Further Characterization of a Chinese Hamster Ovary Cell Mutant Defective in Lanosterol Demethylation†

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ABSTRACT: Sensitive in vitro lanosterol 14α- and 4α-methylsterol oxidase assays, particularly suitable for cell extracts of tissue culture cells, were developed and validated. Using these assays, we showed that the biochemical lesion of mutant 215, a cholesterol-requiring Chinese hamster ovary cell auxotroph isolated and partially characterized previously [Chang, T. Y., Telakowski, C., Vanden Heuvel, W., Alberts, A. W., & Vagelos, P. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 832–836], was localized at the 4α-methylsterol oxidase enzyme system. The defect in 4α-methylsterol oxidase activity in mutant 215 cells could be demonstrated by using either 4,4-dimethylcholestanol or 4α-methylcholestanol as the substrate, suggesting that the enzyme systems responsible for 4α-methyl- and 4,4-dimethylsterols may share a common component. However, demethylation of the C-14α methyl group was found to occur at identical rates in wild-type and mutant 215, suggesting that C-14α demethylation and C-4α demethylation may occur by separate enzyme systems. A [^{3}H]dihydrolanosterol incorporation experiment in intact cells of wild-type and mutant 215 supported these conclusions. Despite these results, a [^{14}C]acetate pulse experiment indicated that [^{14}C]lanosterol, instead of its ^{14}C-labeled 14-demethylated sterol derivative(s), accumulated in intact cells of mutant 215. Possible implications of these findings for the mechanisms of lanosterol demethylation reactions are discussed.

Mammalian cells in culture provide an important tool to examine the cholesterol biosynthetic pathway and its regulation because cellular growth conditions can easily be controlled and manipulated. Chinese hamster ovary (CHO)[1] cells have been shown to have an intact cholesterol biosynthetic pathway with operational low-density lipoprotein-mediated regulation (Chang & Limanek, 1980). This suggests that the CHO cell line is a suitable model for further delineating the cholesterol biosynthetic pathway and its intracellular regulation. Moreover, specific mutants in sterol metabolism have been isolated from this cell line (Chang & Limanek, 1980; Chang et al., 1977; Limanek et al., 1978) and should serve as useful biological tools for such studies. One such mutant, designated mutant 215, was isolated and partially characterized previously (Chang et al., 1977). Mutant 215 was shown to be a cholesterol auxotroph: when cholesterol was not supplied exogenously to the medium, mutant 215 cells died within 3 days, while in the same medium wild-type cells continued normal growth. Pulse studies using [1-^{14}C]acetate, [2-^{3}H]mevalonate, and [^{3}H]squalene all showed that mutant 215 was capable of synthesizing intermediates in the cholesterol biosynthetic pathway up to and including lanosterol but was incapable of synthesizing cholesterol. These experiments suggested that the lesion in mutant 215 was at the stage of lanosterol demethylation (Chang et al., 1977).

Early work in lanosterol metabolism established that C-14α demethylation occurs prior to any demethylation at the C-4 position (Gautschi & Bloch, 1958). C-14α demethylation is currently believed to occur by enzymatic deformylation; this process requires molecular oxygen and NADPH (Akhtar et al., 1978; Alexander et al., 1972; Mitropolous et al., 1976). In addition, at least some of the steps of this process are carbon monoxide sensitive, and cytochrome P-450 is probably a component of these enzymatic systems (Gibbons & Mitropolous, 1973a,b; Gibbons et al., 1979). Following loss of the 14α-methyl group, several nuclear double-bond migrations occur before the 4,4-*gem*-dimethyl groups (C-30 and C-31) are removed in consecutive oxidative decarboxylations. Previous studies have established that oxidative attack at the 4,4-*gem*-dimethyl groups occurs stereospecifically on the 4α-methyl group by a mixed function oxidase (4α-methylsterol oxidase, requiring molecular oxygen and NADPH or NADH) (Miller et al., 1971; Miller & Gaylor, 1970a,b; Rahman et al., 1970). This enzyme system oxidizes the 4α-methyl group to the corresponding 4α-carboxylic acid (Miller & Gaylor, 1970a,b). Decarboxylation of the 4α-carboxylic acid occurs by a NAD$^+$-dependent decarboxylase (Rahimtula & Gaylor, 1972). An epimerase (Gaylor et al., 1966) then epimerizes the 4β-methyl group to the 4α position for oxidative demethylation by the same process. Finally, the 3-keto group is reduced to the 3β-alcohol after each decarboxylation. The point at which the Δ^{24} double bond is reduced is not yet well established.

In this report, we describe our efforts to further elucidate the biochemical lesion of mutant 215 cells by using in vitro enzyme assays of 4α-methylsterol oxidase and lanosterol 14α-demethylase. We compare results of these assays with results of [^{14}C]acetate and [^{3}H]dihydrolanosterol pulses performed on intact cells and discuss possible implications of our findings.

Materials and Methods

Materials

Lipids and other biochemicals were from Sigma except where noted below. Radioactive chemicals were from New England Nuclear except where noted below. [1-^{14}C]Acetate was from Amersham-Searle. Triton WR-1339 was from Ruger Chemical Co., Irvington-on-Hudson, NY. Protosol was

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[1] Abbreviations: CHO cells, Chinese hamster ovary cells; DeL, delipidated fetal calf serum; FCS, fetal calf serum; GLC, gas–liquid chromatography; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; PBS, phosphate-buffered saline; TLC, thin-layer chromatography.

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Table I: R_f Values of Various Lipid Standards on Several TLC Systems[a]

lipid standard	R_f value				
	system I	system II	system III	system IV	system V
desmosteryl 3β-acetate					0.14
cholesteryl 3β-acetate				0.20	0.80
4α-methylcholest-7-enyl 3β-acetate				0.46	
4,4-dimethylcholest-7-enyl 3β-acetate				0.55	0.88
lanosteryl acetate				0.25	0.68
dihydrolanosteryl acetate				0.71	0.96
demosterol	0.35	0.88	0.90	0.00	0.00
cholesterol	0.35	0.88	0.90	0.05	0.08
lanosterol	0.53	0.88	0.90	0.08	0.10
dihydrolanosterol	0.53	0.88	0.90	0.19	0.28
4α-methylcholestanol	0.42	0.88	0.90		
4,4-dimethylcholestanol	0.53	0.88	0.90		
3β-hydroxycholestan-4α-oic acid	0.00	0.30	0.45		
3β-hydroxy-4β-methylcholestan-4α-oic acid	0.00	0.44	0.80		

[a] TLC plates and solvents used for systems I–V were described under Materials and Methods.

from New England Nuclear. Nanograde organic solvents were from Mallinckrodt. Fetal calf serum (FCS) and α-MEM (minimum Eagle's medium) were from M. A. Bioproducts. All other chemicals were of analytical grade.

