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Bioconversion of Tyrosine into the Propylhygric Acid Moiety of Lincomycin*

D. F. Witz, E. J. Hessler, and T. L. Miller

ABSTRACT: *Streptomyces lincolnensis* has been shown to accumulate propyl- and ethylproline in media that are sulfur limited. These two compounds are proposed as intermediates to the propylhygric and ethylhygric acid moieties of lincomycin (Ia) and 4′-depropyl-4′-ethyllincomycin (Ib), respectively.

L-Tyrosine or L-dihydroxyphenylalanine addition to cultures stimulates propylproline and ethylproline production. This observation led to the hypothesis that the amino acid portions of Ia and Ib are derived from intermediates in the pathway from tyrosine to melanin. Preliminary experiments demonstrated that L-[1-^{14}C]tyrosine and L-[^{15}N]-tyrosine are efficiently incorporated into the propylhygric acid moiety of Ia and the ethylhygric acid moiety of Ib. From the comparison of the incorporations of L-[U-^{14}C]-tyrosine and L-[1-^{14}C]tyrosine into Ia, we conclude that seven carbon atoms of tyrosine are incorporated into the propylhygric acid moiety of Ia. Therefore tyrosine must undergo a cyclization (to form a ring containing nitrogen) and partial degradation (to lose two carbon atoms) to yield ultimately the propylhygric acid moiety of Ia. These results are completely consistent with our hypothesis involving the melanin pathway and furthermore provide evidence for a novel conversion of tyrosine into new amino acids.

Previous knowledge of the structure of lincomycin has been published (Schroeder *et al.*, 1967; Magerlein *et al.*, 1967), and the biosynthesis of lincomycin has been reviewed (Eble, 1967). The experiments of Eble and coworkers with radioactive isotopes indicated that several logical precursors of the propylhygric acid (IIa) moiety of lincomycin (Ia), *e.g.*, L-[U-^{14}C]proline, hydroxy-L-[2-^{14}C]proline, L-[U-^{14}C]-glutamic acid, and 5-amino[4-^{14}C]levulinic acid were not incorporated to any great extent (see Chart I). On the other hand, their studies with radioactive pyruvate indicated that the carboxyl carbon and carbons-2 and -3 of the proline ring were probably derived from this intermediate. Further studies (Argoudelis *et al.*, 1969) showed that both the CCH_3 and NCH_3 group of the IIa moiety were derived from methionine. However, the methylene carbons of the propyl side chain were not derived from methionine. Their methylation studies with the ethylhygric acid (IIb) moiety of 4′-depropyl-4′-ethyllincomycin (Ib) likewise showed that only the CCH_3 and NCH_3 groups were derived from methionine. These results indicated that Ib was not a precursor of Ia, but that both the IIa and IIb moieties were derived in the same manner except from precursors differing by one carbon atom. In the present study we have investigated the role of L-tyrosine as a precursor to the amino acid moieties of Ia and Ib.

Experimental Section

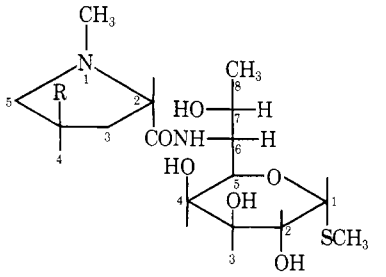
Culture. Streptomyces lincolnensis

Fermentation Procedures. Seed cultures were grown in a medium consisting of dextrose monohydrate (20 g/l.), NZ amine B (Sheffield Chemical) (5 g/l.), and Yeasttolac (A. E. Staley Manufacturing Co.) (10 g/l.). Incubation was at 28° for 48 hr on a rotary shaker (250 rpm). Complex fermentation medium consisting essentially of starch, Pharmamedia (Traders Oil Mill Co.), and inorganic salts was employed for some preparations.

