terase, or of an RNA.....CpCpA pyrophosphorylase (Preiss *et al.*, 1961). The 3′-phosphorylated sequence, in turn, could result from the action of a 3′-specific polynucleotide phosphokinase (an enzyme that has not been reported to date), or a nucleosidase that removes only the terminal nucleoside. It is also possible that the 3′-phosphate in STNV-RNA arises from a specific nuclease acting on a longer RNA. Thus the satellite RNA could be derived from the first part of the tobacco necrosis virus. Regardless of its origin, the finding of a 3′-phosphate on an RNA emphasizes the importance of treating an unknown RNA with phosphatase prior to using any end group methods that require a free periodate-susceptible glycol group.

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Minor Species of Ribonucleic Acid Associated with Rat Liver Mitochondria*

Leonard I. Malkin

ABSTRACT: Species of RNA sedimenting at approximately 20 and 22 S in sucrose–sodium dodecyl sulfate gradients are associated with both rat liver mitochondria and purified cytoplasmic ribosomes. Mitochondria are normally grossly contaminated with cytoplasmic ribosomes and this in itself could explain the presence of these RNAs in mitochrondria preparations. However, a dual localization of these RNAs cannot be excluded. We show here that treatment of mitochondria with low levels of digitonin removes contaminating cytoplasmic ribosomes without affecting mitochondrial integrity. Such treatment also removes the 20S and 22S RNAs, lending strong support to the argument that these RNAs are nonmitochondrial and arise from contamination of mitochondria by microsomal membrane-bound cytoplasmic ribosomes or ribosomal subunits.

Various size classes of RNA are observed in preparations from mitochondria of several animal species. Among these are the mitochondria-specific RNAs, presumably from mitochondrial ribosomes, which, in rat liver preparations, sediment at 13 and 16 S in sucrose gradients (Borst and Grivell, 1971; Bartoov *et al.*, 1970). In addition, there are the 18S and 28S RNAs which arise from microsomal membrane-bound cytoplasmic ribosomes that contaminate mitochondria preparations (Malkin, 1971). Attardi and Attardi (1971) have described two other species of RNA from HeLa cell mitochondria which sediment at approximately 21 and 23 S and whose labeling is insensitive to ethidium bromide. In earlier work, these RNA peaks were poorly resolved but radioactive RNA from the 21S to 23S region of sucrose gradients was shown not to hybridize with mitochondrial DNA (Attardi and Attardi, 1969). Thus it appears likely that these RNAs are not transcribed on mitochondrial DNA. However, this says nothing about their location in the cell since conceivably they could be made elsewhere and then transported to the mitochondria. Recent work (Swanson, 1971) has demonstrated that isolated intact mitochondria can incorporate high molecular weight polyribonucleotides from the surrounding medium into the mitochondrial inner compartment. We have recently shown that the 20S and 22S RNAs found in rat liver mitochondria preparations can be demonstrated in cytoplasmic ribosomes free of mitochondria (Malkin, 1971). These results indicated that these RNAs are nonmitochondrial and arise from contamination of mitochondria by cytoplasmic ribosomes. However, a dual localization of these RNAs cannot be ruled out, *i.e.*, they may be

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associated both with mitochondria and cytoplasmic ribosomes. We have attacked this problem by preparing *intact* mitochondria essentially free of cytoplasmic ribosomes and ribosomal subunits by a method involving the use of low concentrations of digitonin. RNA prepared from these mitochondria show only the 13S and 16S mitochondrial RNAs along with a very small amount of 28S RNA and a barely detectable shoulder in the 18S region. The 20S and 22S RNAs are removed by the digitonin treatment, lending strong support against the argument of dual localization and for the argument that these RNAs are not normally associated with mitochondria except insofar as the mitochondria are contaminated with cytoplasmic ribosomes or ribosomal subunits.