Methods

Cells. CHO cells were grown as monolayers in F-12 or α-MEM medium plus 10% FCS as previously described (Chang & Limanek, 1980; Chang et al., 1977; Limanek et al., 1978). In several experiments, cells were grown in α-MEM medium supplemented with 8% newborn calf serum (NBS) and 2% FCS. F-12 medium supplemented with 10% delipidated fetal calf serum DeL) was also used in several experiments. DeL was prepared according to published procedures (Cham & Knowles, 1976). Mutant 215 was isolated from 20 × 10^6 mutagenized CHO cells by the bromodeoxyuridine light enrichment technique as previously described (Chang et al., 1977; Chang & Vagelos, 1976). Before the enrichment step, cells were grown in F-12 + 10% DeL for 24 h to create a large difference in growth rates between the normal (wild-type) cells and lipid-requiring auxotroph candidates (Chang & Vagelos, 1976). Putative revertants of mutant 215 were isolated by the following procedure: Mutant 215 was recloned once; the single clone was allowed to grow to approximately 100 × 10^6 cells in size. These cells were then plated at 1 × 10^6 cells in 5 mL of F-12 + 10% FCS per dish in 50 Corning 100-mm dishes for 2 days. Afterward, medium was sucked off, and cells were rinsed with 1 × 5 mL of phosphate-buffered saline (PBS) and switched to grow in 5 mL per dish of F-12 + 10% DeL for 10 days. Medium was changed once very 2 days. Afterward, revertant clone candidates (≥200 cells per clone) were visualized by the naked eye, verified under the microscope, and picked with glass cloning cylinders (Bellco Glass Inc., catalog no. 2090-00808). These clones were tested individually for ability to grow in F-12 + 10% DeL on sparse cell density. Approximately 40 clones were found to exhibit healthy growth in this medium: the reversion frequency was thus estimated to be approximately 0.8 × 10^{-6}. One of these revertants (designated revertant 40) was recloned twice before biochemical analysis. The doubling time of revertant 40 in F-12 + 10% DeL was found to be approximately 25–30 h, instead of the 20–24 h found for wild-type CHO cells (Chang & Limanek, 1980).

$^{14}CO_2$ Release from Whole Cell [2-^{14}C]Pyruvate Pulse. Stock cells grown in F-12 plus 10% FCS were trypsinized and replated with 10 mL of F-12 plus 10% FCS in 25-cm^2 flasks. After 2 days, the medium was drained off. The cells were rinsed with 5 mL of PBS, fed with 7 mL of F-12 plus 10% DeL medium, and grown for 24 h. The medium was removed, and 0.92 mL per flask of fresh F-12 ($NaHCO_3$ and pyruvate deleted) + 10% DeL medium containing 10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes) buffer (pH 7.4) was added. [2-^{14}C]Pyruvate solution (80 μL) was then added to each flask to give 0.5 μCi/mL (4.5 μCi/mmol) in the medium. Flasks were capped with 5-mL-size glass scintillation vials containing 100 μL of protosol on filter paper (flasks and vials were joined by rubber hoses) and then incubated at 37 °C for 20, 40, or 60 min. The rate of $^{14}CO_2$ production was found to be linear within the time range. The pulse was terminated by injection of 100 μL of 1 M citric acid to the pulse medium. Flasks were incubated at 37 °C for 90 min. A control experiment using $NaH^{14}CO_3$ showed that this procedure collected 85–90% of the $^{14}CO_2$ released upon citric acid addition. The scintillation vials were then removed from the flasks, and 3.5 mL of Biofluor (New England Nuclear) was added. Vials were allowed to stand at 4 °C for 12 h and then counted.

Thin-Layer Chromatography. Separation of C_{27} sterols from C_{28}, C_{29}, and C_{30} sterols was accomplished on LK5D preactivated silica gel G plates (Whatman, 20 × 20 cm) with methylene chloride/ethyl acetate (97:3 v/v) as the mobile phase (TLC system I; Table I) (Chang et al., 1977; Yamamoto & Bloch, 1970). This system does not distinguish C_{30} sterols from C_{29} or C_{28} sterols, no does it distinguish between the Δ^{24} and dihydro forms of C_{27} or C_{28-30} sterols. 3β-Hydroxy-4β-methylcholestan-4α-oic acid was separated from 4,4-dimethylcholestanol[2] and other sterols on LK5D plates with chloroform/methanol/acetic acid (95:4:1 v/v) as the mobile phase (TLC system II; Table I) (Miller & Gaylor, 1970a,b). 3β-Hydroxycholestan-4α-oic acid was separated from 4α-methylcholestanol and other sterols on LK5D plates with hexane/ether/acetic acid (40:60:5 v/v) as the mobile phase (TLC system III; Table I) (Miller & Gaylor, 1970a,b). The acetylated derivatives of lanosterol, dihydrolanosterol, 4,4-dimethylcholest-7(8),24-dienol, 4α-methylcholest-7(8),24-dienol, and cholesterol were separated on alumina G plates (Analtech, 20 × 20 cm) which were activated for 1 h at 100 °C and then twice prechromatographed with 10% $AgNO_3$ in

[2] Because sterol nomenclature is extremely complex, simplified names of compounds will be used in this paper except where ambiguities may arise. All sterols used in this paper contain 5α-hydrogen and 3β-alcohol stereochemistry. In addition, it should be noted that, with the exception of the 4,4-dimethyl[30,31-$^{14}C_2$]cholest-7-en-3β-ol, the position of the nuclear double bond (i.e., Δ^7 or Δ^8) of sterols described in this paper has not yet been determined.