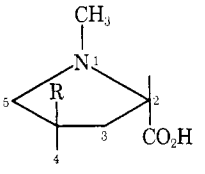
Chemically defined medium (CDM) (glucose, 30 g/l.; trisodium citrate, 3 g/l.; $ZnSO_4 \cdot H_2O$, 1 mg/l.; $FeSO_4 \cdot 7H_2O$, 1 mg/l.; $MgSO_4 \cdot 7H_2O$, 1 g/l.; NaCl, 0.5 g/l.; NH_4NO_3,

* From the Research Laboratories, The Upjohn Company, Kalamazoo, Michigan. *Received October 5, 1970.*

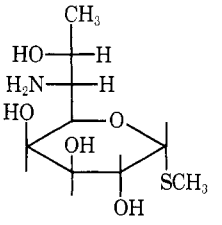
CHART I



Ia, R = $CH_2CH_2CH_3$
b, R = CH_2CH_3



IIa, R = $CH_2CH_2CH_3$
b, R = CH_2CH_3



III

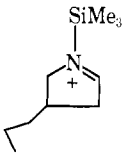
2 g/l.; and K_2HPO_4, 2.5 g/l.) was used in the preparation of washed cells. This medium was inoculated (2% v/v) with washed (three times with sterile water) seed culture and was incubated at 28° on a rotary shaker at 250 rpm (2-in. stroke). Cells from 1 l. of CDM were routinely harvested at 4 days by centrifugation and were washed three times with 200 ml of 0.05 M potassium phosphate buffer (pH 7.0).

Washed Cell Reaction Mixtures. The incubation medium consisted of glucose (18 g/l.), $MgCl_2 \cdot 6H_2O$ (1.02 g/l.), and K_2SO_4 (1.74 g/l.) in 0.05 M potassium phosphate buffer (pH 7.0). The reaction mixtures were incubated at 28° at 250 rpm in 500-ml erlenmeyer flasks (wide mouth) containing either 10 or 20 ml of medium and 1 g of wet packed cells (0.1–0.15 g dry weight) per 10 ml of medium. Generally, 50–100 μg of lincomycin/ml was produced in a 20–24-hr incubation period.

Separation, Detection, and Identification of Propyl- and Ethylproline. Propyl- and ethylproline were isolated from washed cell reaction mixtures by extracting 10 ml of the supernatant solution with 25 ml of butanol and 15 ml of 50% (w/w) potassium carbonate solution. Propyl- and ethylproline were separated by thin-layer chromatography: silica gel GF, 250 μ, 2.5 × 10 cm; 1-butanol–acetic acid–water (80:20:20, v/v). The prolines were detected with a ninhydrin spray consisting of 1-butanol–acetic acid–85% phosphoric acid–ninhydrin (47.5 ml:1.5 ml:1.0 ml:0.15 g); a characteristic pink color was formed after gentle warming of the thin-layer chromatographic plate. This system can detect levels of propylproline as low as 0.1 μg/spot.

For purposes of identification of the propylproline a large-scale butanol extraction of complex fermentation medium was made. The butanol extract was evaporated to dryness, the residue was dissolved in 95% ethanol and this solution was applied to two 20 × 40 cm × 1.5 mm silica gel GF plates. The same solvent system was employed as described above. The two bands corresponding to propyl- and ethylproline were partially separated by this procedure. Both preparations as well as an authentic sample of propylproline were subjected to gas–liquid phase chromatography–mass spectrometry. The propyl- and ethylproline

CHART II



184
$M^+ = 301$

preparations were silylated with *N,O*-bis(trimethylsilyl)-acetamide and chromatographed on a column of 3% OV-1, 6 ft × 3 mm, 30 cc/min of nitrogen, 100–120 mesh of gas Chrom Q at 115°. Glc–mass spectral analysis with the above column and conditions showed that the component corresponding to propylproline had mass spectrum identical with that of authentic propylproline. Signals at *m/e* 301 (M^+) and 184 were present for the propylproline $(Me_4Si)_2$ derivative as shown in Chart II.

The component corresponding to ethylproline showed the corresponding signals at *m/e* 287 (M^+) and 170; however, a signal at *m/e* 184 indicated contamination by propylproline.

Labeling Experiments. COUNTING PROCEDURES. The scintillation solvent was composed of toluene–dioxane–methanol–Liquefluor–naphthalene (300 ml:250 ml:210 ml:50 ml:73 g). The sample was dissolved in 0.5 ml of water and the scintillation solvent (15 ml) added for counting. In general, four samples were counted to a minimum of 10,000 counts. The conversion of counts per minute into disintegrations per minute was made by the internal standard method with [^{14}C]toluene. Counting was performed on a Packard liquid scintillation spectrometer, Model 2211.