Methods

Preparation of Mitochondria and Fractionation with Digitonin. All procedures for the isolation and fractionation of mitochondria were carried out at 0–4°. Male rats (Charles River Laboratories, CD strain), 125–200 g, were sacrificed by a blow to the head. Livers were removed, rinsed under cold tap water, and placed in approximately 2 volumes (per liver wet weight) of sterile buffer I (0.002 M Hepes[1]-HCl, pH 7.4, 0.07 M sucrose, 0.22 M D-mannitol, 0.001 M EDTA, and 0.5 mg/ml of bovine serum albumin). After mincing with scissors, the livers were washed twice by settling and decantation and then homogenized in 2 volumes of buffer I using a motor-driven Teflon-glass homogenizer with a clearance of approximately 0.025 cm running at 500 rpm. Only two up-and-down strokes were used. The homogenate was diluted with another 6 volumes of buffer I and then centrifuged at 1400 × g_{max} for 6 min in the 276a rotor of an International centrifuge. The supernatant solution was carefully decanted through two layers of cheesecloth and centrifuged again as above (two low-speed centrifugations were found to be necessary to remove any traces of erythrocytes from the subsequent mitochondria pellet). The supernatant solution from the second centrifugation was placed in 50-ml polycarbonate tubes (clear tubes aid in the separation of opaque supernatants from pellets) and the mitochondria were pelleted by centrifuging at 4300 × g_{max} for 10 min in a Sorvall centrifuge (relatively low speeds were used to minimize contamination by microsomes and lysosomes). The supernatant solutions were decanted, the loose layer on top of the pellets retained, and the pellets made into a paste with a "cold finger" (test tube filled with ice) followed by adding buffer I to one-half the original volume. Final resuspension was effected by slowly drawing up the suspension in a precooled, wide-bore 10-ml pipet and allowing the suspension to flow out by gravity (all resuspensions of mitochondria were carried out in this manner to minimize damage by shear forces). The mitochondria were pelleted again at 4300 × g_{max} for 10 min, resuspended in buffer I to one-quarter the original volume, and centrifuged at 10,800 × g_{max} for 10 min to obtain the final pellet (P_1). At this stage, the loose layer on top of the pellet was decanted along with the supernatant solution. All subsequent procedures involved the use of buffer D, which is buffer I minus EDTA. The mitochondria were resuspended in a small volume of buffer D and the concentration adjusted to 100 mg/ml. Digitonin fractionation was carried out exactly as described (Schnaitman and Greenawalt, 1968) varying only the digitonin:protein ratio. The mitochondria were incubated with digitonin for 15 min at 0° and recovered by centrifugation at 9700 × g_{max} for 10 min. After resuspension in buffer D and recentrifugation as above, the mitochondria pellets were resuspended in buffer D for enzyme assays and respiration parameter studies. These resuspended mitochondria are referred to in Table II as Pellet. The supernatants from the digitonin washings were combined and are referred to in Table II as Combined Supernatant Solutions. As a control, mitochondria were taken through the digitonin procedure without digitonin and are referred to as "washed" mitochondria. Specifically, these mitochondria were washed twice by resuspension and centrifugation at 9700 × g_{max} for 10 min, the first time with 8 ml of buffer D/100 mg of protein and the second time with 3 ml of buffer D/100 mg of protein.

Enzyme Assays. The ER-specific glucose 6-phosphatase was assayed according to de Duve *et al.* (1955). Adenylate kinase was assayed spectrophotometrically as previously described (Schnaitman and Greenawalt, 1968). One unit is that amount of enzyme causing an increase of 0.001 ODU at 340 mμ/min at 25°. All enzyme assays were shown to be linear with respect to time and the enzyme concentrations used and were unaffected by the presence of added digitonin in concentrations up to 0.1 mg/assay tube. Protein was estimated by the method of Lowry *et al.* (1951).

Respiration Parameters. Respiratory control ratios, P:O ratios, and specific O_2 consumption were measured polarographically as described (Malkin, 1970) using 5 mM succinate as the substrate, ADP as the phosphate acceptor, and the "Mg^{2+} medium" described previously (Schnaitman and Greenawalt, 1968).

Preparation of RNA. Mitochondria pellets were resuspended in buffer BN (0.01 M Bicine-HCl (pH 7.4)–0.15 M NaCl) containing 0.1% SDS and extracted twice with triisopropylnaphthalenesulfonate and phenol–cresol at 0–4° as described (Bishop *et al.*, 1967) except that SDS was added to a final concentration of 0.1% to the first aqueous phase and to the final aqueous phase prior to precipitation with ethanol. The ethanol-precipitated RNA was washed twice by centrifugation with cold 70% ethanol–0.1 M sodium acetate and then dissolved in buffer BN containing 0.01 M $MgCl_2$ and 20 μg/ml of ribonuclease-free deoxyribonuclease (Worthington Biochemicals). After incubating at room temperature for 15 min, SDS was added to a final concentration of 0.1% and the RNA was precipitated by adding 2 volumes of 100% ethanol. After standing at −20° for at least 1 hr, the precipitated RNA was washed by centrifugation once with cold 70% ethanol–0.1 M sodium acetate and once with cold 100% ethanol. The final pellet was dissolved in buffer BN containing 1% SDS and analyzed as described in Figure 1.