acetonitrile (w/v). Plates were used shortly after prechromatography. These plates were developed by using hexane/benzene (85:15 v/v) at 4 °C (TLC system IV; Table I) (Gibbons et al., 1973; Kammereck et al., 1967). The acetylated derivatives of lanosterol, dihydrolanosterol, 4,4-dimethylcholest-7(8)-enol, 4α-methylcholest-7(8)-enol, desmosterol, and cholesterol were separated on identically prepared alumina G plates developed in hexane/benzene (80:20 v/v) at 4 °C (TLC system V; Table I) (Kammereck et al., 1967). Sterol acetylation was carried out by the addition of 0.5 mL of nanograde benzene, 0.2 mL of dry pyridine, and 0.3 mL of acetic anhydride to between 0.1 and 1.0 mg of the free sterol followed by at least a 2-h incubation at room temperature (Miller & Gaylor, 1970a,b). The acetylation reaction was stopped by evaporating samples to dryness under N_2. Yield of the acetylated derivative was 80–90%. Methyl esters of fatty acids and of the sterol carboxylic acid derivatives were prepared by using diazomethane following published procedures (Schlenk & Gellerman, 1960). Free fatty acids and fatty acid methyl ester derivatives were analyzed as previously described (Chang et al., 1979).

Migration of nonradioactive authentic external standard compounds on silica gel TLC plates was determined by iodine staining or by spraying with 0.017% rhodamine 6G in 90% ethanol, followed by viewing and marking under long-wavelength ultraviolet light. Migration of nonradioactive standard compounds on the $AgNO_3$-impregnated alumina G (TLC systems IV and V) was visualized by spraying with rhodamine 6G as described above. Following determination of external standard TLC migrations, the appropriate radioactive bands were scraped into individual 5-mL capacity scintillation vials, 3.5 mL of scintillation fluid was added, and each vial was counted by using a Packard liquid scintillation counter. In all experiments, the entire chromatogram was also cut into small segments (20 1-cm bands for LK5D silica plates; 37 0.5-cm bands for alumina plates) and counted to assure no radioactivity was present outside the substrate and product bands.

Saponification. Samples were saponified as previously described (Chang et al., 1979). Each sample was then concentrated under N_2 to less than 0.5 mL; the volume was then brought to 1.0 mL with methanol before extraction.

Gas–Liquid Chromatography (GLC). GLC analysis of sterols was as previously described (Change et al., 1977, 1979).

Protein Determination. Unless otherwise indicated, protein was quantitated by using the microbiuret method (Munkres & Richards, 1965).

4-Methylsterol Oxidase Assay in Vitro. (a) Substrate Solubilization. Radioactive sterols were adjusted to the desired specific radioactivity for substrate used with nonradioactive sterols and then were dissolved in 1 mL of acetone. The acetone solution was added dropwise at room temperature to an aqueous solution of Triton WR-1339 containing a 400-fold excess of detergent to substrate by weight. Acetone was then evaporated from the solution by concentrating it to one-half of its original aqueous volume under N_2. The solution was then reconstituted to its original aqueous volume with water. Substrate solutions were made fresh for each assay and kept at room temperature until used.

(b) Whole Cell Homogenate Preparation. CHO cells were grown as monolayers in α-MEM plus 10% FCS and harvested by procedures previously described for the oxidosqualene cyclase assay in vitro (Chang et al., 1979). The pelleted cells were then resuspended in hypotonic buffer (1 mM $MgCl_2$ and 1 mM Tris-HCl, pH 7.6) to approximately 30 mg of protein/mL and allowed to swell at room temperature for 15 min. Cells were checked for swelling under a phase contrast microscope and then broken with 200 strokes of a tight-fitting Dounce homogenizer. Complete cell breakage was checked under a phase contrast microscope. Cold hypertonic buffer (250 mM KH_2PO_4, 150 mM EDTA, 385 mM KCl, and 50 mM dithiothreitol, pH 7.4) was added immediately (1:4 v/v, hypertonic buffer/hypotonic buffer). Small aliquots of the cell homogenate were then taken for protein determination.

(c) Reaction Incubation. Aliquots of the cell homogenate were transferred to 15-mL round-bottom screw-capped extraction tubes at 0 °C. To each tube was also added 130 μL of an NADPH-regenerating system consisting of 1.26 μmol of β-$NADP^+$, 25.0 μmol of glucose 6-phosphate, and 3.8 units of yeast glucose-6-phosphate dehydrogenase. The reaction was started by addition of 100 μL of Triton WR-1339 solubilized substrate (25 μg/tube, 1.4 mCi/mmol) to give a final reaction volume of 0.73 mL. Tubes were incubated in a 37 °C water bath with shaking for the desired reaction time. The reaction was stopped by addition of 0.5 mL of 20% KOH in methanol. Tubes were then saponified at 80 °C for 1 h and then acidified with concentrated HCl. Lipids were isolated from each tube with five sequential 2-mL petroleum ether extractions. The pooled petroleum ether fraction was concentrated under N_2 to 3 mL and washed 3 times with 1 mL of 5 mM HCl. Extraction recovery of radiolabeled lipid averaged 90–98%. The samples were analyzed by using TLC system II or III (Table I). The only labeled products found were identified by comigration with authentic lipid external standards (and by other methods; see Results), as the substrate and as the carboxylic acid product. Nanomoles of carboxylic acid formed were calculated according to the formula: nanomoles of substrate per tube × counts per minute in product band ÷ [(counts per minute in substrate band + counts per minute in product band)].

Dihydrolanosterol 14α-Demethylase Assay in Vitro. Cell homogenate and substrate preparations and reaction initiation, incubation, and termination were identical with those described for the 4-methylsterol oxidase assay above except that 5.0 μmol of β-$NADP^+$ was added per tube and Δ^8-[^{3}H]dihydrolanosterol was used as substrate. After reaction termination, the tubes were saponified and then extracted with petroleum ether as described for the 4-methylsterol oxidase assay. The pooled petroleum ether extracts were thn washed 2 times with 1 mL of 3% $NaHCO_3$, 2 times with 1 mL of a saturated aqueous solution of NaCl, and 2 times with 1 mL of water. Extraction recovery of radiolabeled lipid averaged 90–98%. Samples were analyzed by TLC system IV (Table I). The only radiolabeled products found were identified by comigration with authentic lipid external standards as the acetate derivatives of the substrate, dihydrolanosterol, and of the reaction product, 4,4-dimethylcholest-7(8)-enol. The reaction product was quantitated by using the formula described for the 4-methylsterol oxidase assay.

