harvested bacteria incubated to reduce the ATP level less extensively appears to be somewhat greater (as much as twofold) than that found in the "aged" bacteria used in these experiments.

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References

Amesz, J. (1963), *Biochim. Biophys. Acta 66*, 22.
Baltscheffsky, M. (1961), *Acta Chem. Scand. 15*, 215.
Bauchop, T., and Elsden, S. R. (1960), *J. Gen. Physiol. 23*, 457.
Bergmeyer, H. U., Ed. (1963), *in* Methods in Enzymatic Analysis, New York, N. Y., Academic, p 565.
Bose, S. K., and Gest, H. (1962), *Nature 195*, 1168.
Chance, B., and Nishimura, M. (1960), *Proc. Natl. Acad. Sci. U. S. 46*, 19.
Cohen-Bazire, G., and Kunisawa, R. (1963), *J. Cell Biol. 16*, 401.
Cohen-Bazire, G., Sistrom, W. R., and Stanier, R. Y. (1957), *J. Cell. Comp. Physiol. 49*, 25.
Cole, H. A., Wimpenny, J. W. T., and Hughes, D. E. (1967), *Biochim. Biophys. Acta 143*, 445.
Eisenhardt, R. H., and Rosenthal, O. (1968), *Biochemistry 7*, 1327.
Geller, D. M. (1962), *J. Biol. Chem. 237*, 2947.
Gibson, J., and Morita, S. (1967), *J. Bacteriol. 93*, 1544.
Hickman, D. D., and Frenkel, A. W. (1959), *J. Biophys. Biochem. Cytol. 6*, 277.
Hind, G., and Olson, J. M. (1968), *Ann. Rev. Plant Physiol. 19*, 249.
Holt, S. C., and Marr, A. G. (1965), *J. Bacteriol. 89*, 1402.
Nishimura, M. (1962), *Biochim. Biophys. Acta 57*, 96.
Ramirez, J., and Smith, L. (1968), *Biochim. Biophys. Acta 153*, 466.
Senez, J. C. (1962), *Bacteriol. Rev. 26*, 95.
Smith, L., and Baltscheffsky, M. (1959), *J. Biol. Chem. 243*, 1575.

Studies on the Biosynthesis of Lincomycin. IV. The Origin of Methyl Groups*

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ABSTRACT: The bioorgin of the methyl groups of lincomycin has been studied. Using radioactive techniques and mass spectroscopy it was determined that in addition to the SCH_3 and NCH_3 groups of this antibiotic, the CCH_3 group present in the amino acid moiety is derived from C_1 donors. The results obtained present a unique example of biological methylation reactions on nitrogen, sulfur, and carbon occurring in the same biological system.

Lincomycin (Ia), as has been reported (Mason *et al.*, 1962; Herr and Bergy, 1962; Hoeksema *et al.*, 1964), is an antibiotic produced by an actinomycete designated *Streptomyces lincolnensis* var. *lincolnensis*. Soon after the discovery of lincomycin it was observed (Argoudelis *et al.*, 1965) that fermentations of *S. lincolnensis* produce 4′-depropyl-4′-ethyllincomycin (Ib) (U-21,699) in addition to lincomycin. Vapor-phase chromatography indicated that the composition of the mixture of antibiotics produced by *S. lincolnensis* is *ca.* 95% lincomycin and 5% 4′-depropyl-4′-ethyllincomycin. One of the characteristic structural features of lincomycin and 4′-depropyl-4′-ethyllincomycin is the presence of NCH_3, SCH_3, and two CCH_3 groups. As part of our work on the biosynthesis of the antibiotics produced by *S. lincolnensis* we have examined the bioorigin of the methyl groups present in the lincomycin molecule. The present paper describes the results of this study.

Experimental Section

Counting Procedures. Radioactivity was determined with an automatic Packard Tri-Carb liquid scintillation spectrometer, Model 3000 (Packard Instrument Co., Inc.).

