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Studies on Bile Acids. Some Observations on the Intracellular Localization of Major Bile Acids in Rat Liver*

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ABSTRACT: The subcellular distribution of major bile acids in rat liver has been studied by the application of recently developed gas–liquid partition chromatographic methods. The relative concentrations of several bile acids in rat portal blood and liver homogenate resembled each other very closely except for chenodeoxycholic acid. The concentration of chenodeoxycholic acid in liver is significantly higher than in portal blood.

The cytoplasmic compartment accounts for approximately 70% of the bile acids with more than 50% for each individual bile acid studied. The ratio of cholic/deoxycholic in each subcellular fraction revealed the existence of a relatively larger proportion of deoxycholic acid in the mitochondrial and microsomal fractions compared to that in the cytoplasmic fraction (1.7 and 1.3 *vs.* 5.1). Since the enzymes concerned with hydroxylation (7α-hydroxylase) and conjugation are located in microsomes and partly in the mitochondria, there seems to be a relationship between localization of bile acids in these subcellular particles and their functional role.

The primary bile acids, cholic[1] and chenodeoxycholic, are known to be principal end products of cholesterol catabolism, in which the liver occupies a focal position (Bergström *et al.*, 1960). In the rat, deoxycholic, a secondary bile acid generated by bacterial action in the gut, upon reaching the liver *via* the enterohepatic circulation, is rapidly hydroxylated to cholic acid by a 7α-hydroxylating system (Bergström *et al.*, 1953a,b).

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[1] The systematic nomenclature of bile acids referred to in this report by trivial names are as follows: lithocholic acid, 3α-hydroxy-5β-cholanoic acid; hyodeoxycholic acid, 3α,6α-dihydroxy-5β-cholanoic acid; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanoic acid; ursodeoxycholic acid, 3α,7β-dihydroxy-5β-cholanoic acid; deoxycholic acid, 3α,12α-dihydroxy-5β-cholanoic acid; cholic acid, 3α,7α,12α-trihydroxy-5β-cholanoic acid; α-muricholic acid, 3α,6β,7α-trihydroxy-5β-cholanoic acid; β-muricholic acid, 3α,6β,7β-trihydroxy-5β-cholanoic acid.

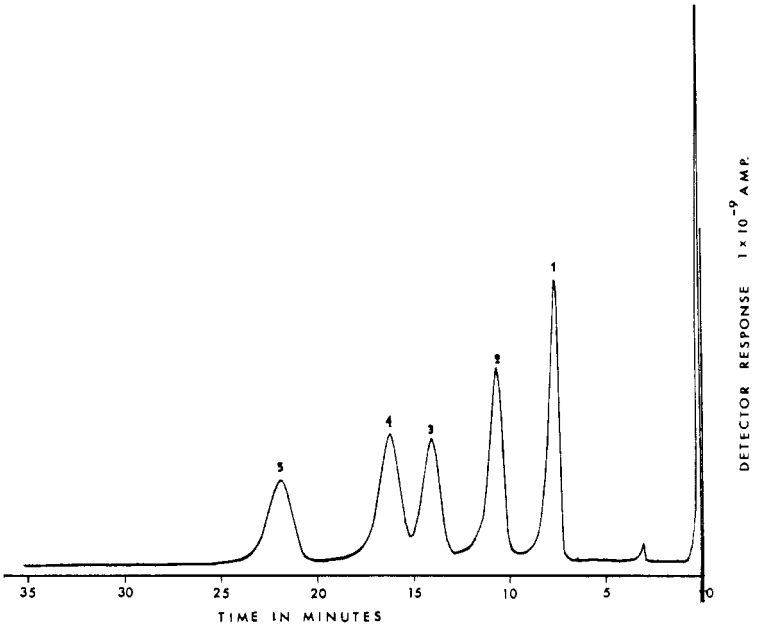

FIGURE 1: Gas–liquid partition chromatography of bile acid standards in the form of methyl ester trifluoroacetates on FS-1265/SE30/NGS (Okishio and Nair, 1966). Column temperature, 210°; (1) lithocholic acid; (2) deoxycholic acid; (3) chenodeoxycholic acid; (4) hyodeoxycholic acid; and (5) cholic acid.