Compounds. Unlabeled and radioactive 4α-methyl-5α-[2,4-^{3}H]cholestan-3β-ol, unlabeled and radioactive 4,4-dimethyl-5α-[2-^{3}H]cholestan-3β-ol, and 3β-hydroxy-4β-methylcholestan-4α-oic acid were synthesized according to published procedures (Nelson et al., 1976; Sharpless et al., 1968, 1969) by Dr. Thomas Spencer, Department of Chemistry, Dartmouth College. Purities of these compounds were found to be greater than 98% by TLC analyses. 4,4-Dimethyl-5α-[30,31-$^{14}C_2$]cholest-7-en-3β-ol (containing approximately 8% Δ^8 isomer) was the gift of Dr. James L. Gaylor, Department of Biochemistry, University of Missouri

Table II: Intact Cell [^{14}C]Acetate Incorporation into ^{14}C-Labeled Squalene, Sterols, and Fatty Acids[a]

cell type	dpm incorporated × 10^{-3}/mg of protein					
	squalene	C_{28-30} sterols	C_{27} sterols	total sterol	total fatty acid	% desaturation[b]
wild type	5.1	24.8 (24.2)	27.8 (75.8)[c]	102.6	823	17.7
mutant 215	2.7	41.5 (94.1)	2.6 (5.9)	44.1	1602	15.3

[a] Cells grown in F-12 plus 10% FCS were trypsinized and replated with 30 mL of F-12 plus 10% FCS in 150-cm^2 flasks at 0.96 × 10^6 cells per flask for wild-type cells and 1.56 × 10^6 cells per flask for mutant 215 cells. After 2 days, the medium was drained off. The cells were rinsed with 10 mL of PBS, fed with 30 mL of F-12 plus 10% DeL medium, and grown for 24 h. The medium was then removed, and 4.2 mL of F-12 plus 10% DeL medium containing sodium [1-^{14}C]acetate (31.25 μCi/mL, 23.4 mCi/mmol was added. After the cells were incubated at 37 °C for 3 h, the pulse medium was removed, and cells were washed 6 times with 5 mL of cold PBS. Cells were dissolved in 3 mL of 3 M KOH. [1,2-$^3H_2$]Cholesterol (125 000 dpm/tube) and [9-^{3}H]oleic acid (45 000 dpm/tube) were added as internal standards. The ^{14}C-saponifiable and nonsaponifiable lipid fractions were extracted and collected as described (Chang et al., 1979). Sterols and fatty acids were analyzed on TLC as described under Materials and Methods. Percent desaturation analysis was accomplished by methylation of the fatty acid fraction followed by TLC analysis according to procedures described previously (Chang et al., 1979; Chang & Vegelos, 1976). Each value represents the average of results from duplicate flashes. Variation between duplicates was within 7% of the mean. [b] Percent desaturation is expressed as radioactivity in monounsaturated fatty acids divided by radioactivity in total fatty acids (Gibbons et al., 1979; Limanek et al., 1978). [c] Values in parentheses represent percent of total [^{14}C]sterols found as C_{27} sterols or as C_{28}–C_{30} sterols.

Medical School at Columbia. Unlabeled Δ^8-dihydrolanosterol was isolated from Sigma practical grade lanosterol by TLC of the free sterols on TLC system V. The dihydrolanosterol band was visualized by staining with rhodamine 6G. Purity of this compound was approximately 98% by TLC and GLC analyses. Its structural identity was established by GLC–mass spectrometry (Chang et al., 1977).

Δ^8-[^{3}H]Dihydrolanosterol Synthesis and Purification. [^{3}H]Dihydrolanosterol was prepared biosynthetically according to published procedures from [5-^{3}H]mevalonate (Miller et al., 1967). Labeled dihydrolanosterol was separated from contaminating labeled lanosterol and the Δ^{24} and dihydro forms of labeled C_{29} and C_{28} sterols by acetylation followed by TLC on system IV. The dihydrolanosteryl acetate was located by comigration with unlabeled dihydrolanosteryl acetate. The dihydrolanosteryl acetate band was then scraped, extracted with 3 × 2 mL of ethyl acetate, and washed sequentially with 3% $NaHCO_3$ and H_2O. The purified dihydrolanosteryl acetate was saponified and extracted in nanograde petroleum ether. The regenerated dihydrolanosterol was reacetylated and analyzed on TLC system IV and was found to be 98% pure.

Results

[^{14}C]Sterol Formation in Intact Cells from [^{14}C]Acetate. Analysis of the nonsaponifiable fraction from a 3-h [^{14}C]-acetate pulse in mutant 215 and wild-type cells grown in DeL medium is shown in Table II. While 75.8% of the total [^{14}C]sterols was found as C_{27} sterol(s) in wild-type cells, the mutant cells produced only a small amount of ^{14}C-labeled C_{27} sterol(s) (5.9%); most of the [^{14}C]sterols accumulated in the mutant cells as C_{28}–C_{30} sterols (94.1%). Further analyses of the C_{28}–C_{30} sterol band and the C_{27} sterol band were carried out by extraction of the sterols into organic solvent (see Materials and Methods) followed by acetylation and argentation TLC analysis on TLC system V. As seen in Figure 1a, for both cell types about 70% of the radioactivity in the C_{28}–C_{30} sterol band is [^{14}C]lanosterol; the remaining radioactivity is tentatively assigned as labeled 4,4-dimethylcholest-7,24-dien-3β-ol and 4α-methylcholest-7,24-dien-3β-ol.[3] Neither cell type produces any [^{14}C]dihydrolanosterol or other 4,4-dimethylsterols with a reduced Δ^{24} bond. In Figure 1b, analysis of the C_{27} sterols showed that the major [^{14}C]sterol formed in both cell types is cholesterol rather than desmosterol,