Procedures described by E. Rapkin (Packard Technical Bulletin, No. 6, March 1963) were generally used. Specifically, samples for counting were prepared by mixing 0.5-ml aliquots of the aqueous solution of substances to be counted with 15 ml of a scintillator consisting of 200 ml of xylene, 600 ml of dioxane, 600 ml of methyl Cellosolve, 14 g of 2,5-diphenyloxazole, 700 mg of bis[2-(5-phenyloxazolyl)-1-benzene], and 112 g of naphthalene. Xylene and methyl Cellosolve were purified by procedures outlined in Weissberger (1956). Naphthalene was recrystallized from absolute ethanol. Sample glass vials were supplied by Packard Instrument Co., Inc.

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Fermentation Procedures. Seed cultures of *S. lincolnensis* var. *lincolnensis* were prepared in a medium consisting of glucose monohydrate (Cerelose; 10 g/l.), *N*-Z-amine B (Sheffield Chemicals, Norwich, N. Y.; 5 g/l.), and Yeastolac (10 g/l.). The cultures were incubated at 28° for 48 hr on a rotary shaker. A fermentation medium consisting of glucose (30 g/l.), sodium citrate (3 g/l.), $ZnSO_4 \cdot 7H_2O$ (0.001 g/l.), $FeSO_4 \cdot 7H_2O$ (0.001 g/l.), $MgSO_4$ (1 g/l.), K_2HPO_4 (2.5 g/l.), NaCl (0.5 g/l.), and NH_4NO_3 (2.0 g/l.) was inoculated at a rate of 5% (v/v) with the 48-hr medium. The fermentation beers were harvested after 144 hr. Antibiotic titers were measured by disk plate activity using *S. lutea* as assay organism (Hanka *et al.*, 1962).

Addition of Radioactive Precursors. The precursors were added to the broth after 24-hr fermentation at a concentration of 180 μCi/l. The broths were harvested after 120- or 144-hr fermentation when antibiotic titers averaged 600 μg/ml of lincomycin.

Isolation of Antibiotics. Crystalline lincomycin hydrochloride was isolated by the procedure of Herr and Bergy (1962). Separation of 4′-depropyl-4′-ethyllincomycin was achieved by the methods described by Argoudelis *et al.* (1965).

Degradation of Lincomycin. ISOLATION OF α-METHYL THIOLINCOSAMINIDE (II) AND *trans*-4-*n*-PROPYL-L-HYGRIC ACID HYDROCHLORIDE (IIIa). The procedure described by Schroeder *et al.* (1967) was used for the hydrazinolysis of lincomycin to α-methyl thiolincosaminide and *trans*-4-*n*-propyl-L-hygric acid hydrazide. The latter was converted into the hydrochloride salt by hydrolysis with 6 N aqueous hydrochloric acid (Schroeder *et al.*, 1967).

Degradation of α-Methyl Thiolincosaminide. ISOLATION OF 2,4-DINITROPHENYL METHYL SULFIDE. α-Methyl thiolincosaminide (500 mg) was dissolved in 10 ml of 5 N aqueous sulfuric acid. The solution was kept at reflux for 45 min under a continuous stream of nitrogen. Volatile materials were passed through a mixture of 3 ml of absolute ethanol and 0.3 ml of 3.3 N aqueous sodium hydroxide solution. The ethanolic solution containing methylmercaptan was then mixed with a solution of 200 mg of 2,4-dinitrochlorobenzene in 3 ml of absolute methanol. Crystalline 2,4-dinitrophenyl methyl sulfide, precipitated immediately, was isolated by filtration and recrystallized from 95% ethanol.

Decarboxylation of trans-4-n-Propyl-L-hygric Acid (IIIa) by Lead Tetraacetate. *trans*-4-*n*-Propyl-L-hygric acid hydrochloride (IIIa) (100 mg) was added to a solution of 450 mg of lead tetraacetate in 25 ml of benzene. The mixture was heated at 50° for 30 min. The evolved carbon dioxide was carried by nitrogen to a trap containing 100 ml of saturated aqueous barium hydroxide solution. Barium carbonate was isolated by filtration, washed with water, and dried.