Results

Table I shows some respiration parameters of digitonin-treated and untreated mitochondria. Of particular importance is the fact that only at ratios of 2.5 and 5.0 mg of digitonin per 100 mg of protein do the respiratory control ratios decrease significantly. Respiratory control ratios are a highly regarded criterion of mitochondrial integrity (Johnson and Lardy, 1967). It should be emphasized that we are using a medium previously shown to give no respiratory control at all with mitochondria lacking outer membranes (Schnaitman and Greenawalt, 1968). Thus the high respiratory control

[1] Abbreviations used are: Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; SDS, sodium dodecyl sulfate; ADP, adenosine 5′-diphosphate; ER, endoplasmic reticulum.

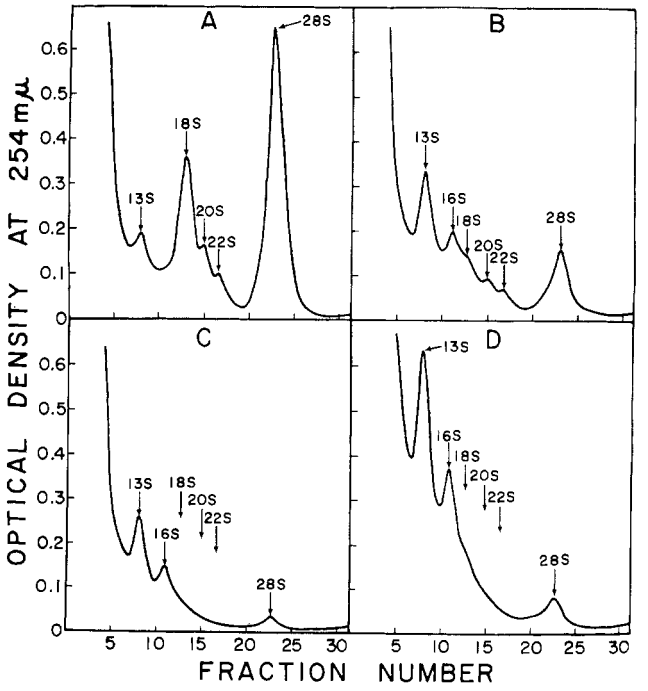

FIGURE 1: Sucrose gradient analyses of mitochondrial RNA prepared as described in Methods. Samples (0.2 ml) were layered over 17-ml linear sucrose gradients (10–25% (w/v) sucrose in buffer BN containing 0.1% SDS) and centrifuged in the Spinco SW27 rotor at 26,000 rpm for 15 hr at 15°. Optical density at 254 mμ was monitored with an ISCO uv analyzer. All S values are approximate and are based on assigning values of 18 and 28 S to cytoplasmic RNAs. (A) RNA from untreated mitochondria (P_1); (B) RNA from "washed" mitochondria; (C, D) RNA from mitochondria treated with 1.0 mg of digitonin/100 mg of protein.

ratios of mitochondria treated with 1.0 mg or less of digitonin per 100 mg of protein are strongly indicative of undamaged outer membranes. As a further test, adenylylate kinase was assayed as an indicator of mitochondrial integrity as it has been shown that this soluble intermembrane enzyme is the first to be released when the mitochondrial outer membrane is ruptured by digitonin (Schnaitman and Greenawalt, 1968). The specific activity of adenylylate kinase in "washed" mitochondria is 2379 units/mg and in mitochondria treated with 0.5 mg of digitonin/100 mg of protein, 2638 units/mg.

TABLE I: Respiration Parameters of Digitonin-Treated Mitochondria.

mg of Digitonin/100 mg of Protein	Respiratory Control Ratio	P:O Ratio	Sp O_2 Consumption[a]
0	6.3	2.4	70.6
0.1	6.6	2.4	65.6
0.25	6.1	2.6	60.7
0.5	7.2	2.4	76.9
1.0	5.9	2.4	75.3
2.5	4.8	2.4	69.2
5.0	4.0	2.4	54.4

[a] nmoles of O_2/min per mg at 25° in the presence of ADP.