Since several such functions in bile acid metabolism have been assigned to the liver, it is most desirable to study the localization of bile acids among liver subcellular fractions, as indicated in a recent review by Danielsson (1963).

The recent development of specific techniques for the isolation and gas chromatographic separation of bile acids from serum (Sandberg *et al.*, 1965) and cellular material (T. Okishio, P. P. Nair, and M. Gordon, in preparation, 1966) enabled us to extend our studies to the liver and its subcellular fractions. In the present paper we describe our findings on the intracellular localization of some major bile acids in rat liver which follows a pattern consistent with the known functional role of the subcellular fractions. A preliminary report of this work has appeared earlier (Okishio and Nair, 1965).

Experimental Section

Animals. Adult male rats of the Wistar strain weighing about 250–300 g were used in all experiments. They were fed a standard diet of Purina Laboratory Chow and water *ad libitum*. Rats were deprived of food for 20 hr and sacrificed by exsanguination from the abdominal aorta under light ether anesthesia, except when portal vein blood samples were required. The portal vein was cannulated with a polyethylene cannula close to the liver, and about 4.0 ml of blood was drawn into a heparinized centrifuge tube. Rats on antibiotic therapy received 125 mg of terramycin (Pfizer) and 200 mg of succinylsulfathiazole twice daily for 3 consecutive days by stomach tube. Following 3 days of therapy, animals were sacrificed as described before.

Isolation of Liver Cell Components. Fractionation of rat liver cell components in isotonic 0.25 M sucrose was carried out according to the procedures of Schneider and Hogeboom (1950) and Hogeboom (1955). Succinoxidase assays were performed on subcellular fractions by the manometric technique of Potter (1957).

Isolation of Bile Acids. Tissue homogenates or subcellular fractions were initially lyophilized and then refluxed for 30 min with 100 ml of 95% ethanol containing 0.1% ammonium hydroxide. The residue was reextracted with the same volume of fresh solvent and filtered. The material left on the filter paper was washed with 50 ml of fresh extractant. The extracts were pooled and evaporated to dryness under reduced pressure.

The bile acids in the residue were dissolved in 3.0–7.0 ml of 0.1 N NaOH (pH 11) and quantitatively transferred to an Amberlyst A-26 anion-exchange resin column with the addition of 35 ml of water. All subsequent steps in the purification except chromatography on alumina were performed as described in an earlier publication from this laboratory (Sandberg *et al.*, 1965).

Aluminum Oxide Chromatography. Since the isolation of lithocholic acid was not studied in detail in the earlier publication (Sandberg *et al.*, 1965), we reinvestigated this aspect and obtained quantitative recoveries by modifying the aluminum oxide chromatographic procedure. Bile acid methyl esters were quantitatively transferred to an aluminum oxide column with about 10 ml of benzene–hexane (1:9, v/v) and washed with

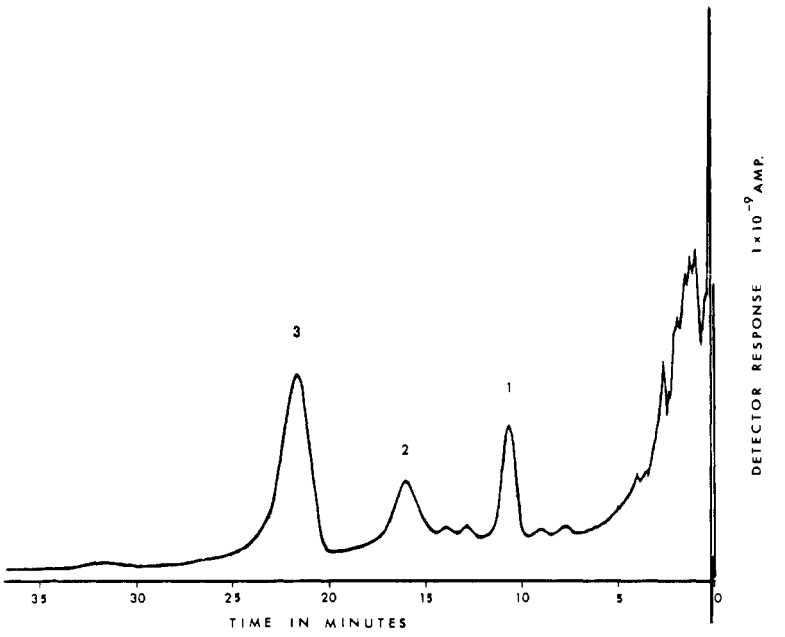

FIGURE 2: Gas–liquid partition chromatography of bile acids from rat liver homogenate. Conditions are the same as in Figure 1; (1) deoxycholic acid; (2) hyodeoxycholic acid; and (3) cholic acid.

about 40 ml of the same solvent mixture. Lithocholic acid methyl ester was eluted with 70 ml of benzene and all the other bile acid methyl esters with 40 ml of methanol–acetone (1:9, v/v).