Permanganate Oxidation of trans-4-n-Propyl-L-hygric Acid (IIIa). ISOLATION OF D-(+)-*n*-PROPYLSUCCINIC ACID (IV). The procedure described by Magerlein *et al.* (1967) was followed. The obtained material was purified by silica gel chromatography using benzene–methanol–acetic acid (10:2:1, v/v) as the solvent. Purification was followed by thin-layer chromatography (silica gel) using benzene–methanol–glacial acetic acid (100:2:1, v/v) or benzene–methanol–glacial acetic acid (100:20:10, v/v) as the solvent systems and DL-propylsuccinic acid (Magerlein *et al.*, 1967) as control.

For identification of the spots, the plates were heated at 50° for 30 min and then sprayed with a 2% solution of bromophenol blue.

Decarboxylation of D-(+)-*n-Propylsuccinic Acid* (IV). D-(+)-*n*-Propylsuccinic acid (100 mg) was dissolved in 20 ml of benzene containing 0.5 ml of pyridine and 400 mg of lead tetraacetate. The mixture was kept at reflux for 3 hr under a stream of dry nitrogen. The carbon dioxide evolved was carried into a solution of saturated aqueous barium hydroxide solution (100 ml). Barium carbonate was isolated by filtration, washed with water, and dried.

Kuhn–Roth Oxidations. Kuhn–Roth oxidations of α-methyl thiolincosaminide, *trans*-4-*n*-propyl-L-hygric acid, and D-(+)-*n*-propylsuccinic acid were run by Huffman Laboratories, Inc. The obtained sodium acetate was transformed to crystalline *S*-benzylisothiouronium acetate (mp 133).

Decarboxylation of Sodium Acetate. A mixture of 30 mg of sodium acetate and 90 mg of sodium hydroxide was heated at 380° for 20 min. The reaction mixture was cooled and dissolved in 10 ml of water. The solution was then mixed with 5 ml of 2 N aqueous hydrochloric acid. Carbon dioxide evolved was carried by a stream of nitrogen to a saturated aqueous solution of barium hydroxide. Barium carbonate was isolated by filtration, washed with water, and dried.

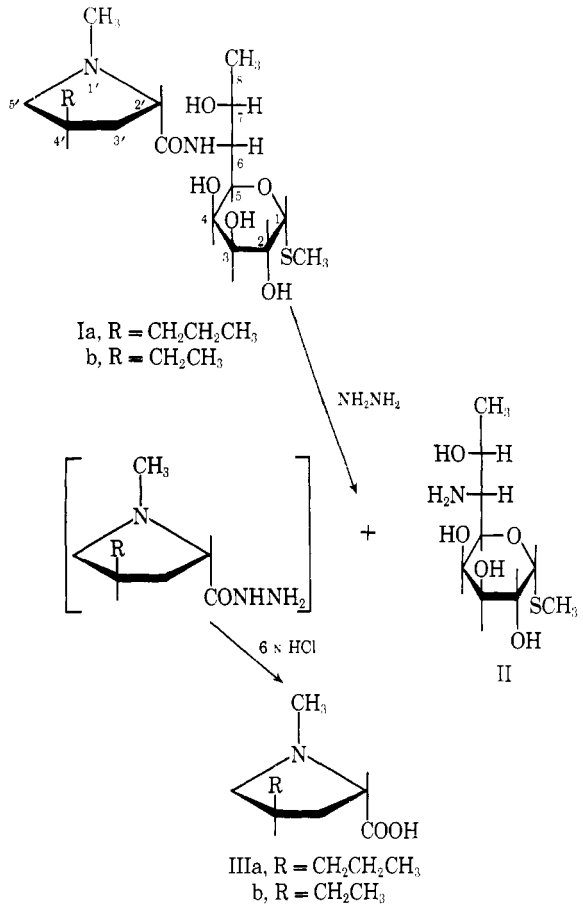
TABLE I: Incorporation of C_1 Donors.