TABLE II: Distribution of Protein and Glucose 6-Phosphatase after Digitonin Treatment.

Sample		% Protein	Enzyme Activity: Sp Act.[c]	Enzyme Activity: % Total Recovered Units	Recov of Total Units[d]
"Washed" mitochondria	P[a]	91.2	0.016	54.3	91.4
	S[b]	8.8	0.141	45.7	
0.1 mg of digitonin/100 mg of protein	P	91.2	0.016	55.6	86.3
	S	8.8	0.129	44.4	
0.25 mg of digitonin/100 mg of protein	P	90.3	0.013	39.9	98.6
	S	9.7	0.179	60.1	
0.5 mg of digitonin/100 mg of protein	P	88.9	0.012	31.5	92.5
	S	11.1	0.180	68.5	
1.0 mg of digitonin/100 mg of protein	P	88.9	0.010	28.2	104.4
	S	11.1	0.199	71.8	
2.5 mg of digitonin/100 mg of protein	P	87.7	0.011	32.2	102.0
	S	12.3	0.167	67.8	
5.0 mg of digitonin/100 mg of protein	P	87.2	0.010	30.6	108.2
	S	12.8	0.162	69.4	

[a] Pellet. [b] Combined supernatant solutions. [c] Units per milligram of protein; the specific activity of untreated mitochondria is 0.029. [d] Based on untreated mitochondria.

The recovery of total units in the digitonin-treated mitochondria is 97.3% compared to "washed" mitochondria.

These results indicate that the mitochondrial outer membrane does not become "leaky" when exposed to low levels of digitonin, a conclusion supported by the work of Loewenstein *et al.* (1970).

Table II shows the distribution of protein concentration and glucose 6-phosphatase activity after treating mitochondria with various concentrations of digitonin. Of particular importance is the per cent of total recovered units in each fraction. By taking mitochondria through the digitonin procedure without digitonin ("washed" mitochondria), about 45% of the glucose 6-phosphatase activity is removed from the mitochondria pellet. However, digitonin treatment improves on this. At ratios of 0.5–5.0 mg of digitonin/100 mg of protein, about 70% of the glucose 6-phosphatase is removed (the enzyme units lost from the pellet are recovered in the combined supernatant solutions as shown in the last column of Table II). Loewenstein *et al.* (1970) also reported residual glucose 6-phosphatase activity after treating mitochondria with low levels of digitonin using a method slightly different from that described here. Even at much higher digitonin concentrations, sufficient to strip off the mitochondrial outer membrane (12–20 mg of digitonin/100 mg of protein), glucose 6-phosphatase activity still remains with the inner membrane plus matrix fraction (Schnaitman and Greenawalt, 1968). These results, however, are equivocal in regard to the effect of digitonin on the cytoplasmic ribosomes bound to

the "rough" ER. First, both rough and smooth ER have glucose 6-phosphatase activity and "smooth" ER contains no bound ribosomes (Leskes *et al.*, 1971). Second, glucose 6-phosphatase is very tightly bound to the ER and its attachment may be much less susceptible to digitonin action than the attachment of cytoplasmic ribosomes. Either or both of these points can explain why digitonin treatment can remove cytoplasmic ribosomes without removing all glucose 6-phosphatase activity from mitochondria preparations. The fact that low levels of digitonin do remove essentially all cytoplasmic ribosomes is shown in Figure 1. Figure 1A shows RNA prepared from untreated mitochondria (P_1). The 13S mitochondrial RNA is apparent but by far the major components present are the 18S and 28S RNAs from contaminating cytoplasmic ribosomes. Also clearly seen are the 20S and 22S RNAs. Figure 1B shows RNA prepared from "washed" mitochondria (digitonin control). The level of contamination by cytoplasmic RNA has been reduced considerably, allowing one to see clearly the 16S mitochondrial RNA, but the 18S, 20S, 22S, and 28S components are still apparent. Figure 1C shows RNA from mitochondria treated with 1.0 mg of digitonin/100 mg of protein. Here the only high molecular weight RNAs seen are the mitochondrial 13S and 16S RNAs along with a small amount of 28S material. Figure 1D displays the same RNA preparation as in Figure 1C but with more material on the gradient to better see minor peaks. A barely detectable shoulder is now seen in the 18S region but clearly the 20S and 22S RNAs have been completely removed.