(1) CONVERSION OF L-[1-^{14}C]TYROSINE INTO LINCOMYCIN. Fermentation. A culture in complex medium (10 ml) was incubated at 28° in a 125-ml erlenmeyer flask on a rotary shaker (250 rpm). The L-[1-^{14}C]tyrosine (New England Nuclear Corp.) was added to the medium at 3 days (25 μCi/20 mg of tyrosine).

Isolation. After 10 days the culture was centrifuged and the clear aqueous phase decanted. The mycelia were shaken with two 7-ml portions of water and centrifuged. The total aqueous phase (20 ml) was adjusted to pH 2, and extracted with 10 ml of chloroform to remove lipids. To the aqueous phase was added 2.2 g of potassium carbonate and the solution was extracted with four 25-ml portions of methylene chloride. The methylene chloride extract was dried over magnesium sulfate, filtered, and concentrated to dryness to give 27 mg of an oil. A thin-layer chromatography of this material was run in methyl ethyl ketone–acetone–water (75:25:10, v/v) which is known to separate Ib from Ia. This plate was scanned with a Vanguard thin-layer chromatography plate scanner (Model 885) and gave a shoulder for Ib and a peak for Ia.

The material (25.2 mg) was then diluted with Ia (4.4847 g containing less than 0.5% Ib) and the mixture dissolved in 4.5 ml of water and 25 μl of 6 N HCl. This was crystallized by addition of 50 ml of acetone. The solid was recrystallized four times in the same manner to yield 2.83 g of material. The incorporation of L-[1-^{14}C]tyrosine was 14.1%.

(2) POSITION OF LABEL IN IA FROM L-[1-^{14}C]TYROSINE. The procedure used for the conversion of Ia into IIa has been described (Argoudelis *et al.*, 1969) (Scheme I).

Compound Ia from the above experiment (933 mg, 742 dpm/μmole) was converted into the free base; this was

SCHEME I

$$\text{Ia} \xrightarrow[2.\ H^+]{1.\ NH_2NH_2} \text{IIa} \xrightarrow{Pb(OAc)_4} CO_2$$

dissolved in 8.1 ml of hydrazine hydrate and refluxed for 21 hr.

The mixture was concentrated to dryness and the residue was leached with acetonitrile. The acetonitrile-soluble portion (416 mg) was dissolved in 3 ml of 6 N HCl and refluxed to hydrolyze the hydrazide. The residue after concentration was crystallized from acetonitrile to yield 96.3 mg of IIa hydrochloride. This (91 mg) was dissolved in 20 ml of benzene and 462 mg of lead tetraacetate. The system was quickly sealed and a stream of N_2 bubbled through the reaction mixture and into a saturated, filtered barium hydroxide solution. The benzene mixture was heated to *ca.* 60° for 40 min. The precipitated barium carbonate was collected by filtration to yield 42 mg (48%).

Four samples of this were carefully weighed and added to a flask with a center well containing 2:1 methyl Cellosolve–ethanolamine. The flasks were capped with a septum and 5.0 ml of concentrated perchloric acid added to each. These were swirled overnight at room temperature. The ethanolamine–CO_2 solutions were each counted by liquid scintillation to give an average of 718 dpm/μmole of barium carbonate. The specific activity of the original lincomycin was 742 dpm/μmole. It was concluded that 96% of the radioactivity was in the carboxyl carbon of the IIa moiety of Ia.

(3) INCORPORATION OF ^{15}N FROM TYROSINE INTO LINCOMYCIN. Fermentation. A culture in complex medium (10 ml) was incubated at 28° in a 125-ml erlenmeyer flask on a rotary shaker (250 rpm). At 3 days 20 mg of L-[^{15}N]tyrosine (95% ^{15}N excess) (Schwarz BioResearch, Inc.) was added to the culture.

Isolation. After 10 days the 10-ml culture was adjusted to pH 2, 25 ml of carbon tetrachloride added, and centrifuged. The aqueous phase was treated with 13 g of potassium carbonate, then extracted with three 50-ml portions of methylene chloride. The methylene chloride was dried over magnesium sulfate, filtered, and concentrated to dryness to yield 22.3 mg. The lincomycin Ia to Ib ratio was determined to be 91.3:8.7 by gas–liquid chromatography. Mass spectral data are reported in the discussion. The per cent incorporation of [^{15}N]tyrosine can be calculated as follows: (1) determination of the number of millimmoles of Ia and Ib containing ^{15}N from the gas–liquid chromatography–mass spectral data; and (2) correcting for the 95% ^{15}N level in tyrosine. Thus the per cent incorporation is: Ia, 7.0%; Ib, 0.7%.