	% Incorp	Ratio of Sp Act. (α-methyl thiolincosaminide/*trans*-4-*n*-propyl-L-hygric acid)
L-[*methyl*-^{14}C]Methionine	17.8	0.46
[2-^{14}C]Glycine	5.8	0.40
L-[U-^{14}C]Serine	2.3	0.47

Discussion and Results

Work reported from several laboratories (Whalley, 1963) shows that the origin of methyl group attached to oxygen or nitrogen as well as many of those attached to carbon is the C_1 metabolic pool and that these methyl groups are attached to the appropriate receptor centers through transmethylation from C_1 donor systems like methionine. When L-[*methyl*-^{14}C]-methionine, [2-^{14}C]glycine, or L-[U-^{14}C]serine was added to fermentations of *S. lincolnensis*, a high degree of incorporation of radioactivity into lincomycin was observed (Table I). The radioactive lincomycin, obtained from fermentations of *S. lincolnensis* containing L-[*methyl*-^{14}C]methionine, was degraded (Scheme I) to α-methyl thiolincosaminide (II) and to *trans*-4-*n*-propyl-L-hygric acid (IIIa) and the specific activity of these compounds was determined. As indicated in Table I the ratio of the specific activity of α-methyl thiolincosaminide to that of *trans*-4-*n*-propyl-L-hygric acid is *ca.* 0.45, *i.e.*, the amino acid is twice as radioactive as α-methyl thiolincosaminide. Acid hydrolysis of α-methyl thiolincosaminide (II) yielded methylmercaptan which was isolated as the 2,4-dinitro-

SCHEME I



phenyl methyl sulfide (Scheme II). This compound contained 91% of the radioactivity present in α-methyl thiolincosaminide. Kuhn–Roth oxidation of α-methyl thiolincosaminide afforded radioinactive acetic acid. These results indicate that of the two methyl groups present in α-methyl thiolincosaminide, only the SCH_3 originates from methionine.

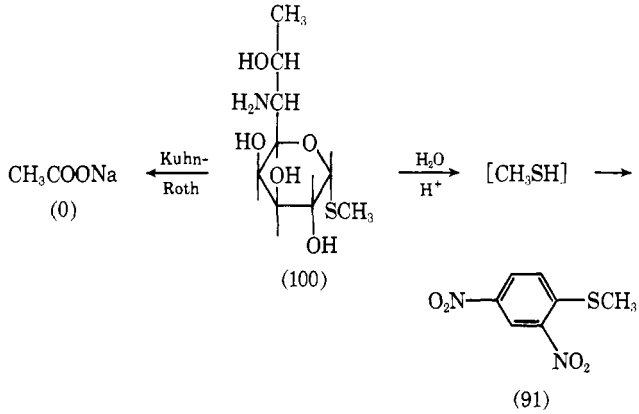
Oxidative decarboxylation of *trans*-4-*n*-propyl-L-hygric acid (IIIa) using lead tetraacetate (Roth, 1961) gave radioinactive carbon dioxide (Scheme III). On the other hand, permanganate oxidation of IIIa (Magerlein *et al.*, 1967) gave D-(+)-*n*-propylsuccinic acid (IV) containing 47% of the radioactivity present in IIIa. This indicates that the NCH_3 group contains the remaining 53% and thus also originates from methionine.

Kuhn–Roth oxidation of *trans*-4-*n*-propyl-L-hygric acid (IIIa) yielded sodium acetate containing 44% of the radioactivity present in IIIa. Sodium hydroxide fusion gave radioinactive carbon dioxide indicating that the methyl group of the propyl side chain of IIIa is derived from methionine. Furthermore lead tetracetate decarboxylation (Grob *et al.*, 1958) of D-(+)-*n*-propylsuccinic acid (IV) gave inactive carbon dioxide while Kuhn–Roth oxidation afforded highly active sodium acetate containing all the activity in the methyl group.