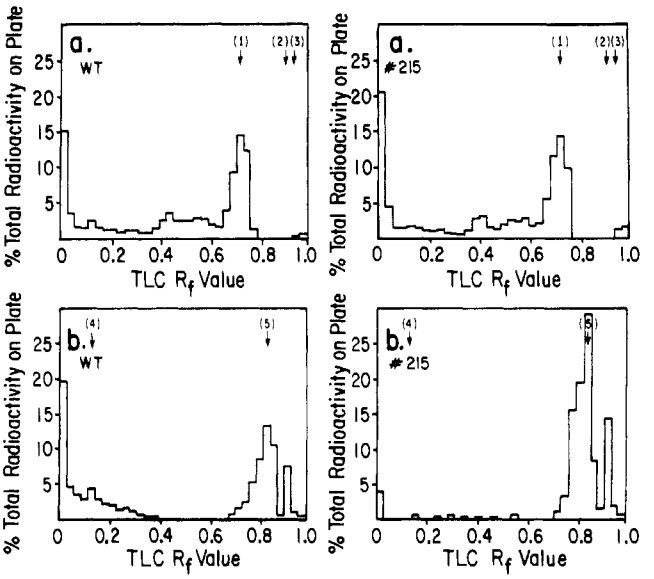

FIGURE 1: Identification of acetylated ^{14}C-labeled C_{28}–C_{30} sterols (a) and acetylated ^{14}C-labeled C_{27} sterols (b) after a [^{14}C]acetate pulse in wild-type and mutant 215 cells by argentation TLC (system V). The nonsaponifiable lipid fraction isolated after whole-cell [^{14}C]acetate incorporation (as described in Table II) was chromatographed on TLC system I. The C_{27} sterol band and the C_{28}, C_{29}, and C_{30} sterol bands were scraped into separate tubes and extracted with ethyl acetate. The ethyl acetate extract was washed 2 times with H_2O. A small aliquot of each extract was counted to determine radioactivity. Aliquots containing at least 5000 dpm of [^{14}C]sterol were then transferred to conical extraction tubes and blown to dryness under N_2. The contents of each tube were then acetylated, chromatographed on TLC system V, and analyzed as described under Materials and Methods. The migrations of acetylated authentic external standards are indicated as numbered arrows: (1) lanosterol; (2) 4,4-[^{14}C]dimethylcholest-7-enol; (3) dihydrolanosterol; (4) desmosterol; (5) cholesterol.

showing that the Δ^{24}-sterol reductase activity (Steinberg & Avigan, 1969) of mutant 215 cells is probably intact. These data also suggest that desmosterol rather than C_{28}, C_{29}, or C_{30} sterols may be the preferred substrate of Δ^{24}-sterol reductase in CHO cells. Figure 1b shows that an unidentified labeled compound of high R_f value (≥0.9) is found upon analysis of the C_{27} sterol band in both wild-type and mutant 215. The identity of this labeled compound is unknown at present.

Data from the [^{14}C]acetate pulse experiment are consistent with previous work (Chang et al., 1977) localizing the lesion of mutant 215 to the state of lanosterol demethylation. In addition, these data show that [^{14}C]sterol formation from [^{14}C]acetate is diminished in the mutant, while ^{14}C-labeled

[3] This tentative assignment was made by extrapolating TLC migrations of these compounds on a TLC system described by Gibbons et al. (1973) to TLC system V.

Table III: Accumulation of Sterol Metabolites in [^{3}H]Dihydrolanosterol Incorporation in Intact Cells of Wild-Type, Mutant 215, and Revertant 40 Cells[a]

cell type	% of incorporated [^{3}H]sterol			amount of total [^{3}H]sterol incorporated (ng/mg of protein)
	4,4-dimethyl[^{3}H]-cholest-7-enol	4α-methyl[^{3}H]-cholest-7-enol	[^{3}H]cholesterol	
wild type	2.88	9.66	5.57	0.18
mutant 215	3.79	4.90	1.05	0.18
revertant 40	3.14	6.71	5.74	0.20

[a] Cells grown in F-12 plus 10% FCS were trypsinized and replated with 20 mL of F-12 plus 10% FCS in 75-cm^2 flasks at 0.48 × 10^6 cells per flask for wild-type, 0.66 × 10^6 cells per flask for revertant 40, and 0.78 × 10^6 cells per flask for mutant 215 cells. After 60 h, the medium was drained off. The cells were rinsed with 9 mL of PBS, fed with 10 mL of F-12 plus 10% DeL medium, and grown for 24 h. The medium was then removed, and 5 mL of F-12 plus 10% DeL medium containing [^{3}H]dihydrolanosterol (16 μg/mL, 9.9 mCi/mmol) was added. After the cells were incubated for 12 h, the medium was removed, and cells were washed 6 times with 5 mL of cold PBS. Cells were dissolved in 2 mL of 1.5 M KOH. The resulting cell extract was saponified, extracted, washed, acetylated, and chromatographed on TLC system IV exactly as described under Materials and Methods for the 14α-demethylase assay in vitro. Total cellular incorporation of [^{3}H]dihydrolanosterol was approximately 0.3% of the total [^{3}H]dihydrolanosterol in the medium for three cell types. Each value represents the average of results from duplicate flasks. Variation between duplicates was within 15% of the mean.

fatty acid formation from [^{14}C]acetate is higher in mutant 215 than in wild-type cells. The percent desaturation of ^{14}C-labeled fatty acids is found to be very similar in the mutant and wild-type cells.

Respiratory System of Mutant 215 Is Intact. The possibility existed that mutant 215 cells might contain an unknown defect in the respiratory chain (Gaylor et al., 1966; Miller et al., 1967) which might cause a pleiotropic abnormality in sterol demethylation activities. Such pleiotropic mutants have been isolated from yeasts (Bard et al., 1974; Gollub et al., 1974; Karst & Lacroute, 1973; Resnick & Mortimer, 1966; Wada et al., 1969). To test this possiblity, wild-type and mutant 215 cells grown in DeL medium were pulsed with [2-^{14}C]pyruvate, and $^{14}CO_2$ was collected (see Materials and Methods). The results of this experiment showed that mutant 215 and wild-type cells have identical rates of $^{14}CO_2$ production from [2-^{14}C]pyruvate (4.8 nmol of $^{14}CO_2$ (mg of protein)$^{-1}$ h^{-1} for both cell types), indicating that the lesion in mutant 215 is not related to any abnormality in the respiratory system. Also, TLC analyses of [^{14}C]sterol and ^{14}C-labeled fatty acid formations from this [^{14}C]pyruvate pulse yielded data very similar to those of the [^{14}C]acetate pulse presented in Table II, showing that the metabolism between pyruvate and cytosolic acetyl-CoA is not impaired in the mutant.