Discussion

Previous work has shown that the 20S and 22S RNAs cannot be considered mitochondria specific because they are also found associated with purified cytoplasmic ribosomes (Malkin, 1971). The salient point to which this present work addresses itself is the question of dual localization, *i.e.*, are the 20S and 22S RNAs also mitochondrial RNAs in the sense that they are located within the mitochondria. The results show clearly that this is not so. Of particular importance is the evidence related to the integrity of the mitochondrial outer membrane, since one could argue that the digitonin treatment altered the membrane in such a way as to allow the 20S and 22S RNAs to leak out. The sensitivity of respiratory control ratios and adenylylate kinase leakage as indicators of damaged outer membranes argue very strongly against this.

The point of maintaining mitochondrial integrity while removing cytoplasmic ribosome contamination emphasizes the uniqueness of the digitonin procedure. Another procedure has been devised to remove contaminating ribosomes from rat liver mitochondria but the method involves exposure of the mitochondria to hypertonic sucrose, and when taken from hypertonic to isotonic sucrose, the mitochondria were observed to have ruptured or missing outer membranes (Bartoov *et al.*, 1970). In addition, when mitochondria were purified with the hypertonic sucrose method, the high molecular weight mitochondrial RNAs could not be extracted and could only be demonstrated by mixing RNA precursor-labeled mitochondria with unlabeled postmitochondrial supernatant containing cytoplasmic ribosomes (Bartoov *et al.*, 1970). Thus the maintenance of mitochondrial integrity by the digitonin procedure not only is of obvious importance to the study of mitochondrial functions that require intactness, but apparently is also necessary for the preparation of mitochondrial RNA from cytoplasmic ribosome-free mitochondria.

The RNA profile in Figure 1B indicates that repeated washings of the mitochondria may be sufficient to remove all cytoplasmic RNA contamination without the necessity of treating with digitonin. However, recent work (L. I. Malkin, 1971, unpublished data) has shown that washing P_1 as much as five times with buffer I (20 ml/100 mg of protein) not only does not reduce the level of cytoplasmic RNA contamination to that seen with the digitonin-treated material but also results in a lower yield of RNA and a lower 16S:13S ratio indicating more extensive degradation of the 16S RNA. That the 16S RNA even from the digitonin-treated mitochondria is degraded to some extent is indicated by a 16S:13S molar ratio of less than 0.5 although it is somewhat difficult to quantitate the RNA profiles. In any case, the molar ratio is certainly less than 1, a ratio observed by others in mitochondrial RNA preparations from rat liver (Bartoov *et al.*, 1970), BHK cells (Dubin and Montenecourt, 1970), *Xenopus laevis* oocytes (Swanson and Dawid, 1970), and HeLa cells (Attardi and Attardi, 1971). It should be mentioned however that the RNA preparations from mammalian cell mitochondria referred to above were always contaminated to a large extent with cytoplasmic ribosomal RNAs which may have acted as competitive substrates for ribonucleases thus protecting the mitochondrial RNAs. The especial sensitivity of the 16S RNA to nuclease action may be due to its more open conformation as indicated by a discrepancy between its sedimentation and electrophoretic properties (*cf.* Attardi and Attardi, 1971, for discussion and references).

The mechanism by which digitonin removes cytoplasmic ribosomes from mitochondria preparations most probably involves an interaction of digitonin, not with the mitochondrial membranes (as indicated by the preservation of mitochondrial integrity), but rather with the rough ER, since it is known that ribosomes bound to the rough ER, as opposed to unbound ribosomes, will sediment under conditions that will also sediment mitochondria and will grossly contaminate mitochondria bands in isopycnic sucrose gradients (Scornik *et al.*, 1967).

We have also observed another manifestation of this apparent differential effect of digitonin. When mitochondria are treated with 0.5 or 1.0 mg of digitonin per 100 mg of protein, conditions which leave the mitochondria intact, over 90% of a lysosomal-specific enzyme, β-*N*-acetylglucosaminidase (assayed as previously described (Malkin, 1970)) is removed from the mitochondria. This confirms and complements work by Loewenstein *et al.* (1970) who, using a slightly different procedure, demonstrated the removal of another lysosomal-specific enzyme, acid phosphatase, from rat liver mitochondria preparations.