(4) COMPARISON OF L-[1-^{14}C]TYROSINE AND L-[U-^{14}C]TYROSINE INCORPORATIONS INTO LINCOMYCIN. Washed cell reaction mixture. Two flasks each containing 2 g of wet cells and 20 ml of reaction mixture were used; one (flask C-U) received L-[U-^{14}C]tyrosine and the other (flask C-1) L-[1-^{14}C]tyrosine (New England Nuclear Corp.). Both flasks received about 4 μCi/1.0 mg of L-tyrosine. The reaction mixtures were incubated for 20 hr as previously described.

Isolation. Approximately 1 mg of lincomycin was produced. To flask C-U was added 1.00740 g of pure Ia hydrochloride, and to flask C-1 was added 1.01272 g of pure Ia hydrochloride. The mixture was shaken at room temperature for 2.5 hr to equilibrate the lincomycin in the cells with the lincomycin in the solution. The lincomycin in each flask was then isolated by adding 33 g of potassium carbonate followed by extraction with four 50-ml portions of methylene chloride. The methylene chloride was dried over magnesium sulfate, filtered, and concentrated to dryness. The residues were separately crystallized and recrystallized four times from acetone–water as the hydrochloride salt to yield: C-U, 780 mg; C-1, 750 mg. These were each counted by liquid scintillation. The incorporation for C-1 was 14.48% and for C-U was 10.7%. The number of carbon atoms incorporated is $9 \times 10.7/14.48 = 6.65$ carbon atoms.

The error assignments of the counting results were obtained as number of counts/(number of counts)$^{1/2}$. To estimate the errors of the final answer the counting results were substituted as maximum and minimum values. Thus, 14.48% had a range of 15.1–13.9 and 10.7% had a range of 11.2–10.3% and the number of carbon atoms incorporated had a final range of 6.15–7.15 or 6.65 ± 0.5 carbon atoms.

(5) CONFIRMATION OF POSITION OF RADIOACTIVITY IN LINCOMYCIN FROM L-[U-^{14}C]TYROSINE. The Ia hydrochloride produced from L-[U-^{14}C]tyrosine (1 mmole, 452 dpm/μmole) was converted into the free base. This was refluxed with 6 ml of hydrazine hydrate for 21 hr. The mixture was concentrated to dryness under reduced pressure and the residue leached with five 10-ml portions of acetonitrile. The residual solid was crystallized from dimethylformamide to give 82 mg of crude methylthiolincosaminide (III). This was recrystallized three times. Its infrared spectrum was identical with authentic methylthiolincosaminide and a mixture melting point was undepressed. The material was counted to give 1.6 dpm of radioactivity/μmole. This corresponds to 0.4% of the radioactivity in lincomycin. Therefore, at least 99.6% of the radioactivity in the lincomycin sample is in the IIa moiety.

