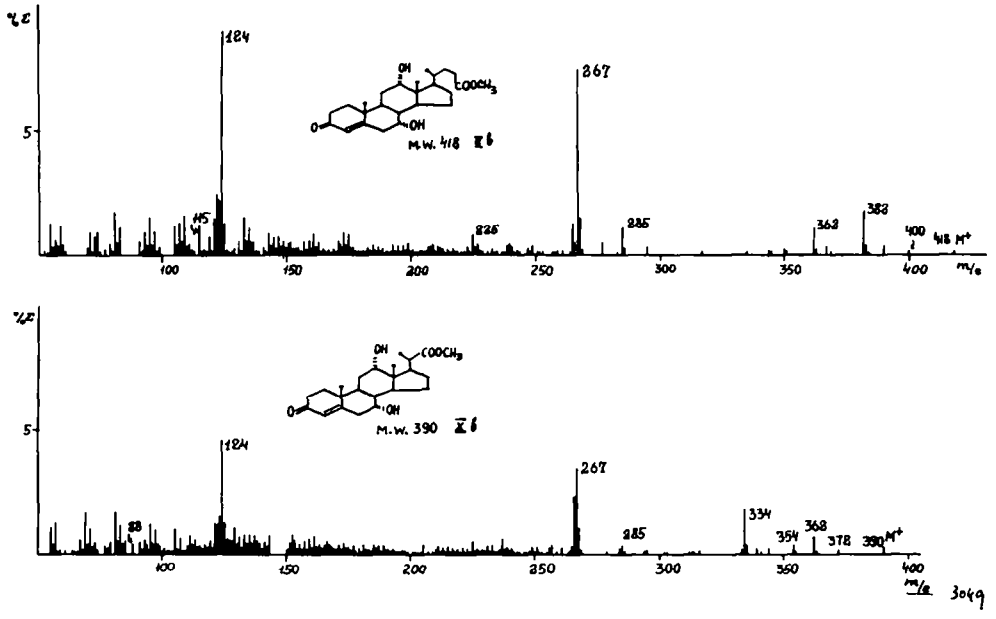
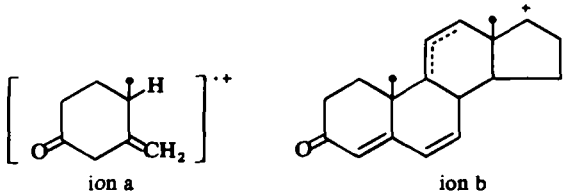


FIG. 1 Mass spectra of: (a) methyl 7α,12α-dihydroxy-3-oxo-Δ^4-cholenate (IIb); (b) methyl 7α,12α-dihydroxy-3-oxo-Δ^4-bisnorcholenate (Xb).

A comparison of the mass spectra of IIb and Xb (Fig. 1a, b) shows that these esters have similar fragmentation patterns under electron impact. In the case of Xb the molecular peak (*m/e* 390) and the peaks of the fragments containing the side chain (M-H_2O, *m/e* 372 and M-2H_2O, *m/e* 354) are shifted by 28 mass units to the low mass region, whereas the peaks deprived of the side chain (ion a, *m/e* 124 and ion b, *m/e* 267) have the same *m/e* values in the spectra of IIb and Xb. This suggests that IIb



ion a ion b

and Xb have similar structures and differ only in the size of the side chain. In contrast to IIb, the loss of the side chain from the ions M-H_2O and M-$2H_2O$ proceeds in two directions in the case of Xb. One consists in the simple cleavage of the 17-2O bond giving rise to the ions with *m/e* 285 and 267, respectively. On the other hand, the same process accompanied by the migration of an H-atom to the neutral fragment leads to the *m/e* 284 and 266 ions. The peaks at *m/e* 284 and 266 are practically absent in the spectra of the derivatives of 12α-hydroxy-Δ^4-3-oxocholenic acid.[1] Finally, the cleavage of the 17–20 bond accompanied by the localization of the charge on the side chain fragment and the migration of H-atom to the charged fragment leads to the *m/e* 88 $(CH_3CH_2COOCH_3)^+$ ion[2] which also characterizes the size of the side chain.