3-Hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) Reductase Activities in Wild-Type and Mutant 215 Cell Extracts. HMG-CoA reductase activities in cell extracts of wild-type and mutant 215 cells were measured by using methods described previously (Chang & Limanek, 1980; Limanek et al., 1978). Cell extracts of wild-type and mutant 215 cells grown in FCS or DeL were found to contain the same HMG-CoA reductase activities (data not shown). These results suggest that neither the defect nor manifestations of the defect in mutant 215 caused or were caused by abnormal HMG-CoA reductase levels.

Metabolism of [^{3}H]Dihydrolanosterol in Intact Cells. Because authentic C_{27}, C_{29}, and C_{30} sterol standards without the Δ^{24} bond were available, a whole-cell [^{3}H]dihydrolanosterol incorporation experiment was performed. The results of this experiment (Table III) show that mutant 215 accumulates slightly more ^{3}H-labeled 4,4-dimethylsterol than the wild type, while accumulating only about one-half as much 4α-methylsterol and less than 20% as much cholesterol as the wild type, suggesting that both 4,4-dimethylsterol and 4α-methylsterol demethylation reactions are defective in the mutant. Data presented in this table also show that revertant 40, a spontaneous revertant of mutant 215 (see Materials and Methods), has essentially the same [^{3}H]cholesterol accumulation as wild-type cells, suggesting that the abnormalities in sterol demethylation seen in the mutant are probably due to a single gene defect. As a control, the total amount of [^{3}H]sterol incorporated into wild-type, mutant 215, and revertant 40 cells was measured and found to be essentially identical (Table III, last column).

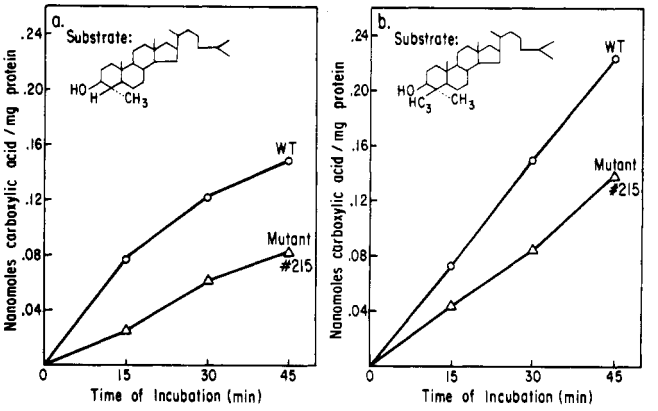

FIGURE 2: 4-Methylsterol oxidase activities using 4α-methylcholestanol (a) or 4,4-dimethylcholestanol (b) as substrate in cell homogenates of wild-type (○) and mutant 215 cells (△). Cell growth, homogenate preparation, and 4-methylsterol oxidase assay were described under Materials and Methods. Each point represents the average of results from duplicate assays. Variation between duplicates was within 7% of the mean.

4-Methylsterol Oxidase Assay in Vitro. The establishment of several in vitro 4-methylsterol oxidase assays has been reported (Brady et al., 1976; Gaylor et al., 1975; Swindell & Gaylor, 1968). Most of them require coupling of an aerobic oxidation step to the anaerobic decarboxylation step (to produce $^{14}CO_2$). Such assays are dependent upon two enzyme activities, the oxidase and the decarboxylase. We sought to develop a new 4-methylsterol oxidase assay not dependent upon the decarboxylase activity yet sensitive enough to be practical for tissue culture work. This assay takes advantage of the fact that 4-methylsterol oxidase requires reduced pyridine nucleotide while the 4α-carboxylic acid decarboxylase requires NAD^+ (Miller & Gaylor, 1970a,b). The oxidation and decarboxylation reactions are uncoupled by excluding NAD^+ from the reaction mixture (Miller & Gaylor, 1970a,b). The product of this reaction is thus the 4α-carboxylic acid, which is easily separable from the substrate sterol by TLC. Incubations of 4,4-dimethyl- or 4α-methylcholestanol with CHO cell homogenates (or crude microsomal preparations) with NADPH in the absence of NAD^+ produced a radioactive fraction extractable from the reaction mixture by petroleum

ether only after acidification of the mixture. The amount of radioactivity in this acid fraction was shown to be fairly linearly dependent upon the length of incubation time for up to 45 min (Figure 2). TLC analyses of the acid fraction produced by the 4,4-dimethyl or the 4α-methyl substrate showed that a single product was produced from each substrate. The TLC mobilities of each product using cell extracts from both cell types were very similar to the mobilities reported by Gaylor's group (Miller & Gaylor, 1970a,b) for the 4α-carboxylic acids of similar sterol substrates.[4] Treatment of this acid fraction with diazomethane produced a compound of higher TLC mobility (using solvent systems II and III, respectively); the acidic sterol exhibiting original TLC mobility was regenerated with approximately 80% recovery by subsequent saponification of this compound (data not shown). These experiments provide strong evidence that the radioactivity in the acid fraction which accumulates during incubation is a carboxylic acid. Accumulation of radioactivity in the acid fraction was shown to be dependent upon the presence of Triton WR-1339 (data not shown), a nonionic detergent known to be necessary for 4-methylsterol oxidase activity (Brady et al., 1976; Miller et al., 1967, 1971; Miller & Gaylor, 1970a,b). Addition of excess NAD^+ to the reaction mixture caused a marked decrease (approximately 50%) of radioactivity in the acid fraction produced from 4α-[^{3}H]methylcholestanol substrate (data not shown). All of the data are consistent with the conclusion that this acidic fraction corresponds to the 4α-carboxylic acid derivatives of 4α-methylsterol produced by microsomal 4-methylsterol oxidase activity. A cell fractionation study using cell homogenates of both wild-type and mutant 215 cells showed that this 4-methylsterol oxidase activity, assayed by the accumulation of 4α-[^{3}H]carboxylic acid, is localized in the crude microsome with more than 90% recovery in activity. This finding is consistent with the fact that 4-methylsterol oxidase is known to be a microsomal enzyme (Brady et al., 1976; Miller et al., 1967, 1971; Miller & Gaylor, 1970a,b).