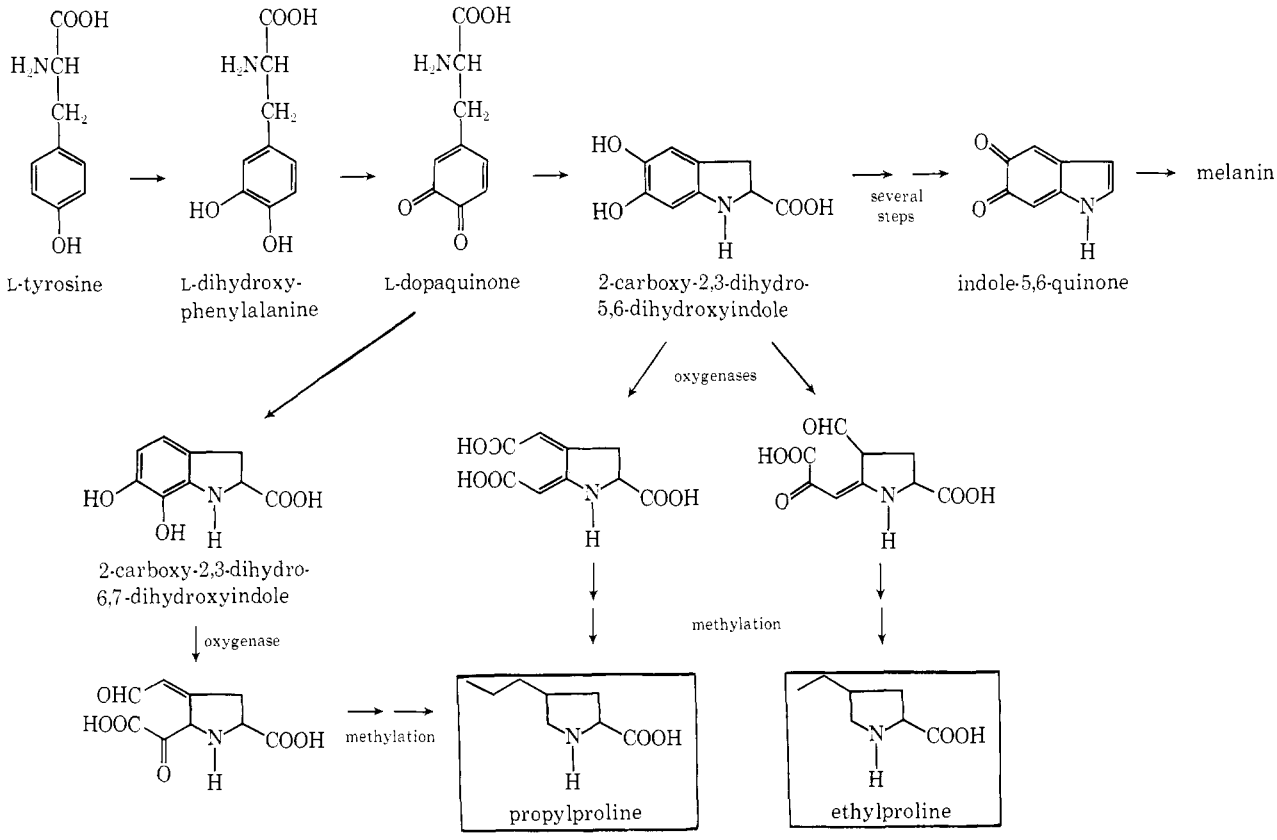
Discussion and Results

The structures of the two lincomycins (Ia and Ib), their respective amino acid moieties, and the sugar moiety, methylthiolincosaminide, are shown in Chart I. Since ^{14}C-labeled IIa was not incorporated into lincomycin (A. D. Argoudelis, 1968, personal communication), a study to determine if propylproline is the precursor of the amino acid moiety of lincomycin was initiated. The first approach was to determine if propylproline could be detected in washed cell reaction mixtures that were sulfur limited, *i.e.*, unable to synthesize the sugar moiety, methylthiolincosaminide (III), of lincomycin. It was readily shown that sulfur-deficient cells produced components that reacted with ninhydrin forming a pink complex somewhat similar to that formed with the amino acid, proline (Troll and Lindsay, 1955). Using a thin-layer chromatography system for separation and a ninhydrin spray for detection, a butanol extract of the washed cell system gave two components with the color characteristic of propylproline. One component had an R_F value identical to that of 4(*R*)-propyl-(*S*)-proline, while the other had a slightly smaller R_F value identical with that of authentic 4(*R*)-ethyl-(*S*)-proline.

The two components were separated by preparative thin-layer chromatography; one component was identified as propylproline by gas–liquid chromatography–mass spectrometry. The other component was tentatively assigned as ethylproline. These results provide circumstantial evidence that propylproline is a presursor of the IIa moiety and that ethylproline is a precursor of the IIb moiety.

It was known that *S. lincolnensis* produces a dark melanin-like pigment in media containing tyrosine. An inspection of the melanin pathway (shown in Scheme II) (Lerner, 1953) revealed that five-membered N-containing ring compounds

SCHEME II: Pathway of Melanin Formation Showing Several Hypothetical Divergenes to Propyl- and Ethylproline.



are intermediates. Therefore, the effects of L-tyrosine and L-dihydroxyphenylalanine on the production of propyl- and ethylproline were tested. Both compounds stimulated the production of propyl- and ethylproline with L-dihydroxyphenylalanine being more effective. Therefore, we hypothesized that propylproline and thus the IIa moiety of lincomycin are derived from intermediates on the pathway for converting L-tyrosine into melanin. Scheme II shows the melanin pathway along with the proposed reactions leading to propyl- and ethylproline.