Gas–Liquid Partition Chromatography. For quantitative purposes bile acids were converted to their methyl ester trifluoroacetates and chromatographed on a triple component column consisting of a mixture of FS-1265 (QF-1, Dow Corning Corp.), neopentyl glycol succinate, and SE30 (General Electric) phases (Okishio and Nair, 1966). Bile acids were identified by comparison with standard gas chromatographic retention data of their methyl esters and methyl ester trifluoroacetates on a column with FS-1265 alone or on the triple component column (Okishio and Nair, 1966). Experimental conditions for gas chromatography were the same as those described in previous publications (Sandberg *et al.*, 1965; Okishio and Nair, 1966).

Results

Figure 1 represents a gas chromatographic tracing of a standard mixture of five bile acids in the form of methyl ester trifluoroacetates. The major bile acids isolated from a homogenate of rat liver and processed as described under "Experimental Section" give a clear-cut chromatographic tracing (Figure 2) from which peak areas obtained by triangulation could be compared with those from known standards. Other bile acids which are present in smaller amounts are quantitated from a second run where the principal peaks are overloaded.

The distribution of succinoxidase activity among the subcellular fractions of rat liver is summarized in Table I. Approximately 50–60% of total oxygen uptake of a 10% homogenate of liver resided in the mitochondrial fraction with the nuclear and microsomal fractions accounting for 15 and 6% of the total activity. However, the recovery of total succinoxidase activity averaged only about 78% of that in a total homogenate, resulting in the slightly lower values obtained with mitochondria.

TABLE I: Succinoxidase Activity of Rat Liver Cell Fractions.

Cell Fraction	Oxygen Uptake[a] (%)	
	Expt A	Expt B
Nuclear	17.9	12.3
Mitochondrial	50.8	63.9
Microsomal	6.7	5.9
Cytoplasmic	—	—

[a] Results are expressed as per cent of total respiration of a 10% homogenate of rat liver. The incubation medium and experimental conditions are as described by Potter (1957).

Upon comparing the relative concentrations of five different bile acids in rat liver homogenate with those in peripheral and portal blood plasma (Table II), it is seen that portal blood and rat liver homogenate resembled each other closely in all respects except chenodeoxycholic, as would be expected, since this bile acid is known to undergo alterations during enterohepatic

TABLE II: Distribution of Bile Acids in Rat Peripheral and Portal Blood Plasma and in Rat Liver Homogenate.

Bile Acids	Plasma Peripheral (4)[a]		Plasma Portal (4)[a]		Liver Homogenate (6)[a]	
	μg/ml	%	μg/ml	%	μg/g of Liver	%
Lithocholic	—	—	Trace		Trace	
Deoxycholic	0.19	12.3	12.1	14.5	20.0	18.3
Chenodeoxycholic	0.08	4.9	0.38	0.5	2.39	2.2
Hyodeoxycholic	0.58	38.2	10.7	12.8	14.5	13.3
Cholic	0.68	44.6	60.2	72.2	72.2	66.2
Total	1.53	100	83.38	100	109.09	100

[a] Figures within parentheses represent number of animals in each experiment.