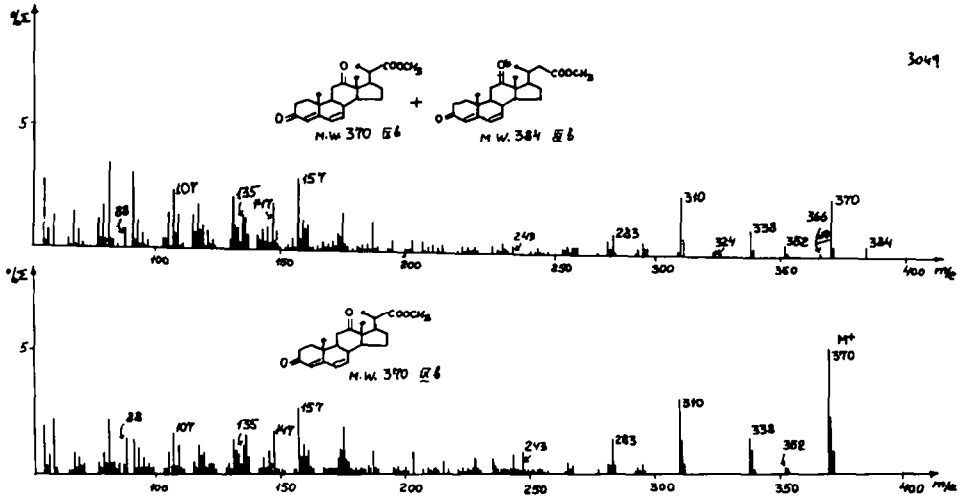
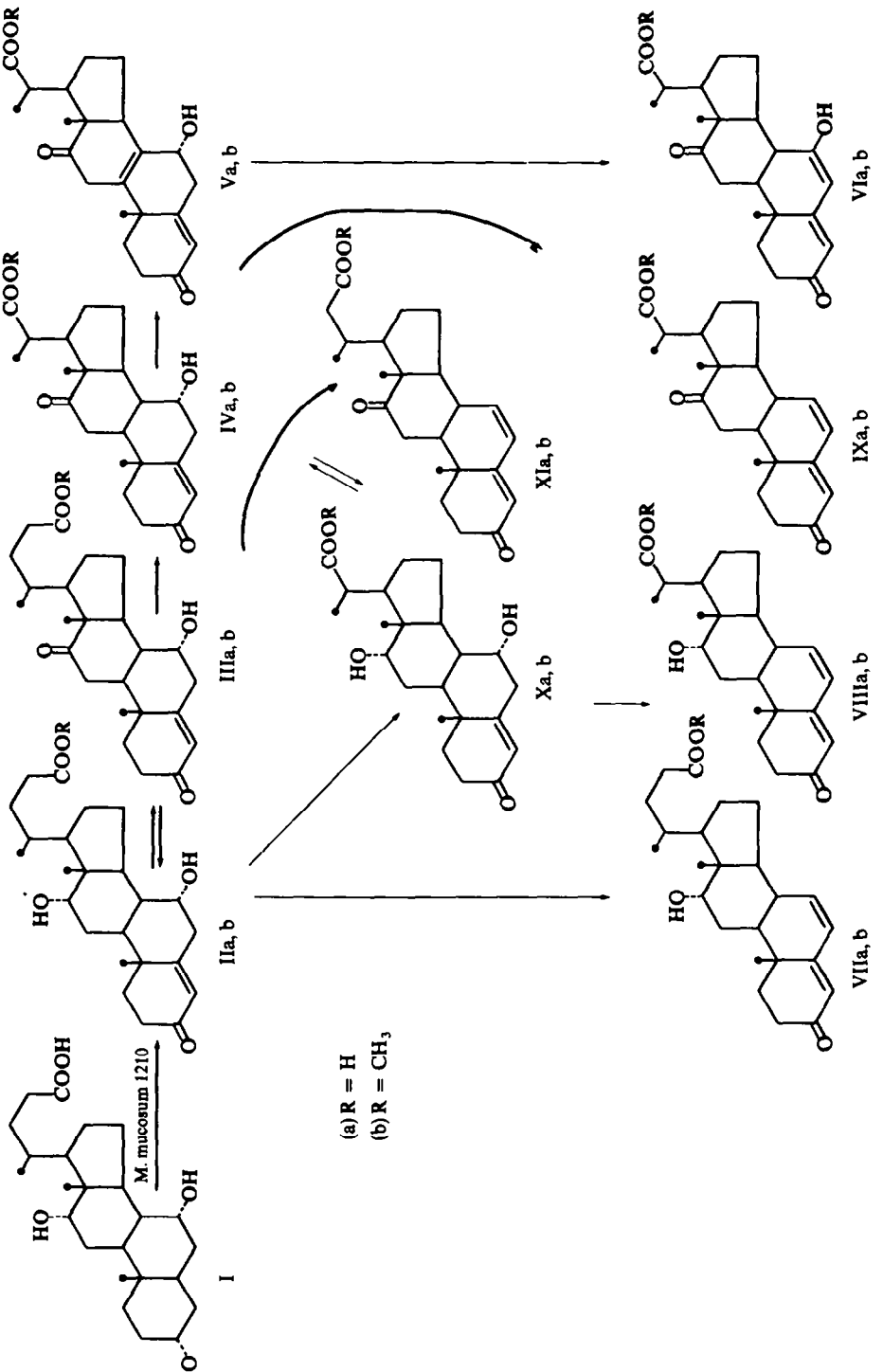