These results show that the SCH_3 group of α-methyl thiolincosaminide and the NCH_3 and CCH_3 groups of *trans*-4-*n*-propyl-L-hygric acid originate from C_1 donors.

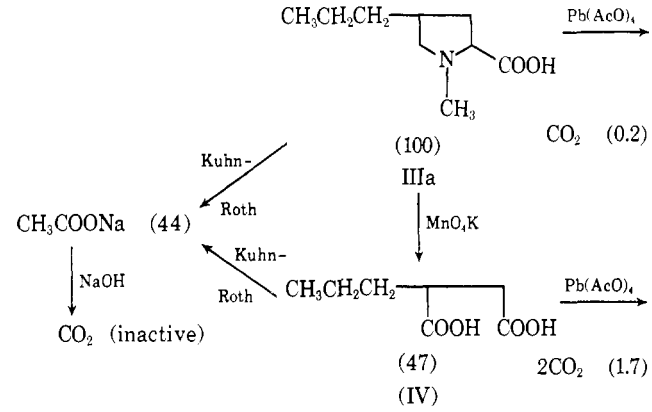
Furthermore, the rate of incorporation of methionine, glycine, and serine suggested that the C_1 fragments are incorporated as methyl groups rather than as oxidized forms. That this is the case was shown by adding L-[CD_3]methionine in

SCHEME II[a]



[a] Numbers in parentheses indicate percentage of radioactivity in the indicated compounds.

SCHEME III[a]

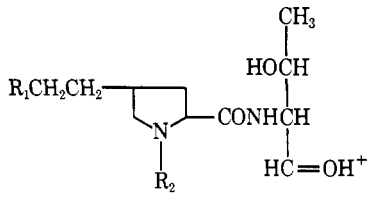


[a] Numbers in parentheses indicate percentage of radioactivity in the indicated compounds.

TABLE II: Mass Spectrum of Lincomycin Obtained by Feeding L-[CD_3]Methionine

Mass Ions (Ratio)	406 (48)	409 (18)	412 (20)	415 (13)
		↓ −SCH_3, −SCD_3		
	359 (58)	362 (25)	365 (20)	
		↓		
	257 (58)	260 (22)	263 (21)	
		↓		
	126 (57)	129 (24)	132 (20)	

CHART I



IVa, *m/e* 257; $R_1 = CH_3$; $R_2 = CH_3$
b, *m/e* 260; $R_1 = CH_3$; $R_2 = CD_3$ or $R_1 = CD_3$; $R_2 = CH_3$
c, *m/e* 263; $R_1 = CD_3$; $R_2 = CD_3$

fermentation of *S. lincolnensis* and isolating deuterated lincomycin. The mass spectrum of this material (Table II) showed molecular ions at *m/e* 406 (M^+ of regular lincomycin), 409, 412, and 415. This indicates that a maximum of three CD_3 groups was incorporated verifying the radioactive work.

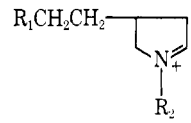
Ions at *m/e* 359, 362, and 365 are derived from the molecular ion by elimination of SCH_3 or SCD_3. Ions at *m/e* 257, 260, and 263 are due to the fragments IVa–c (Chart I). (F. Kagan and M. F. Grostic, private communication).

Finally ions at *m/e* 126, 129, and 132 correspond to fragments Va, b, and c (Chart II) derived from the propyl hygric acid moiety of the lincomycin molecule.

The mass spectral data in combination with the radioactivity studies establish conclusively that in addition to the SCH_3 and the NCH_3 group the CCH_3 group of *trans*-4-*n*-propyl-L-hygric acid originate from the methyl group of methionine and other C_1 donors.