TABLE III: Distribution of Individual Bile Acids among Subcellular Fractions of Rat Liver.[a]

Bile Acids		Cell Fractions			
		Nuclear	Mitochondrial	Microsomal	Cytoplasmic
Deoxycholic (18.3)	μg/g	2.39	2.54	4.26	9.56
	%[b]	12.8	13.5	22.7	51.0
Chenodeoxycholic (2.2)	μg/g	0.44	0.41	0.5	1.35
	%[b]	16.3	15.2	18.5	50.0
Hyodeoxycholic (13.3)	μg/g	1.14	0.9	1.09	7.13
	%[b]	11.1	8.8	10.6	69.5
Cholic (66.2)	μg/g	6.37	4.43	5.69	48.5
	%[b]	9.8	6.8	8.8	74.5
Total	μg/g	10.34	8.28	11.54	66.54
	%[b]	10.7	8.6	11.9	68.8

[a] Mean of six individual experiments. [b] Per cent distribution in the fractions. Figures within parentheses represent percent of each bile acid in total homogenate of liver.

circulation (Mahowald *et al.*, 1957). The concentration of chenodeoxycholic in liver is significantly higher than in portal blood. Cholic acid constitutes about 70% of the bile acids studied in portal blood and in liver homogenate compared to about 45% in peripheral blood, the deficiency being made up by hyodeoxycholic (38%). However, the total of five bile acids in peripheral blood plasma represents only about $^1/_{55}$ of that transported by portal blood.

Table III presents data on the intracellular distribution of each individual bile acid in rat liver. The fact that more than 50% of each bile acid studied is located in the cytoplasmic compartment constitutes a significant observation. The rest of the bile acids are almost equally divided among the particulate elements of the cell with the exception of deoxycholic. Among the nuclear, mitochondrial, and microsomal fractions, almost twice as much deoxycholic is present in the microsomal fraction as in the nuclear and mitochondrial fractions.

The distribution of component bile acids in each individual cell fraction is illustrated in Table IV. Cholic, deoxycholic, and hyodeoxycholic acids, in decreasing order, constitute the major bile acids in total homogenate of liver. From the ratios of cholic/deoxycholic in each individual fraction it is seen that there is a relatively larger proportion of deoxycholic in the mitochondrial and microsomal fractions compared to that in the cytoplasmic compartment (1.7 and 1.3 *vs.* 5.1).

Since both deoxycholic and hyodeoxycholic acids are assumed to be formed by microbial action in the gut, rats were placed on antibiotics *per os* for 3 days to suppress the bacterial flora. The bile acid composition of portal and peripheral blood plasma and liver homogenates from these animals is compared with those from normal animals in Table V. No significant differences in cholic acid are apparent with antibiotic therapy. However, in sharp contrast, there is a total disappearance of deoxycholic from the enterohepatic system in animals treated with antibiotics. In a similar manner, hyodeoxycholic acid levels are significantly

TABLE IV: Component Bile Acid Composition of Individual Rat Liver Subcellular Fractions.[a]

Subcellular Fractions		Deoxy-cholic	Chenodeoxy-cholic	Hyodeoxy-cholic	Cholic	Total	Cholic/Deoxycholic Ratio
Total homogenate	μg/g	20.0	2.39	14.5	72.2	109.09	3.6
	%	18.3	2.2	13.3	66.2	100	
Nuclear	μg/g	2.39	0.44	1.14	6.37	10.34	2.7
	%	23.1	4.3	11.0	61.6	100	
Mitochondrial	μg/g	2.54	0.41	0.9	4.43	8.28	1.7
	%	30.7	4.9	10.9	53.5	100	
Microsomal	μg/g	4.26	0.5	1.09	5.69	11.54	1.3
	%	36.9	4.3	9.5	49.3	100	
Cytoplasmic	μg/g	9.56	1.35	7.13	48.5	66.54	5.1
	%	14.4	2.0	10.7	72.9	100	

[a] Mean of six individual experiments.

lower than those for the normal control group.

Discussion

The nature and composition of liver and portal blood bile acids are the resultant of a number of metabolic processes which include: (1) the biogenesis of the primary bile acids, cholic and chenodeoxycholic; (2) the formation of secondary bile acids by the action of intestinal microflora; and (3) the extent of reabsorption of each bile acid from the gut. The evidence for the synthesis in the liver of the primary bile acids and their conjugation with taurine and glycine are well documented in reviews (Bergström *et al.*, 1960; Bergström and Danielsson, 1963; Danielsson, 1963).