FIG. 2 Mass spectra of: (a) the of methyl 3,12-dioxo-$\Delta^{4,6}$-bisnor-choladienate (IXb) and methyl 3,12-dioxo-$\Delta^{4,6}$-norcholadienate (XIb); (b) methyl 3,12-dioxo-$\Delta^{4,6}$-bisnorcholadienate (IXb). 12-Ketogroups are missing in the formulas.

Methyl 7-hydroxy-3,12-dioxo-$\Delta^{4,6}$-bisnorcholadienate (VIb) and the substance which turned out to be the mixture of known methyl 3,12-dioxo-$\Delta^{4,6}$-bisnorcholadienate (IXb, mol. wt 370) and a methyl ester with the mol. wt of 384 were isolated from the $CHCl_3$-extract of the culture medium. The mass spectrum of this mixture (Fig. 2a) contains, besides the peaks characteristic of the ester IXb,[1] the peaks at *m/e* 384, 366 (384-H_2O) and 324 which are shifted by 14 mass units in the high mass region when compared with the corresponding peaks in the spectrum of the pure ester IXb (370, M^+; 352, M-H_2O; 310). The UV spectrum of the substance (λ_{max} 280 mμ) is characteristic of the $\Delta^{4,6}$-3-oxo-grouping. The IR spectrum reveals the bands of unconjugated ketone, ester bond and $\Delta^{4,6}$-3-oxo-grouping whereas the band of hydroxy group is absent. This means that the ester with the mol wt of 384 has the structure of methyl 3,12-dioxo-$\Delta^{4,6}$-norcholadienate (XIb). Consequently, together with β-oxidation, α-oxidation also takes place. Hitherto only β-oxidation of the side chain of bile acids has been reported.[3–5]

Scheme of cholic acid transformation by the culture *Mycobacterium mucosum* 1210



In the previous paper[1] we proposed a scheme for the enzymatic transformation of cholic acid via the acid IIa although it had not been isolated. Now this suggestion has been confirmed. Naturally, it would be of interest to establish the consequence of the enzymic oxidation of cholic acid. For this purpose the microbiological transformation of the acids IIa and IIIa (obtained by saponification of the esters IIb and IIIb respectively) by the culture *Mycobacterium mucosum* 1210 has been studied under the conditions used for the transformation of cholic acid itself.[1] The acid IIa was converted to the acids IIIa, IVa and Va in complete agreement with the suggested scheme. The microbiological transformation of the acid IIIa resulted in the formation of the acids IIa, IVa and Xa. The presence of the acid IIa supports the reversible process IIa $\rightleftharpoons$ IIIa which is in agreement with previous data.[6] Since saponification of the ester Xb yielded only the dehydration product, ester VIIIb, we investigated the transformation of the ester Xb. The expected ester IVb and Vb were found in the culture medium. The fermentation of the ester IVb resulted in the esters Vb and Xb. It is quite possible that the compounds with the $\Delta^{4,6}$-3-oxo-grouping (the acids VIa, VIIa, VIIIa and IXa) are artefacts since dehydration of Δ^4-7α-hydroxy-3-oxo-steroid acids (or their esters) proceeds readily and can take place even during treatment of the reaction mixture. Indeed, the Δ^4-7α-hydroxy-3-oxo-compounds are dehydrated on heating in the acid or alkaline medium for 5 min and dehydration in the acid medium proceeds even at room temperature. These compounds are practically absent after fermentation of the esters, in particular, of the esters IVb and Xb (when isolation of products is from a neutral medium). In contrast to other 7α-hydroxy-3-oxo-compounds, the ester Vb is not dehydrated; in all attempts only the isomeric ester VIb was obtained.