4-Methylsterol Oxidase Activity of Wild-Type and Mutant 215 Cell Extracts. As shown in Figure 2a,b the 4-methylsterol oxidase activity in cell extracts of mutant 215 using 4,4-dimethyl[2-^{3}H]cholestanol or 4α-methyl[2,4-^{3}H]cholestanol as the substrate was found to be defective as compared to that of the wild type.[5] Similar results were seen in three separate experiments. Temperature sensitivity curves of 4-methylsterol oxidase activity using 4,4-[^{3}H]dimethylcholestanol as the substrate were determined for wild-type and mutant 215 cell homogenates. No difference between wild-type and mutant 215 was detected at 50 °C; both activities were found to decay in first-order kinetics with a half-life of approximately 20 min.

Sterol 14α-Demethylase Activities of Wild-Type and Mutant 215 Cell Extracts. Lanosterol 14α-demethylase activity has been assayed by Mitropolous, Gibbons, and co-workers (Gibbons et al., 1979; Mitropolous et al., 1976) by measuring the rate of [^{14}C]formic acid release from [32-^{14}C]dihydrolanosterol. An alternative 14α-demethylase assay using the rate of 4,4-[^{3}H]dimethylcholest-7(8)-en-3β-ol formation from [^{3}H]dihydrolanosterol was developed in this laboratory. A TLC system to separate the acetate derivatives of the substrate, dihydrolanosterol, and the product, 4,4-dimethylcholest-7-(8)-en-3β-ol, was optimized. The identity of the reaction product was confirmed by showing it comigrated with authentic 4,4-dimethylcholest-7-en-3β-ol on TLC systems IV and V. The reaction was shown to be linear for up to 45 min. Figure 3 shows that cell extracts of wild type and mutant 215 contain very similar sterol 14α-demethylation activities. Similar results were obtained in two separate experiments.

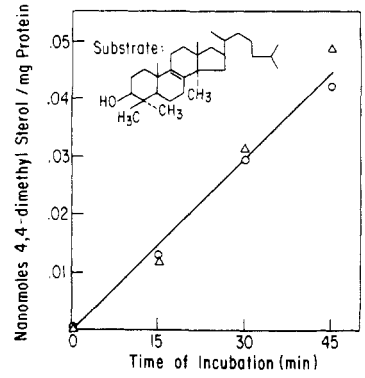

FIGURE 3: Lanosterol 14α-demethylase activity in cell homogenates of wild-type (○) and mutant 215 (△) cells. 14α-Demethylase was assayed by using the procedure described under Materials and Methods. Cells were grown in α-MEM plus 8% NBS and 2% FCS. Each point represents the average of duplicate assays. Variation between duplicates was within 10% of the mean.

Discussion

The spontaneous reversion frequency of mutant 215 cells to cells capable of growth in delipidated serum medium was found to be approximately 0.8×10^{-6}. One spontaneous revertant, revertant 40, was further analyzed to ensure that putative revertants had regained full cholesterol biosynthetic capacity. Revertant 40 was shown to have regained normal ability to convert labeled dihydrolanosterol to labeled cholesterol (Table III). These findings suggest that the sterol demethylation lesion in mutant 215 is a single gene defect.

Evidence presented in this paper suggests that the inability of mutant 215 cells to synthesize cholesterol is due to a defective demethylation mechanism for 4,4-dimethyl- and 4α-methylsterols. With the assumption that the lesion in mutant 215 is a single gene defect, the finding that microsomal 4α-methylsterol oxidase activities on 4,4-[^{3}H]dimethylcholestanol or 4α-[^{3}H]methylcholestanol were similarly defective in mutant 215 suggests that these enzyme activities share a common component which is defective in the mutant. The work of Gaylor and co-workers [for a review, see Gaylor (1977)] has shown that the rat liver 4α-methylsterol oxidases using 4,4-dimethyl and 4α-methyl substrates exhibit identical sensitivity to heat, glycothiocholate, trypsin, dithiothreitol, and cyanide, suggesting that the enzyme systems utilizing the two substrates may share some common components. Our results are consistent with this view. At present, it is not clear whether mutant 215 cells produce a defective protein component necessary for the C-4 demethylation reaction or produce such a protein component in lesser quantity.

We have also developed an in vitro assay for measuring lanosterol 14α-demethylase activity with labeled dihydrolanosterol as the substrate. Using this assay, we showed (Figure 3) that cell extracts of wild-type and mutant 215 cells possess very similar lanosterol 14α-demethylation activities. These data suggest that the lesion in mutant 215 cells only involves C-4 demethylation reactions and does not directly involve C-14 demethylation reactions. Previously, work by other investigators (Gibbons & Mitropolous, 1973a,b, 1974) has shown that the enzyme components catalyzing the C-14α

[4] The identity of 3β-hydroxy-4β-methyl[2-^{3}H]cholestan-4α-oic acid was further established by its comigration in TLC system III (Table I) with the chemically synthesized standard before and after methylation by diazomethane.

[5] Similar results were obtained by using the substrate containing the Δ^7 double bond (4,4-dimethyl[30,30-$^{14}C_2$]cholest-7-en-3β-ol) for measuring methylsterol oxidase activities in wild-type and mutant 215 cell extracts.

demethylation reactions are subject to inhibition by carbon monoxide and have different cofactor requirements (strictly NADPH, not NADH) than the steps in C-4α demethylation reactions. Our findings are consistent with the view that C-4 and C-14 demethylation pathways utilize different enzyme systems.