Among several chemical alterations effected by the action of intestinal microorganisms on primary bile acids, the elimination of the 7α-hydroxyl group and the hydrolysis of conjugated bile acids seem to be the most important ones. Thus, removal of the 7α-hydroxyl group from cholic and chenodeoxycholic has been shown to give rise to deoxycholic and lithocholic in the rat (Norman and Sjövall, 1958, 1960). In surgically jaundiced animals, further metabolic products have been identified, of which 3α,6β,12α-trihydroxy-5β-cholanoic acid was derived from deoxycholic acid (Ratliff *et al.*, 1959). The same group of workers have reported the isolation of chenodeoxycholic, 3α,6β-dihydroxy-5β-cholanoic acid, and α- and β-muricholic acids as metabolites of lithocholic acid after its intraperitoneal administration (Thomas *et al.*, 1964).

In our studies, cholic and deoxycholic acids together formed almost 90% of the total bile acids in portal blood and liver homogenate, and in relative concentrations, paralleled each other. This is in sharp contrast to the predominance of cholic and chenodeoxycholic acids (ratio 8/2) in rat bile (Eriksson, 1957a,b).

TABLE V: Bile Acid Composition of Blood and Liver from Rats Fed Antibiotics.

	Antibiotic Fed[a]		
Bile Acids	Portal Blood Plasma (μg/ml) (4)[b]	Peripheral Blood Plasma (μg/ml) (4)[b]	Liver (μg/g wet wt) (4)[b]
Lithocholic	—	—	—
Deoxycholic	—	—	—
Chenodeoxycholic	3.73	0.02	0.87
Hyodeoxycholic	1.97	0.01	1.16
Cholic	71.2	1.46	76.0
Total	76.90	1.49	78.03

[a] Terramycin (125 mg) (Pfizer) and 200 mg of succinylsulfathiazole were administered orally twice a day for 3 consecutive days to rats with an average weight of 300 g. [b] Figures within parentheses represent the number of animals in each experiment.

The absence of deoxycholic acid is attributable to the presence of an active 7α-hydroxylating system in rat liver which hydroxylates deoxycholic to cholic acid before being excreted into the bile. In separate experiments, not reported here, free bile acids in liver homogenate accounted for less than 5% of those bile acids studied, in contrast to about 15% in rat portal blood, reported by Olivecrona and Sjövall (1959). The relatively insoluble lithocholic acid, as would be expected, is not present in any detectable amounts because it is poorly reabsorbed. The tentative identification of

significant amounts of hyodeoxycholic acid (12–13% in portal blood and liver) is supported by its reported presence in rat bile (Lin *et al.*, 1963) and rat feces (Roscoe and Fahrenbach, 1963; Makita and Wells, 1963), which is contrary to the general belief that this acid is found only in pig bile. The unknown compound upon conversion to the corresponding methyl ester, trifluoroacetyl methyl ester, and trimethylsilyl ether gave retention times identical with those of authentic hyodeoxycholic acid (gas–liquid partition chromatography) on two sets of phases, FS-1265 (Sjövall, 1964) and FS-1265/SE30/NGS (Okishio and Nair, 1966). Purina Laboratory Chow, which contains traces of hyodeoxycholic acid, does not contribute significantly to the total bile acid pattern, since there is no difference between chow-fed rats and rats fed white bread (T. Okishio and P. P. Nair, unpublished observations).

Hyodeoxycholic acid is probably formed by the action of microbial flora in the intestinal tract, because this bile acid disappears almost completely from the portal blood and liver of rats maintained on oral antibiotic therapy (Table V). Palmer (1965) has found small amounts of hyodeoxycholic in rat gallstones, induced by feeding a diet containing 1% lithocholic acid. Since completing this study, Einarsson (1966) has reported the formation of hyodeoxycholic acid from lithocholic acid in the rat, corroborating our findings.

Ursodeoxycholic acid, a metabolite of chenodeoxycholic acid, which has been reported to be present in small amounts in rat bile (Mahowald *et al.*, 1958), was not detected in our extracts. The apparent divergence in our results is probably a reflection of the differences in microbial flora in rat intestinal contents, since the formation of ursodeoxycholic from chenodeoxycholic acid is regarded as a process effected entirely by bacterial enzymes (Van Belle, 1965). Both ursodeoxycholic and hyodeoxycholic acids could be clearly resolved either as methyl esters or as methyl ester trifluoroacetates on our gas–liquid partition chromatographic system (Okishio and Nair, 1966).