According to our hypothesis L-[1-^{14}C]tyrosine should be converted into lincomycin containing the label in the carboxyl carbon. In addition, L-[^{15}N]tyrosine should be converted into lincomycin containing ^{15}N in the proline ring. Two experiments to test these possibilities were performed.

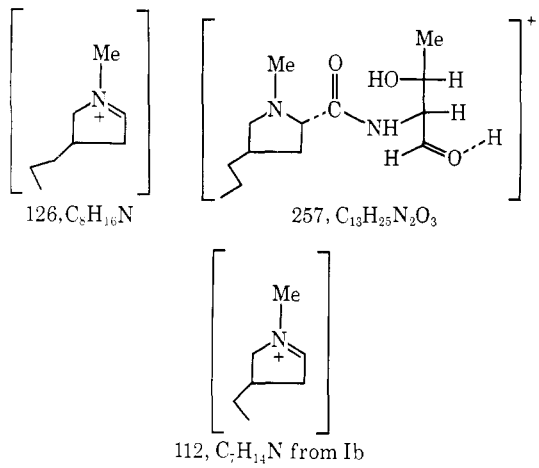
In a preliminary experiment L-[^{14}C]tyrosine was incorporated to the extent of 14.1% into Ia. A thin-layer chromatography of the product was analyzed with a thin-layer chromatography plate scanner; both Ia and Ib were radioactive. The label was shown to be solely in the carboxyl carbon by degradation of the lincomycin according to a known procedure (see Scheme I, Argoudelis *et al.*, 1969).

In a second experiment the L-[^{15}N]tyrosine incorporation into lincomycin was shown by mass spectrometry. The lincomycin was analyzed by high-resolution mass spectrometry on those fragments shown in Chart III. The molecular composition of these fragments has been verified by high-resolution mass spectrometry.

In lincomycin there is no detectable peak corresponding to ^{15}N at *m/e* 127. ($^{12}C_8H_{16}{}^{15}N$ occurs at 127.1253 *m/e*; this is cleanly separated from $^{12}C_7{}^{13}CH_{16}N$ occurring at 127.1316 *m/e* as shown in Figure 1.)

In lincomycin prepared from L-[^{15}N]tyrosine there is a peak at 127.1265 and it was 16.2% of the 126 peak in an average of 10 scans (range 15.4–17.4%). This corresponds to 16.2 atom % excess ^{15}N. In order to ensure that the ^{15}N of the IIa moiety did not arise from randomization, the amount of ^{15}N of the amino sugar moiety was determined by analyzing the *m/e* 257 peak. This fragment contains both nitrogens. We could not use the high-resolution scan since the intensity of the 257 peak is much less than the 126 peak. A medium resolution mass ratio determination was performed. The results are shown in Table I.

CHART III



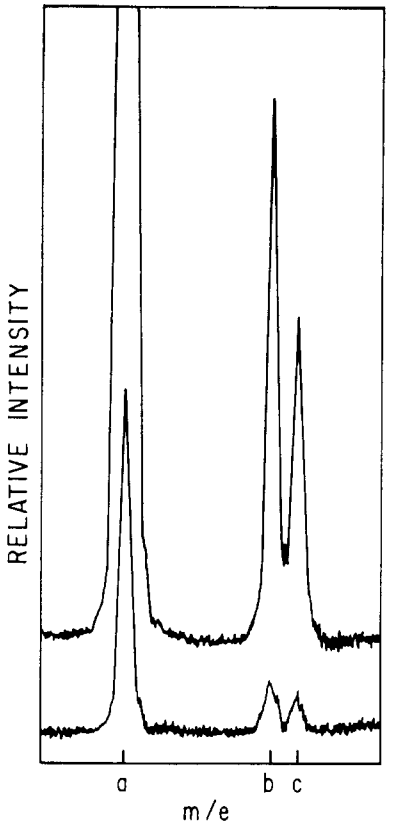


FIGURE 1: High-resolution mass spectrum of [^{15}N]lincomycin. The signals indicated are: (a) $C_8H_{16}N$, found *m/e* 126.1293 (theoretical 126.1283); (b) $C_8H_{16}{}^{15}N$, found *m/e* 127.1265 (theoretical 127.1253); (c) $C_7{}^{13}CH_{16}N$, found *m/e* 127.1334 (theoretical 127.1316).