The whole cell [^{14}C]acetate pulse results (Table II) were not well explained by the conclusion that the defect in mutant 215 was in 4-methylsterol oxidase activity. While such a defect would be expected to be manifested by an accumulation of 4,4-[^{14}C]dimethylcholest-7(8),24-dien-3β-ol in a [^{14}C]acetate pulse, the predominant labeled sterol accumulated in mutant 215 was instead [^{14}C]lanosterol. That lanosterol, instead of its C-14 demethylated sterol derivatives, accumulated in mutant 215 cells grown in serum-free medium was previously established by gas–liquid chromatography–mass spectrometry analysis (Chang et al., 1977). The reasons for these findings are not clear; however, at least three possible explanations exist. One possible explanation involves reports by Gibbons et al. (1979) that the initial step in 14α-demethylation is inhibited in vitro by low concentrations of its product, 5α-lanost-8-en-3β,32-diol, and by the corresponding 32-aldehyde (the product of the next step in 14α-demethylation) in rat liver microsome. The 4-methylsterol oxidase defect in mutant 215 could cause an increase in the cellular concentration of intermediate sterols in lanosterol demethylation which might inhibit the initial step in lanosterol demethylation, thereby causing lanosterol accumulation in the mutant. However, comparison of the sterols formed from [^{14}C]acetate in mutant 215 and wild-type cells (Figure 1) does not show accumulation in mutant 215 of any polar or nonpolar lanosterol metabolites to levels above those found in the wild type. Therefore, direct feedback inhibition of 14α-demethylase by a lanosterol metabolite seems an unlikely mechanism for the accumulation of lanosterol in mutant 215. A second explanation for lanosterol accumulation in mutant 215 is that metabolites of lanosterol may be produced in intact cells only as protein–sterol complexes which do not dissociate appreciably to give free, unbound sterol intermediates. If the efficiency of a sterol demethylation reaction in intact cells depends on rapid release of its reaction product, which in turn is controlled by the rate of successive demethylation reactions, a lesion in mutant 215 at 4α-methylsterol oxidase would lead to an initial backup of partially demethylated sterol intermediates, saturating the enzymes in the demethylation pathway with bound sterols. The net effect in mutant 215 would be a slower release of the reaction product from the enzyme catalyzing the 14α-demethylation reactions, which would lead to accumulation of lanosterol. In otherwords, our results are consistent with the hypothesis that the enzymes catalyzing the successive C-14α and C-4α demethylation steps are tightly associated with one another and highly organized in the microsomal membrane; the sterol metabolites of lanosterol destined to be demethylated are rapidly transferred as sterol–protein complexes within the multienzyme unit without being efficiently released as unbound, free sterols. Further experiments, including purification and reconstitution of the enzyme components of lanosterol demethylation, are needed to test the validity of this hypothesis. A final explanation for lanosterol accumulation in mutant 215, which cannot be entirely eliminated by the data presented in this paper, is that a defective C-14 demethylation system exists in intact cells of mutant 215, but the assay conditions for C-14 demethylation in vitro are not suitable to demonstrate such a defect. This possibility can be explored more effectively in the future through the isolation and characterization of additional mutants in the lanosterol demethylation pathway.

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Phosphorus-31 Nuclear Magnetic Resonance of Double- and Triple-Helical Nucleic Acids. Phosphorus-31 Chemical Shifts as a Probe of Phosphorus–Oxygen Ester Bond Torsional Angles†

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ABSTRACT: The temperature dependence to the ^{31}P NMR spectra of poly[d(GC)]·poly[d(GC)], $d(GC)_4$, phenylalanine tRNA (yeast) and mixtures of poly(A) + oligo(U) is presented. The ^{31}P NMR spectra of mixtures of complementary RNA and of the poly d(GC) self-complementary DNA provide torsional information on the phosphate ester conformation in the double, triple, and "Z" helix. The increasing downfield shift with temperature for the single-strand nucleic acids provides a measure of the change in the phosphate ester conformation in the single helix to coil conversion. A separate upfield peak (20–26% of the total phosphates) is observed at lower temperatures in the oligo(U)·poly(A) mixtures which is assigned to the double helix/triple helix. Proton NMR and UV spectra confirm the presence of the multistrand forms. The ^{31}P chemical shift for the double helix/triple helix is 0.2–0.5 ppm upfield from the chemical shift for the single helix which in turn is 1.0 ppm upfield from the chemical shift for the random coil conformation.

We have recently proposed that ^{31}P chemical shifts in phosphate esters may serve as a direct probe of P–O ester bond torsional angles. Both theoretical considerations [Gorenstein & Kar, 1975; see also Gorenstein (1975, 1978, 1981) and Pradd et al. (1979)] and direct experimental tests of this hypothesis [Gorenstein et al., 1976; Gorenstein & Luxon, 1979; see also Patel (1979a,b) and Gueron & Shulman (1975)] confirm that the ^{31}P signal of a phosphate diester monoanion in a gauche,gauche (g,g)[1] conformation (as found in the helix state) should resonate several parts per million upfield from a diester in a nongauche conformation (as found in the random coil state).

These conclusions prove to be especially significant since other spectroscopic probes fail to provide detailed conformational information on the phosphate ester bonds in the nucleic acids. Since it is now believed that of the six torsional angles that largely define the conformational structure of nucleic acids, the two P–O ester torsional angles provide the main conformational flexibility to the nucleic acid backbone (Kim et al., 1973; Sundaralingam, 1969). Thus ^{31}P NMR can monitor the "helix–coil" transitions in single-stranded nucleic acids (Gorenstein et al., 1976; Haasnoot & Altona, 1979). A large (0.7–1.3-ppm) downfield shift for a wide structural range of nucleic acids was observed (Gorenstein et al., 1976) upon raising the temperature. At low temperature the nucleic acids will exist largely in a base stacked, helical conformation with the phosphate ester predominantly in the g,g conformation, while at higher temperatures the nucleic acids will largely exist in random coil, unstacked conformations with the phosphate ester in an increased proportion of nongauche (i.e., g,t, etc.) conformations [see, for example, reviews such as Ts'o (1975)].

Earlier ^{31}P NMR[2] model studies were conducted on a number of simple ribo- and deoxyribodinucleoside monophosphates and homopolyribonucleic acids [see also Akasaka et al. (1975) and Cozzone & Jardetzky (1976)]. In the present work we extend these ^{31}P NMR studies on single-stranded nucleic acids to double- and triple-stranded nucleic acids. In

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[1] We should mention that for purposes of conveniently describing the torsional dependence to chemical shifts, we generally make no distinction between torsional angles +60° (+g) or −60° (−g). In addition, ω,ω' torsional angles of g,t (60°,180°), $-g,t$, t,g, and $t,-g$ will often be grouped together as g,t. Similarly, g,g includes conformers $-g,-g$, $g,-g$, and $-g,g$, although the latter two conformers do have ^{31}P chemical shifts that are different from g,g (Gorenstein & Kar, 1975).

[2] Abbreviations: NMR, nuclear magnetic resonance; ORD, optical rotatory dispersion; CD, circular dichroism, EDTA, (ethylenedinitrilo)-tetraacetic acid; poly(A), poly(adenylic acid); oligo(U), oligo(uridylic acid); poly[d(GC)], poly(deoxyguanidylcytidylic acid); bp, base pair.

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