One could therefore suppose that the low intensity of the peak M-H_2O in the mass spectrum of the ester Vb[1] is also due to a partial isomerization of the latter under mass spectrometric conditions. In this connection one should note the absence of 7-oxo-compounds among fermentation products. We also failed to obtain them by chemical oxidation. For example, the ester IVb was prepared by oxidation of the ester Xb with sodium bichromate in acetic acid, but we could not oxidize the 7α-hydroxy group in the ester IVb.

Based on the data obtained we put forward the following scheme of the conversion of cholic acid by the culture *Mycobacterium mucosum* 1210.

EXPERIMENTAL

M.ps were determined on a Koffler block. UV spectra were taken in alcohol solns on spectrophotometer SF-4; IR-spectra in paste with vaseline oil on spectrophotometer Hilger H-800.

Mass spectra were taken on commercial instruments MX-1303 and MX-1306 provided with the glass system for direct inlet of the sample into the ion source close to ionization chamber[7] and stabilization of the inlet temp ($\pm 1°$) at ionizing voltages 24–30 V (MX-1303) and 50 V (MX-1306) and temperatures 180° (IIb, Xb) and 170° (the mixture of the esters IXb and XIb).

Alumina of III and IV degree of activity according to Brockman was used for chromatography. The following systems were used for separation of the methyl esters on the paper (chromatographic paper c): A: hexane–benzene–methanol–water 6:6:2:1; B: hexane–benzene–methanol–water 20:2:2:1.

The fermentation process has been described. After ethereal extraction, the culture medium was twice extracted with a double volume of EtOAc and twice with a double volume of $CHCl_3$. The EtOAc extract

was evaporated to a volume of 50 ml and the residue obtained was methylated with an etheral soln of diazomethane. The mixture of methyl esters was separated on a thin layer of alumina in the systems EtOAc–ether (4:3, 5:2) and pure EtOAc. The following compounds were isolated as the main components of the mixture.

Methyl 7α,12α-dihydroxy-3-oxo-Δ⁴-bisnorcholenate (Xb). A compound (97 mg), m.p. 210–215° was isolated (R_f 0·20 in the system A). After two crystallizations from EtOAc 56 mg of Xb was obtained, m.p. 217–219°, λ_{max} 242 mμ, IR spectrum: 3450, 1739, 1670 and 1631 cm^{-1}.

Methyl 7α,12α-dihydroxy-3-oxo-Δ⁴-cholenate (IIb). A compound (33 mg), m.p. 187–192° was isolated (R_f 0·34 in the system A). Double crystallization from EtOAc yielded 16 mg of IIb, m.p. 194–196°; λ_{max} 242 mμ; IR spectrum: 3400, 1740, 1670 and 1630 cm^{-1}.

In addition, 25 mg of a substance, m.p. 275–280° and 17 mg of another, m.p. 248–253° was isolated. After two crystallizations of the first substance from EtOAc–EtOH, 14 mg of crystalline Vb was obtained. After two crystallizations of the second substance from EtOAc and EtOAc–EtOH 7 mg of a compound, m.p. 256–258° identical with IVb was obtained.

The chloroform extract was evaporated to dryness *in vacuo* and the residual dark-brown oil (158 mg) methylated and 50 mg of the methyl esters separated on a thin layer of alumina in the systems EtOAc–ether (2:1, 1:6) and pure ether. A compound (17 mg), m.p. 230–233° was isolated which after two crystallizations from EtOAc yielded 8 mg of a compound, m.p. 239–241° identical with VIb. In addition, 6 mg of a compound, m.p. 185–195° was isolated which after two crystallizations from EtOAc–ether yielded 2·5 mg of a compound, m.p. 210–215°; λ_{max} 280 mμ; IR spectrum: 1720, 1700, 1655, 1612 and 1580 cm^{-1}.

Saponification of the ester IIIb. 2% K_2CO_3 aq (0·6 ml) was added to a soln of IIIb (8 mg) in 1·6 ml EtOH and the mixture was carefully heated for 30 min. After evaporation of EtOH *in vacuo* and acidification with 2N HCl, the colourless ppt was extracted with EtOAc. After usual treatment, crystallization from EtOAc–ether and two fold crystallization from EtOAc, IIIa, m.p. 222–225° was isolated.