The data show that approximately 0.3 atom % excess ^{15}N is present in the amino sugar and we concluded that randomization of the ^{15}N occurred only to a very small extent.

The amount of ^{15}N in Ib was estimated by gas–liquid chromatography–mass spectrometry. The ratios of 112:113 were determined to be 100:26 from Ib isolated from the [^{15}N]tyrosine experiment; unlabeled Ib gave a 112:113 ratio of 8% (due to the natural abundance of ^{13}C, ^{15}N, and ^{2}H), therefore approximately 18 atom % excess ^{15}N into the proline ring of Ib occurred.

This same procedure gave 17 atom % excess ^{15}N into the proline ring of Ia. Although this procedure is less accurate, it is still indicative of ^{15}N incorporation. These experiments prove that the [C-1] carbon and the nitrogen of tyrosine are efficiently incorporated into the IIa and IIb moiety of Ia and Ib.

TABLE I: Incorporation of ^{15}N into Lincomycin.

	Peak Height Ratio	Isotopic Correction[a]	^{15}N Atom % Excess
257:258	100:28.3	15.3	13.0
126:127	100:22.0	9.3	12.7

[a] Beynon and Williams (1963), p 19 for the 126:127 ratio and p 123 for the 257:258 ratio.

TABLE II: Incorporation of [^{14}C]Tyrosines into Lincomycin.

	dpm Added	dpm in Lincomycin	% Incorpn
L-[1-^{14}C]Tyrosine added	9,242,042	1,274,780	14.48
L-[U-^{14}C]Tyrosine added	8,802,967	989,347	10.70
Number of carbon atoms	$9 \times 10.70/14.48 = 6.65$ ($\pm$ 0.5)		

We reasoned that by comparing the label incorporations of L-[1-^{14}C]tyrosine and L-[U-^{14}C]tyrosine we could obtain the total number of carbon atoms of tyrosine incorporated into the IIa moiety. We felt that this experiment would prove whether or not a cyclized intermediate of tyrosine is required for the biosynthesis of the IIa moiety.

Two washed cell experiments were set up; one contained L-[1-^{14}C]tyrosine and the other contained L-[U-^{14}C]tyrosine. The lincomycin was isolated from each one by standard methods. Table II shows the results.

These data indicate that seven of the nine carbon atoms of tyrosine are incorporated into the IIa moiety of lincomycin. The other two carbon atoms of IIa are known to arise from methionine. The lincomycin from L-[U-^{14}C]tyrosine was shown to contain at least 99.6% of the radioactivity in the IIa moiety.

We conclude that tyrosine must undergo a cyclization to form a five-membered ring containing nitrogen followed by partial degradation (removing two carbons) to yield ultimately the IIa moiety. Scheme II shows several hypothetical pathways to propyl- and ethylproline from five-membered nitrogen-containing ring compounds that might originate from the melanin pathway. Two possibilities exist for cyclization of L-dopaquinone: the 2-carboxy-2,3-dihydro-5,6-dihydroxyindole and the 2-carboxy-2,3-dihydro-6,7-dihydroxyindole. However, direct cyclization of L-tyrosine followed by hydroxylation should not be overlooked. This could result in the 2-carboxy-2,3-dihydro-6,7-dihydroxyindole which then would be converted into propylproline. We have speculated that one or more oxygenases might function in these aromatic ring cleavages in reactions similar to those previously described (Hayaishi, 1966; Hayaishi and Nozaki, 1969).