Finally, one must consider the nature of the 20S and 22S RNAs. It is doubtful that either species is related to a 21S RNA from BHK cells described by Dubin and Czaplicki (1970). Their RNA was labeled in the presence of a level of actinomycin D that prevented the labeling of cytoplasmic rRNA and in addition was observed only in pulse-labeled preparations. More recent work (Dubin, 1971) has shown that its synthesis is inhibited by a high concentration of ethidium bromide. Dubin's 21S RNA therefore appears to be mitochondrion specific. On the other hand, the 21S and 23S RNAs from HeLa cell mitochondria preparations are probably related to the rat liver 20S and 22S RNAs because they can be detected by optical density and thus are stable species of RNA and they become labeled only when pulse times are long enough to label the 18S and 28S cytoplasmic RNAs. In addition, their labeling is insensitive to ethidium bromide (Attardi and Attardi, 1971). The possibility that these RNAs are

artifacts of isolation must be considered. Our earlier work (Malkin, 1971) indicated that the 22S RNA may be a degradation product of 28S RNA since it is observed in cytoplasmic ribosome preparations only when RNA is prepared by the procedure described in this paper, a procedure which is rather lengthy and which involves an incubation at room temperature under nonprotein denaturing conditions. On the other hand, the 20S RNA was observed as a constant percent component (compared to 18S and 28S RNA) when RNA was prepared by simply dissolving ribosomal pellets in buffer BN containing 1% SDS just prior to sucrose gradient fractionation even though three different ribosome preparations were investigated, each differing in preparation time and lysosome content. These results are similar to those of Attardi and Attardi (1971) who showed that when HeLa cell mitochondria are prepared by a shortened procedure, the 23S peak is no longer seen but the 21S material persists (although reduced in amount relative to the 18S RNA). Experiments are in progress to elucidate these points further.

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Isolation of a Highly Purified Myelin Protein*

J. Gagnon, P. R. Finch, D. D. Wood, and M. A. Moscarello†

ABSTRACT: Myelin was extracted first with mercaptoethanol followed by 0.2 N H_2SO_4. The resulting residue was dissolved in chloroform–methanol (1:1, v/v) containing 5% of 0.1 N HCl and applied to a Sephadex LH-20 column. The major protein fraction was rechromatographed on a similar LH-20 column. The protein was isolated by precipitating with ether and the precipitate was washed with 2-propanol. It migrated as a single component in polyacrylamide gel electrophoresis and appeared to be homogeneous by analytical ultracentrifugation. Amino acid analysis revealed it was relatively high in neutral and low in basic residues. A single N-terminal glycine was found. Of the carbohydrates tested, sialic acid, glucose, mannose, galactose, fucose, and hexosamine, only the latter two were present in appreciable amounts. About 2% of fatty acids was detected, mainly C_{16} and C_{18}. Phosphorus was less than 0.03 μmole/10-mg sample and no glycerol was detected. It was concluded that the fatty acid was not part of triglyceride, phospholipid, sphinogolipid, or galactolipid. Attempts to remove the residual fatty acid by prolonged Soxhlet extraction, treatment with phenol–acetic acid–water containing 2 M urea followed by dialysis, ether extraction of a solution of the protein in 98% formic acid, and charcoal failed to reduce the amount of fatty acid recovered.

At the present time, at least three protein fractions have been isolated from myelin. One of these, a basic protein (also called the encephalitogenic protein), was first isolated by Laatsch *et al.* (1962) and has been subsequently studied in a number of laboratories including our own (Martenson *et al.*, 1969; Lowden *et al.*, 1966; Tomasi and Kornguth, 1967). It has been extensively purified and an amino acid sequence has been reported (Kibler *et al.*, 1969; Eylar, 1970).

The other two protein fractions have been less extensively characterized, largely due to technical difficulties associated with delipidation. One of these protein fractions was first isolated by Folch and Lees (1951) and termed "proteolipid," to denote a mixture of protein and lipid soluble in mixtures of chloroform–methanol. A water-soluble preparation was

* From the Department of Biochemistry, Research Institute, The Hospital for Sick Children, Toronto, Ontario, Canada. *Received March 10, 1971.*