Saponification of the ester IIb. Saponification of IIb was carried out under the conditions used for IIIb and acid IIa, m.p. 229–232° was isolated.

Attempted saponification of the ester Xb. The ester Xb was saponified under conditions used for IIb and IIIb, but a compound, m.p. 156–158° identical with VIIIb was isolated instead of the expected acid.

Oxidation of the ester Xb. A 2·7% soln of $Na_2Cr_2O_7$ in acetic acid (0·1 ml) was added to a soln of Xb (5 mg) in 0·1 ml acetic acid and the mixture was kept at 0° for 7 min. The soln was diluted with a 10-fold volume of water and thoroughly extracted with EtOAc. The extract was washed with water, dried and evaporated to dryness *in vacuo*. The residue was separated on a thin layer of alumina in the system EtOAc–ether 5:2. The ester IVb (m.p. 247–250°) and an insignificant amount of the initial ester was isolated. Treatment of IVb with excess oxidant for 40 min did not yield the expected trioxoester and only the initial IVb was recovered.

Dehydration of the esters IIb *and* IVb *and attempted dehydration of the ester* Vb. One drop conc HCl was added to each of 3 test-tubes containing 0·5 mg of esters IIb, IVb and Vb respectively in 0·2 ml MeOH. The mixtures were boiled for 5–7 min and the compounds obtained separated by paper chromatography in the systems A and B. In the case of the esters IIb and IVb complete dehydration yielded the esters VIIb and IXb respectively. The ester Vb was completely converted to the ester VIb. One drop conc HCl was added to each of 3 test-tubes containing on 0·5 mg of esters IIb, IVb and Vb respectively in 0·2 ml MeOH. The mixtures were kept for ½ hr at room temp. Paper chromatography using systems A and B established that an insignificant part of IIb and IVb was converted to VIIb and IXb respectively, and a portion of Vb was isomerized to VIb.

One drop of 0·1N KOH was added to each of three test-tubes containing on 0·5 mg of IIb, IVb and Vb respectively in 0·2 ml MeOH. The mixtures were boiled for 5–7 min and the products chromatographed on the paper in the systems A and B. Significant portions of IIb, IVb and Vb were converted to VIIb, IXb and VIb, respectively.

Enzymatic transformation of the acid IIIa. 4 Test-tubes each of which contained 2 mg of IIIa in 10 ml of the medium [$(NH_4)_2SO_4$–2·0 g, K_2HPO_4–1·0 g, $MgSO_4 \cdot 7H_2O$–0·5 g, K_2CO_3–1·0 g, $FeCl_3 \cdot 6H_2O$–0·01 g, distilled water 1000 ml, pH 7] were sterilized and inoculated by the culture *Mycobacterium mucosum* 1210 previously grown on agarized medium. The test-tubes were incubated under shaking (160 min^{-1}) at 27°. In 1·5, 3, 5, 10, 24, 30, 40 and 50 hr samples containing 5 ml of the cultural medium were acidified with 2N HCl to pH 2, thoroughly extracted with EtOAc and evaporated to dryness *in vacuo*. The mixtures were methylated and chromatographed. The paper chromatograms in the systems A and B showed that the transformation of the acid IIIa by the culture *Mycobacterium mucosum* 1210 begins 1·5 hr after innoculation and in

40–50 hr the compounds absorbing in UV are practically absent from the cultural medium. On chromatograms intense spots were found on the level of the esters IIb, IVb, Xb and on the level of the initial ester IIIb, as well as less intense spots on the level of the esters Vb and IXb.

Transformation of the acid IIa. The enzymatic transformation of IIa was carried out under similar conditions. On the paper chromatograms of the methyl esters in the systems A and B, intense spots were found on the level of the esters IIIb, IVb, Xb and IIb, as well as the less intense spots on the level of the esters Vb, VIIb and IXb.

Transformation of the ester Xb. The microbiological conversion was carried using the same conditions for the acids IIa and IIIa. A sample containing 5 ml of the cultural medium was thoroughly extracted with EtOAc and evaporated *in vacuo*. The mixture obtained was chromatographed in the systems A and B. The spots on the level of the esters IVb, Vb and the initial ester Xb were found on the chromatograms.

Transformation of the ester IVb. Similar conditions were used in the enzymatic conversion and treatment of the samples. After chromatography the spots on the level of the esters Vb, Xb and the initial ester were found.

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