The studies on subcellular fractions clearly indicate an important role for the cytoplasmic compartment, since more than 50% of the bile acids are found in this part of the cell. Whether this represents an active metabolic or storage phase in the final transport of bile acids into the bile has yet to be elucidated. The ratios of cholic/deoxycholic among the subcellular fractions constitute an important observation in these studies. Higher concentrations of deoxycholic relative to cholic are found in the microsomal and mitochondrial fractions (cholic/deoxycholic, 1.3 and 1.7) compared to that in the cytoplasmic or soluble fraction (cholic/deoxycholic, 5.1). Rat liver is known to have an active 7α-hydroxylating system capable of hydroxylating deoxycholic to cholic in the intact rat (Bergström *et al.*, 1953b), when incubated with rat liver slices (Bergström *et al.*, 1953a) or with fractionated homogenates (Bergström and Gloor, 1954, 1955a,b; Gloor, 1954), where the 7α-hydroxylase system has been located in the microsomes. The higher concentration of deoxycholic relative to cholic in the microsomes is probably a reflection of its functional role in the hydroxylation to cholic acid.

Another reaction catalyzed by the microsomal fraction is the conjugation of bile acids presumably through the formation of the coenzyme A ester which in turn reacts with the appropriate amino acid (Siperstein and Murray, 1956; Bremer, 1955a,b; Bremer and Gloor, 1955). Mitochondria are less active in this respect. Thus, our ratio for cholic/deoxycholic in subcellular fractions is compatible with the functional roles assigned to these organelles. Since those enzymes which are concerned with both 7α-hydroxylation and conjugation are located in the microsomes, they are probably contributory factors regulating the relative amounts of deoxycholic and cholic in this fraction. Deoxycholic upon hydroxylation to cholic is conjugated with taurine to yield taurocholic which, on account of its greater solubility, is released into the cytoplasmic compartment. This is purely speculative, because about half of the total deoxycholic is still located in the cytoplasmic fraction in a conjugated or unconjugated form. The intracellular distribution of bile acids in livers from those forms of life which do not possess a 7α-hydroxylase will probably be helpful in establishing the relationship between localization and functional requirements.

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The Polynucleotide Product of Poly A Polymerase from *Escherichia coli**

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ABSTRACT: Polyriboadenylic acid (poly A) polymerase from *Escherichia coli*, an enzyme found associated with the ribosomes in a crude cell extract, has been studied both in the ribosome-bound state and in a partially purified state. No differences in the enzyme activity or specificity were found between the two states.

Adenosine triphosphate (ATP) is utilized preferentially as a substrate, the incorporation of the other nucleoside triphosphates or adenosine diphosphate (ADP) into acid-precipitable material being much less under all of the conditions studied. The enzyme requires a ribonucleic acid (RNA) primer as well as the divalent cations magnesium and manganese for optimal activity. Nearest neighbor and zone centrifugation analysis show the product of enzyme activity to be a short chain of poly A attached to the 3′ terminus of the primer RNA.

Enzymes which incorporate AMP[1] into chains of polyriboadenylic acid (poly A) have been found in vertebrate tissues as well as in bacteria (Klemperer, 1963; Burdon, 1963a,b; Edmonds and Abrams, 1960, 1962; Venkataraman and Mahler, 1963; August *et al.*, 1962; Gottesman *et al.*, 1962). All of these enzymes require ATP as substrate and a ribonucleic acid (RNA) primer. Their ubiquity suggests a basic but unknown role in nucleic acid metabolism. The provocative observation by August *et al.* (1962) that the poly A polymerase of *Escherichia coli* is apparently associated with the ribosomes has prompted the present investigation to determine whether this enzyme has some function in protein synthesis or the regulation of ribosome activity.

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[1] Abbreviations used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; GMP, guanosine monophosphate; UMP, uridine monophosphate; CMP, cytidine monophosphate; PCA, perchloric acid; A_{260} unit, unit of material which in a 1-ml volume and light path of 1 cm will have an optical density of 1 at 260 mμ; TCA, trichloroacetic acid; $E^{260}_{1\%}$, extinction coefficient of a 1% solution at 260 mμ; TSM, Tris, 0.01 M-succinic acid, 0.003 M-$MgSO_4$, 0.01 M, pH 8.0; SDS, sodium dodecyl sulfate.