The results obtained present a unique example of biological methylation reactions on nitrogen, sulfur, and carbon occurring in the same biological system. Methylation on nitrogen undoubtedly occurs by transmethylation process involving *S*-adenosylmethionine. This mechanism has been well described in the literature (Shapiro and Schlenk, 1965). However the biosynthesis of the SCH_3 group of lincomycin can occur either by transmethylation process (involving *S*-adenosylmethionine) or by "transthiomethylation" process in which the SCH_3 group of methionine is transferred intact to the appropriate acceptor. The latter mechanism has been also considered by Patterson *et al.* (1964) in their studies related with formation of the *S*-ethyl analog of lincomycin by *Streptomyces umbrinus*. It must be pointed out, however, that such a biosynthetic mechanism which involves the intact transfer of the SCH_3 group of methionine with the subsequent formation of methylthio compounds, has not been described in the literature. In regard to the mechanism of biological methylation on carbon, Jaureguiberry *et al.* (1964, 1965) have shown that only two of the three protons of the methionine methyl group were involved in methylation reactions leading to tuberculostearic acid and ergosterol. On the other hand, Tropp *et al.* (1964) found that transmethylation to the 5 position of uridine involves transfer of an intact methyl group. The results obtained in the present study indicate that the terminal CCH_3 group of the amino acid moiety of lincomycin

CHART II



Va, *m/e* 126; $R_1 = CH_3$; $R_2 = CH_3$
b, *m/e* 129; $R_1 = CH_3$; $R_2 = CD_3$; or $R_1 = CD_3$; $R_2 = CH_3$
c, *m/e* 132; $R_1 = CD_3$; $R_2 = CD_3$

results also by transfer of an intact methyl group from methionine by a mechanism involving, most probably, *S*-adenosylmethionine.

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References

Argoudelis, A. D., Fox, J. A., and Eble, T. E. (1965), *Biochemistry 4*, 698.

Grob, C. A., Ohta, M., and Weiss, A. (1958), *Angew. Chem. 70*, 343.

Hanka, L. J., Mason, D. J., Burch, R. M., and Trick, R. W. (1962), *Antimicrobial Agents Chemotherapy*, 565.

Herr, R. R., and Bergy, M. E. (1962), *Antimicrobial Agents Chemotherapy*, 560.

Hoeksema, H., Bannister, B., Birkenmeyer, R. D., Kagan, F., Magerlein, B. J., MacKellar, F. A., Schroeder, W., Slomp, G., and Herr, R. R. (1964), *J. Am. Chem. Soc. 86*, 4223.

Jaureguiberry, G., Law, J. H., McCloskey, J. A., and Lederer, E. (1964), *Compt. Rend. 258*, 3587.

Jaureguiberry, G., Law, J. H., McCloskey, J. A., and Lederer, E. (1965) *Biochemistry 4*, 347.

Magerlein, B. J., Birkenmeyer, R. D., Herr, R. R., and Kagan, F. (1967), *J. Am. Chem. Soc., 89*, 2459.

Mason, D. J., Dietz, A., and DeBoer, C. (1962), *Antimicrobial Agents Chemotherapy*, 554.

Patterson, E. L., Hash, J. H., Lincks, M., Miller, P. A., Bohonos, N. (1964), *Science 146*, 1691.

Roth, H. J. (1961), *Arch. Pharm. 294*, 427.

Schroeder, W., Bannister, B., and Hoeksema, H. (1967), *J. Am. Chem. Soc. 89*, 2448.

Shapiro, S. K., and Schlenk, F. (1965), Transmethylation and Methionine Biosynthesis, Chicago, Ill., University of Chicago.

Tropp, B. E., Law, J. H., and Hayes, J. M. (1964), *Biochemistry 3*, 1837.

Weissberger, A. (1956), Technique of Organic Chemistry, Vol. III, 2nd ed, New York, N. Y., Interscience.

Whalley, W. B. (1963), *in* Biogenesis of Natural Compounds, Bernfeld, P., Ed., New York, N. Y., MacMillan, Chapter 18.