Acknowledgments

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Specificity in the Association of Histones with Deoxyribonucleic Acid. Evidence from Derivative Thermal Denaturation Profiles*

Allen T. Ansevin and Barry W. Brown

ABSTRACT: Progressive stages in the heat denaturation of dehistonized rat thymus DNA reconstituted with each of the five major histone fractions from calf thymus were revealed as distinct peaks in plots of the temperature derivative of hyperchromicity *vs.* temperature. Nucleohistones containing F1, F2a1, F2a2, or F3 in each case increased their ultraviolet absorbance in three or more steps which are presumed to correspond to critical denaturation temperatures for free or weakly complexed DNA and two or more nucleohistone complexes having different binding energies. This is evidence for a more complicated interaction between histones and DNA than previously has been reported. Analysis of the derivative denaturation patterns for these nucleohistones, which were formed in 3.6 M urea solution at low ionic strength, suggests that complexes of the five histone fractions are distinctive with respect to (1) number of complexes formed by the fraction and the weight distribution among these complexes, (2) apparent binding strengths, and (3) complexing efficiency on a weight basis (F1 > F2b > F3 $\simeq$ F2a1 $\simeq$ F2a2). With the exception of the F1 complex, the nucleohistones approximated the stoichiometric relationship: (arginine + lysine) = DNA phosphate.

Reliability of the completely automated spectrophotometer system employed for these studies was demonstrated by determining the melting point and quantity of each component in a mixture of three bacterial DNAs.

Specific associations of histones with DNA have been examined from several aspects. These include chemical investigations of possible differences in the species or tissue distribution of major histone fractions (Stedman and Stedman, 1950; Hnilica, 1966; Hnilica *et al.*, 1966; Fambrough *et al.*, 1968), *in vitro* studies of the biological and biochemical effects of particular histones (Huang and Bonner, 1962; Allfrey *et al.*, 1963, 1965; Hnilica and Billen, 1964; Littau *et al.*, 1965; Liau *et al.*, 1965; Mirsky *et al.*, 1968), physical analysis of the elution of histones from chromatin (Giannoni and Peacocke, 1963; Ohba, 1966; Marushige and Bonner, 1966; Ohlenbusch *et al.*, 1967), and the interaction of histones with DNA (Akinrimisi *et al.*, 1965; Olins, 1969; Johns and Butler, 1964; Johns and Forrester, 1969; Huang *et al.*, 1964; Sponar *et al.*, 1967; Tuan and Bonner, 1969; Shih and Bonner, 1970). Surprisingly little species or tissues pecificity has been observed in the types and distribution of histones (Hnilica, 1967; Fambrough and Bonner, 1969). This has led to theories that other molecules such as RNA (Frenster, 1965a,b; Britten and Davidson, 1969; Bekhor *et al.*, 1969) or acidic proteins (Gilmour and Paul, 1969; Paul and Gilmour, 1968; Wang, 1968; Huang and Huang, 1969) may be primarily responsible for controlling the "readout" of genetic information. Presently, there is little indication that histones bind selectively to specific base sequences. However, the evidence presented here on multiple-stage binding patterns for most major histone fractions, and the results of other studies, indicate that histones show at least some degree of uniqueness in the manner of their attachment to DNA. These differences need to be included among the many factors influencing the unquestionably complex mechanisms for genetic control in advanced organisms.

The present experiments approach the question of whether different histones make specific contributions to nucleoprotein structure by examining the thermal stabilization of DNA complexed with purified preparations of each of the five major histones from calf thymus. A particularly revealing analysis is achieved in these studies by derivative thermal denaturation profiles which show transitions that normally would go undetected. Results from an independent but very similar investigation recently were reported by Shih and Bonner (1970). The present work differs from theirs in the variety of fractions employed, the method for preparing the DNA, and the way in which complexes were formed. All major histones of calf thymus are represented in the present investigation and the DNA has been isolated by a very gentle method. The nucleoprotein complexes were formed by the direct addition of histone to DNA in a medium of low ionic strength. The experiments to follow indicate a greater complexity in the structural organization of nucleohistones than has been demonstrated previously.

Materials and Methods

Equipment. Thermal transitions in DNA and nucleoproteins were observed at 260 nm with an automatic, temperature-programmed, digitally recording spectrophotometer. This

* From the Departments of Physics and Biomathematics, The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston, Houston, Texas 77025. *Received July 16, 1970.* This research was supported in part by U. S. Atomic Energy Commission Contract AT-(40-1)-2832 and Robert A. Welch Foundation Grant G-290. Computer processing of data was supported by U. S. Public Health Service Grant CA 11430 to the Department of Biomathematics of The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston.