Investigation of the Component Reactions of Oxidative Sterol Demethylation. Study of the Aerobic and Anaerobic Processes*

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ABSTRACT: Microsomal enzymes catalyze the oxidative demethylation of lanosterol to yield carbon dioxide from the methyl groups on positions 4α, 4β, and 14α. Others proposed that demethylation is composed of two separate enzymatic processes: aerobic hydroxylation of the methyl group and anaerobic dehydrogenation of the primary alcohol. A procedure has been developed to study separately these aerobic and anaerobic activities. Rat liver microsomes treated with a solution of Triton WR-1339 in buffer do not catalyze the oxidative demethylation of 4,4-dimethyl-5α-cholest-7-en-3β-ol. Addition of pyridine nucleotides completely restores demethylase activity. Reduced pyridine nucleotide is required for the aerobic reaction. Dehydrogenations (and decarboxylation) may occur under anaerobic conditions; oxidized pyridine nucleotide is required. Assay of the aerobic activity by a two-step incubation procedure is described. A normal time course of the aerobic reaction is observed. In the two-step process equal quantities of CO_2 and 4α-methyl-5α-cholest-7-en-3β-ol are formed from 4,4-dimethyl-5α-cholest-7-en-3β-ol. Apparently, complete oxidative cleavage of the methyl group that is hydroxylated first precedes hydroxylation of the other 4-methyl group.

In the terminal reactions of sterol biosynthesis microsomal enzymes catalyze the oxidative demethylation[1] of lanosterol (4,4,14α-trimethyl-5α-cholesta-8,24-dien-3β-ol) and 4,4-dimethyl-5α-cholest-7-en-3β-ol, a synthetic sterol, to yield carbon dioxide from the 4α-, 4β-, and 14α-methyl groups (Lindberg *et al.*, 1963; Gaylor, 1964). Bloch and co-workers have proposed that demethylation is initiated by enzymatic attack of the methyl groups in positions 4α, 4β, and 14α to yield hydroxymethyl intermediates (Olson *et al.*, 1957; Bloch, 1965). Subsequent dehydrogenation of the hydroxymethyl intermediates produces carbon dioxide

$$RCH_3 \xrightarrow{A} RCH_2OH \xrightarrow{B} RCHO \xrightarrow{C} RCOOH \xrightarrow{D} RH + CO_2$$

Little is known about this sequence of reactions because conditions have not been obtained to study the properties of the individual microsomal enzymes. Initial hydroxylation (reaction A) probably requires atmospheric oxygen and a reduced pyridine nucleotide (Olson *et al.*, 1957). Presumably, the alcohol and aldehyde dehydrogenases[2] (B and C, respectively) are pyridine nucleotide dependent enzymes if they are similar to other dehydrogenases (Olson, 1965; Pudles and Bloch, 1960). Possibly, reaction D proceeds noncatalytically (unpublished data cited in Lindberg *et al.*, 1963).

The individual steps catalyzed by these particulate enzymes may be investigated by obtaining appropriate conditions to eliminate concomitant reactions of the substrate or product. For example, to study demethylase activities 4,4-dimethyl-5α-cholest-7-en-3β-ol is used as substrate and well-washed microsomes are used as the source of enzymes. These choices eliminate concomitant reactions of isomerization of 8 to 7 isomers, saturation of the side-chain double bond, and redox at position 3 that are catalyzed by microsomal enzymes (Gaylor *et al.*, 1966a; Avigan *et al.*, 1963; Frantz *et al.*, 1959; Swindell and Gaylor, 1967).

In this initial report, conditions that permit the separate investigation of the aerobic reaction (A) and the anaerobic reactions (B to D) are described. A two-step incubation procedure has been developed to assay the aerobic process. The cofactor requirements of the enzymes and the identification of a monomethylsterol

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[1] Oxidative demethylation of lanosterol is the loss of methyl groups from positions 4α, 4β, and 14α (Olson *et al.*, 1957).

[2] The term hydroxylase in this report refers to the activity associated with the enzymatic hydroxylation of a methylsterol substrate. The use of the term does not suggest knowledge about the actual process of hydroxylation. Dehydrogenase refers to the alcohol and aldehyde dehydrogenases that catalyze the conversion of hydroxymethyl intermediates to carboxylates. Because decarboxylation may occur spontaneously, the term dehydrogenase in this report includes the processes of dehydrogenation and decarboxylation.

product of the two-step incubation procedure are described.

Experimental Procedures

Incubation Procedures. Liver from adult male rats was dissected and suspended in two volumes of 0.1 M phosphate buffer (pH 7.4 and 30 mM nicotinamide). The liver suspension was homogenized in a TenBroeck glass homogenizer. The suspension was centrifuged for 20 min at 10,000*g*. The supernatant fraction was decanted. The supernatant fraction from centrifugation at 10,000*g* then was centrifuged for 45 min at 105,000*g* (No. 40 rotor, Spinco Model L centrifuge, 40,000 rpm). The supernatant fraction from centrifugation at 105,000*g* was decanted and discarded. The precipitate (microsomes) was suspended with homogenization after the addition of fresh buffer. The final volume of the suspension was adjusted to 25% of the volume of supernatant fraction from centrifugation at 10,000*g*. Unless otherwise specified an equal volume of a 20% solution (w/v) of Triton WR-1339 in 0.1 M phosphate buffer was added. The mixture was incubated for 10 min at 37°. Then the mixture was cooled to 4°. An equal volume of cold phosphate buffer was added, and the suspension was centrifuged again for 45 min at 105,000*g*. The precipitate (Triton-treated microsomes) either was suspended in buffer for incubation or frozen as pellets. In either case fresh buffer was added to yield a final volume of 25% of the volume of supernatant fraction from centrifugation at 10,000*g*. When the microsomes were to be frozen all buffers contained 1 mM glutathione (Gaylor *et al.*, 1966b). No loss of activity from frozen samples was observed.

For incubation, 1 ml of the suspension of Triton-treated microsomes (28–43 mg of protein) was added to 100 mμmoles of [30,31-di-^{14}C]4,4-dimethyl-5α-cholest-7-en-3β-ol that was suspended by the aid of Tween 80 (Gaylor, 1964). The final volume of the incubation mixture was 1.6 ml. Additions generally were made 5 min before substrate was added. Incubations in stoppered 50-ml erlenmeyer flasks were for 30 min at 37° under 100% oxygen. Collection and assay of $^{14}CO_2$ were carried out exactly as described previously (Gaylor, 1964). The rate of demethylation was calculated from the amount of $^{14}CO_2$ released and the specific activity of the substrate. For purposes of calculation it was assumed that only one methyl group on position 4 was removed; demethylation rates were calculated accordingly.

The following procedure was used to isolate the product of demethylation. Approximately 1 mg of unlabeled 4α-methyl-5α-cholest-7-en-3β-ol was added to the heat-killed contents of the incubation flasks before saponification and extraction. The monomethylsterols were separated from the large amount of endogenous cholesterol and substrate by chromatography on acid-washed alumina (Brockmann grade V; Gaylor, 1963). 4-Methylsterols were collected and chromatographed on an 8 mm × 6 ft column of 2% SE-30 as described previously (Gaylor *et al.*, 1966b). 4α-Methyl-5α-cholest-7-en-3β-ol and 4,4-dimethyl-5α-cholest-7-en-3β-ol are easily separated by chromatography on alumina and SE-30; the retention times on the latter, relative to cholestane, are 2.55 and 3.39, respectively (Gaylor *et al.*, 1966b). The 4-monomethylsterol was collected during gas–liquid partition chromatography in the range from 2.40 to 2.70 (2.55 ± 0.15) on the relative time scale. 4β-Methyl-5α-cholest-7-en-3β-ol has a relative retention time of 2.75 (unpublished results). Protein was determined by the method of Lowry *et al.* (1951); bovine serum albumin was used as standard.

Materials. The ^{14}C-labeled substrate (41,300 dpm/mg) was prepared from [^{14}C]methyl iodide (New England Nuclear Corp.) exactly as described by Gautschi and Bloch (1958). The physical constants of the compound agreed with reported values. Gas–liquid partition radiochromatographic analysis revealed that the compound was greater than 95% pure 4,4-dimethyl-5α-cholest-7-en-3β-ol, and the compound contained the isomeric 4,4-dimethyl-5α-cholest-8-en-3β-ol as the only contaminant.

4α-Methyl-5α-cholest-7-en-3β-ol was synthesized exactly as described by Wells and Lorah (1960). The physical constants and retention times on gas–liquid partition chromatography agreed with reported values and an authentic sample kindly supplied by Professor Wells.

Initially, the Triton WR-1339 was a gift of the Rohm and Haas Co., Philadelphia, Pa. Recently, the Triton (lot AC 148) was purchased from the Ruger Chemical Co., Irvington-on-Hudson, N. Y.

Tween 80 was purchased from the Nutritional Biochemicals Corp., Cleveland, Ohio. DPN[3] was purchased from P-L Biochemicals, Milwaukee, Wis. Glucose oxidase (purified, type II), TPN, DPNH, TPNH, and isocitric dehydrogenase (purified, type IV) were purchased from Sigma Chemical Co., St. Louis, Mo. β-Hydroxybutyric dehydrogenase (purified, suspension) was obtained from C. F. Boehringer. Common biochemicals were purchased from Calbiochem, Sigma Chemical Co., and Nutritional Biochemicals Corp.

Effect of Triton on Microsomal Composition and Morphology. The relative content of protein and nucleic acid in microsomes was assayed spectrophotometrically. Approximately 7 mg of microsomal protein was added to cuvets and a solution of deoxycholate was added to clear the turbidity. Absorption at 260 and 280 mμ was determined. The approximate protein and nucleic acid content was determined with the nomograph of Adams (distributed by Calbiochem). The ratio of protein to nucleic acid content for untreated microsomes was 6.5; for Triton-treated microsomes the ratio was 14.5.

The relative loss of nucleic acid was confirmed by electron microscopy. Frozen pellets of untreated and Triton-treated microsomes were collected, thawed,

[3] Abbreviations used: DPN, diphosphopyridine nucleotide, oxidized form; TPN, triphosphopyridine nucleotide, oxidized form; reduced forms, DPNH, and TPNH, respectively.

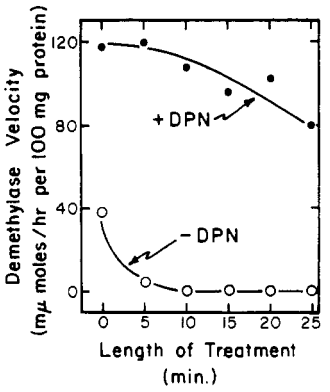

FIGURE 1: Effect of length of Triton treatment. The suspension of treated microsomes was cooled and centrifuged and the resulting microsomes were suspended in fresh buffer. Samples were incubated without (○—○) or with DPN (0.5 mM (●—●)). Each value is the average of results from three separate incubations.

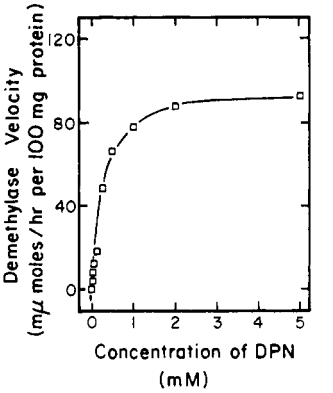

FIGURE 2: Effect of DPN concentration. Each value is the average of results from two separate incubations.

and fixed with buffered osmium tetroxide solution. The materials were dehydrated and embedded. Sections of 300 A thickness were cut, and the sections were doubly stained with uranium acetate and lead citrate. Grossly, the ribosomes of untreated microsomes were sedimented in clusters in the pellet. Triton treatment reduced the relative abundance of ribosomes and the ribosomes still present were not clustered, but these dense granules were distributed throughout the entire pellet.

Results

Effect of Triton and Conditions for Assay of Demethylase Activity. Rat liver microsomes were treated with a 10% solution of Triton in buffer for 5–25 min. In the absence of added DPN the rate of demethylation by untreated microsomes was about 30% of the rate with added DPN (zero time, Figure 1). Exposure of microsomes to Triton further reduced the rate of demethylation to zero. In the presence of DPN, Triton treatment produced only a slight loss of activity. Thus, treatment of rat liver microsomes with 10% Triton for 10 min produced an absolute requirement of the demethylase system for exogenous DPN.

Incubation of Triton-treated microsomes with increasing amounts of DPN yielded increasing amounts of activity (Figure 2). Maximal rates were observed when the concentrations of DPN were 2 mM or more. Lineweaver-Burk plots of the effect of lanosterol concentration on observed demethylation rates yielded straight lines (Gaylor, 1964). The reciprocal plot of the data from Figure 2 did not yield a single straight line.

TPN was substituted for DPN (Table I). The rate of demethylation with TPN was considerably slower than with DPN.

Incubation under an atmosphere of approximately 1% of oxygen did not severely decrease the rate of demethylation (Table II). Addition of glucose and glucose oxidase removed most of the dissolved oxygen, and loss of most of the demethylase activity was observed. When the suspension of microsomes was equilibrated with nitrogen before glucose and glucose oxidase were added, demethylase activity was reduced to zero. Thus, with this technique oxygen was suitably limited to prevent sterol demethylation.

Conditions for the Two-Step Incubation and Assay of the Aerobic Activity. Aerobic incubation of samples of Triton-treated microsomes yielded 180 mμmoles of sterol demethylated per hr (Table III). Another sample of the same microsomal preparation was incubated in two steps. First the samples were incubated aerobically with a TPNH generator. Following the 30-min incubation, the flasks were cooled in an ice bath and nitrogen was bubbled slowly into the suspension of microsomes. The flasks were stoppered. Glucose and glucose oxidase were added, and the samples were incubated for 5 min at 37° to consume residual oxygen. A solution of DPN was injected into each flask, and the suspensions were incubated again for 15–30 min at 37°.

TABLE I: Substitution of TPN for DPN.

Addition (mg)	No. of Samples	Rate of Demethylation (mμmoles/hr per 100 mg of protein)
None	5	0
DPN[a] (1.0)	10	130
TPN (1.0)	5	72.8

[a] This value is the average of data from five samples incubated with 1.0 mg of DPN and five samples incubated with 1.0 mg of DPN plus 1.0 mg of TPNH. Equal activity was observed under each condition.

TABLE II: Production of Anaerobic Conditions.

Addition (mg)	Atmosphere	Buffer Equilibrated with	No. of Samples	Rate of Demethylation (mμmoles/hr per 100 mg of protein)
None	~1% O_2	Air	8	134
Glucose (20) + glucose oxidase (2)[a]	~1% O_2	Air	8	27
Glucose (20) + glucose oxidase (2)[a]	N_2	N_2	8	<1.4

[a] The concentration of oxygen in the buffer was measured (oxygen electrode method) before and during the treatment with glucose oxidase. The concentration of dissolved oxygen was decreased to 5% of the initial concentration within 1 min after the addition of glucose to the microsomal preparation. Relatively inaccurate and difficult analyses of the final concentration of oxygen in the closed system suggest that anaerobic conditions are not obtained, but that the incubation flasks contain as little as 6.6 mμmoles of oxygen in the 1.6 ml of buffer and approximately 50 ml of atmosphere.

A rate of 138 mμmoles/hr of sterol demethylated was observed.

DPN was required for the reactions of step II. Similarly, omission of step II prevented the release of $^{14}CO_2$. As observed previously, no demethylation occurred under anaerobic conditions (Table III). Conversely, oxidized pyridine nucleotide was not required for step I because the generating systems kept the TPN in the reduced form. In addition, preliminary investigation of the reactions of step II with isolated products of step I and fresh microsomes revealed that exogenous reduced pyridine nucleotide was not required for step II. Reduced pyridine nucleotide was required for the enzymatic activity of step I. The aerobic reaction was somewhat more active with DPNH than with TPNH (Table III).

The time course of the aerobic activity was investigated (Figure 3). Reaction velocity was essentially constant through 10 min of incubation. Considerable variation was observed after 10 min of aerobic incubation.

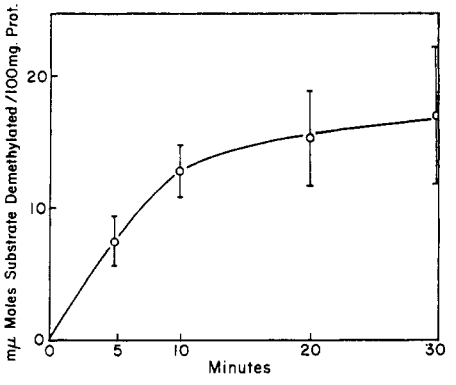

FIGURE 3: Time course of hydroxylation. The length of aerobic incubation was terminated by heating the samples at 80° for 5 min. Fresh Triton-treated microsomes were added to the cooled samples. The results show the means and standard deviations of seven samples each.

Reaction Products and Stoichiometry. Facile identification of the intermediate following aerobic hydroxylation was prevented by extreme lability of the small amount of material. Characterization of the labile intermediate must await synthesis of the two most likely products: 4α-hydroxymethyl-4β-methyl- and 4β-hydroxymethyl-4α-methyl-5α-cholest-7-en-3β-ol.

TABLE III: Two-Step Incubation Conditions and Cofactor Requirements.

Additions			
Step I (aerobic)	Step II (anaerobic)	No. of Samples	Rate of Demethylation (mμmoles/hr per 100 mg of protein)
DPN	Omitted	6	180
TPNH[a]	DPN	5	138
TPNH	None	5	<1.2
TPNH	Omitted		
Omitted	DPN		
None	DPN	2	5.4[b]
TPNH	DPN	6	93[b]
DPNH[c]	DPN	6	113[b]

[a] TPNH generator; 117 μmoles of isocitric acid, 1.3 μmoles of TPNH, and 0.5 mg of isocitric dehydrogenase. [b] Samples were heat denatured after step I. Fresh Triton-treated microsomes were added after the samples were cooled to 4°. [c] DPNH generator; 39.6 μmoles of β-hydroxybutyric acid, 1.3 μmoles of DPNH, and 0.1 mg of β-hydroxybutyric dehydrogenase.

The product of the two-step incubation was identified, and the stoichiometry of $^{14}CO_2$ and [^{14}C]4-monomethylsterol formation was studied with the two-step incubation. The yield of 4-monomethylsterol essentially equalled the yield of $^{14}CO_2$ in all experiments (Table IV).

TABLE IV: Stoichiometric Formation of $^{14}CO_2$ and [^{14}C]4-Methylsterol by the Two-Step Incubation.

Expt	$^{14}CO_2$ (dpm)	[^{14}C]4-Methylsterol[a] (dpm)	(^{14}C in 4-Methylsterol/^{14}C in CO_2) × 100
I	1000	720	72
II	1540	1195	78
III	1240	1060	86

[a] The samples were corrected for "apparent" [^{14}C]4-methylsterol by subtraction of a small amount of ^{14}C radioactivity recovered from an identical sample that was incubated with heat-killed Triton-treated microsomes.

Thus, the stoichiometry suggests that only one of the two methyl groups on position four was hydroxylated and released as CO_2. This lower recovery of the monomethylsterol from the gas–liquid partition chromatographic technique may be ascribed to either minor losses during the extensive chromatography or a very small amount of demethylation of the monomethylsterol in addition to this loss.

In addition to the gas–liquid partition chromatographic behavior of the isolated 4-methylsterol product (Table IV), the labeled compound has been further identified as 4α-methyl-5α-cholest-7-en-3β-ol by additional carrier techniques.

Finally, the two-step incubation was repeated by incubation of [30,31-di-^{14}C]4,4-dimethyl-5α-cholest-7-en-3β-ol that was further labeled with tritium in position 2 (Lindberg *et al.*, 1963). The ratio of ^{3}H to ^{14}C content of the substrate was between 1.87 and 1.90. In three separate incubations the average yield of [^{14}C]4α-methyl-5α-cholest-7-en-3β-ol was 92% of the yield of $^{14}CO_2$. The ratio of ^{3}H to ^{14}C content of the isolated 4α-methyl-5α-cholest-7-en-3β-ol was between 2.85 and 2.96. Thus, an average of 1.5 of the 2 tritiums on position 2 was retained.

Discussion

Demethylation required oxygen (Table II), reduced pyridine nucleotide (Table III), and oxidized pyridine nucleotide (Figures 1 and 2, Tables I and III). The oxygen and reduced pyridine nucleotide must be supplied simultaneously during step I of Table III. As predicted by Olson (1965), oxidized pyridine nucleotide is required for the dehydrogenases (step II reactions). When DPN is added to the aerobic mixture as in the measurement of over-all demethylase activity, addition of exogenous reduced pyridine nucleotide is no longer required (see footnote *a* of Table I). Apparently, the generation of reduced pyridine nucleotide supports the aerobic process. Thus, as suggested by others, the microsomal dehydrogenases may supply reduced pyridine nucleotide for mixed-function oxidases (Mason *et al.*, 1965).

Provided suitably anaerobic conditions are achieved, no hydroxylation occurs during step II (Table II). By maintaining the pyridine nucleotides in the reduced form during step I, no dehydrogenation occurs in step I (Tables I and III). Thus, the two-step incubation may be used to assay the aerobic process of demethylation. When this assay procedure was used, a normal time course of reaction was observed (Figure 3).

Labeled 4α-methyl-5α-cholest-7-en-3β-ol was identified as the product of the two-step incubation. Further, equal amounts of the 4α-methylsterol and carbon dioxide were formed. Thus, only one methyl group was aerobically attacked during the step I process, and the hydroxylated methyl presumably was oxidized during step II. Had both methyl groups been hydroxylated simultaneously in step I, neither stoichiometric amounts of $^{14}CO_2$ and [^{14}C]monomethylsterol would have been formed nor would 4α-methyl-5α-cholest-7-en-3β-ol have been isolated.

Identification of the 4-monomethylsterol product demonstrates that conditions have been obtained to study demethylation of only one of the 4-methyl groups. Previously, it has been possible to investigate only complete demethylation to C_{27}-sterol products (Gaylor, 1964). Stepwise demethylation was confirmed recently. In the absence of added TPNH, stoichiometric amounts of $^{14}CO_2$ and [^{14}C]4α-methyl-5α-cholest-7-en-3-one were formed from 4,4-dimethyl-5α-cholest-7-en-3β-ol by this system (Swindell and Gaylor, 1967).

Although ^{14}C radioactivity of the 4-monomethylsterol remained with the 4α epimer (Table IV) either of the epimeric 4-methylsterols may have been formed and only the 4α-methyl compound was isolated. Chromatography on the acid-washed alumina may have effected epimerization. Acid-catalyzed epimerization of similar 4β- to 4α-methylsterol ketones was reported recently (Sanghvi *et al.*, 1967).

Acknowledgment

The authors acknowledge the careful preparation of [30,31-di-^{14}C]4,4-dimethyl-5α-cholest-7-en-3β-ol by Mr. A. J. Green. The electron microscopy was most thoughtfully carried out by a professor of this university.

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New Procedures for the Preparation of Complexes of Ribosomes with Polyuridylic Acid*

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ABSTRACT: A new method for the isolation of complexes of polyuridylic acid (poly U) with ribosomes is presented. Under certain conditions of salt and pH, free poly U is adsorbed by polyacrylamide gels while ribosome-bound poly U is not. Using columns of polyacrylamide, complexes of poly U with ribosomes can be separated from free poly U in a matter of minutes. Evidence which indicates that this column technique and the customary sucrose gradient procedure yield similar complexes is presented.

The interaction of poly U[1] with ribosomes has been investigated extensively (Logan and Whitmore, 1966; Moore, 1966a,b; for a review of earlier work see Singer and Leder, 1966). Studies of the properties of the resulting complex are, however, hampered by several factors. The customary method for separating free poly U from ribosome-bound poly U, namely, sucrose density gradient centrifugation, is usually more time consuming than is desirable and the isolated ribosome–poly U complex is contaminated with large amounts of sucrose.

In the course of studying poly U–ribosome complexes as substrates for *Escherichia coli* RNase II (Spahr, 1964; Singer and Tolbert, 1965) we have developed a rapid column technique by which free poly U and ribosome-bound poly U can be separated in a matter of minutes. The method is based on the fact that under certain conditions of pH and salt, free poly U is adsorbed to polyacrylamide gels while poly U bound to ribosomes is not. The action of RNase II on poly U–ribosome complexes obtained by these procedures will be considered in a subsequent publication.

Experimental Procedure

Materials. Copolymers of acrylamide and methylenebisacrylamide (Bio-Gels) were obtained from Bio-Rad Laboratories, Richmond, Calif., and are designated Bio-Gel P-30 and P-100. Blue Dextran 2000 was obtained from Pharmacia, Uppsala, Sweden. [^{14}C]- and [^{3}H]phenylalanines were purchased from the New England Nuclear Corp.

E. coli strain MRE-600 was the kind gift of Dr. H. E. Wade. This mutant is devoid of RNase I (Spahr and Hollingworth, 1961; Cammack and Wade, 1965;

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[1] Abbreviations used: A, U, G, C, and I represent the residues of adenylic, uridylic, guanylic, cytidylic, and inosinic acids, respectively, in polyribonucleotide chains. For example, poly A is polyadenylic acid, and poly UC is a copolymer of uridylic and cytidylic acids. Similarly, poly-4-*N*-methyl C represents poly-N^4-methylcytidylic acid; poly-6-*N*-methyl A, poly-N^6-methyladenylic acid; and poly-6,6-*N*-dimethyl A, poly-N^6,N^6-dimethyladenylic acid. 5′-UMP, uridine 5′-monophosphate; GTP, guanosine triphosphate.