TRANSFER RNA ASSOCIATED WITH
RAT LIVER PLASMA MEMBRANES

BY

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ABSTRACT

Plasma membranes were isolated by differential and gradient centrifugation of rat liver homogenates. The isolation was monitored by assay of the plasma membrane marker enzymes 5'-nucleotidase, Mg^{2+} activated ATPase and alkaline p-nitrophenyl phosphatase. Gross contamination was estimated by electron microscopy and assay of the endoplasmic reticulum marker enzyme glucose-6-phosphatase. RNA was extracted from purified plasma membranes and aminoacylated with radioactive amino acids. The amino acid acceptor activity of this RNA could not be attributed to contamination with transfer RNA from other cell fractions or adherence of cytoplasmic low molecular weight RNA during the isolation. This is the first demonstration that transfer RNA is associated with purified plasma membranes.

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INTRODUCTION

Initial studies on plasma membranes were concerned mainly with physiological measurements of their barrier properties such as permeability, excitation and transport, with the intention of obtaining information about the mechanisms of these processes. The membrane was regarded as a fairly uniform structural entity as exemplified by the Davson-Danielli model which describes it as a bimolecular layer of phospholipid sandwiched by protein aggregates. In addition, artificial lipid membranes or black lipid films were subjected to permeability measurements and structural analysis, and gained acceptance as experimental models for biological membranes.

It became apparent that studies of the plasma membrane by these methods did not yield results that would adequately describe the many functions which were assigned to it. Among these are facilitated diffusion, active transport, secretion, adhesion, contact inhibition, information transfer and intercellular communication. In the early 1960's plasma membranes were isolated first from erythrocytes and then from rat liver, thereby allowing their characterization as discreet cellular organelles.

Biochemical studies indicated that the membrane was composed primarily of protein and phospholipid, but also contained significant amounts of carbohydrate, cholesterol and ribonucleic acid (RNA). Furthermore, several enzyme activities were found to be associated with plasma membranes, strongly implicating a dynamic functional organization. A major portion of plasma membrane research to date has been concerned with determination of chemical and enzymatic composition.

Further studies have led to the understanding of mechanisms by which the plasma membrane, in coordination with other cellular constituents, is able to act as a transducer of information between the external milieu and intracellular systems. One such example is Sutherland's study on the mechanism of hormone action in which it was determined that the plasma membrane, through the action of adenylate cyclase, transfers the hormonal message into the cell in the form of cyclic adenosine monophosphate. Another is the participation of the plasma membrane in the immune response where information on antigenic determinants crosses the membrane and results in antibody synthesis and cell proliferation. On the basis of such studies it has been suggested that the membrane might play a role in the storage of acquired information.

One perplexing question in the functional characterization of plasma membranes lies in the presence of RNA in purified preparations. Since nucleic acids in general are the ultimate source of information transfer, storage and retrieval in the cell, their association with the plasma membrane at least implies a functional significance. Furthermore, transfer RNA of cellular origin has been found in various strains of RNA tumor viruses. These viruses bud from the cell surface upon maturation, incorporating a segment of the plasma membrane. The possibility exists that the tRNAs present in these virions may be tRNA which is associated with the plasma membrane.

RNA has been reported as a constituent of plasma membranes by nearly every investigator who has isolated them. Its presence is considered by some to be an artifact of isolation and by others a component of a liporibonucleoprotein complex, an integral part of the membrane. To date however, no biological function has been attributed to the plasma membrane RNA population.

As an initial step in assigning a biological activity to plasma membrane associated RNA, it is the purpose of this investigation to determine if this RNA will accept amino acids; that is to detect tRNA associated with plasma membranes.

LITERATURE REVIEW

Transfer RNA (tRNA) is best known for its role in ribosomal protein synthesis. Aminoacyl tRNA is hydrogen bonded through codon-anticodon interactions to messenger RNA at a ribosomal site. Its amino acid is enzymatically transfered to the carboxy terminus of the growing polypeptide. Esterification of the correct amino acid to the 3' terminal adenylic acid residue of its corresponding tRNA is catalyzed by a tRNA synthetase(E.C. 6.1.1) which is specific for a particular amino acid-tRNA pair(1).

Transfer RNA is involved in other activities in biological systems. Glycyl tRNA in Staphylococcus Aureus acts as an intermediate in the ribosome independent incorporation of glycine into the cell wall glycopeptide(2). The tRNA donates its amino acid to a phospholipid intermediate which functions in peptidoglycan synthesis(3). There is at least one instance where an isoaccepting glycyl tRNA is able to function in cell wall synthesis but not in protein synthesis(4). Participation of tRNA in peptidoglycan synthesis of bacterial cell walls is also known for other aminoacyl tRNAs (5,6).

Aminoacylphosphatidylglycerol synthesis is also mediated by tRNA. Lysyl tRNA provides its amino acid for the synthesis of O-L-lysyl phosphatidylglycerol in a ribosome independent system. The reaction is unaffected by inhibitors of protein synthesis and differs from peptidoglycan synthesis by the formation of an O-ester linkage between the amino acid and phosphatidylglycerol(7).

Another function of tRNA is in the enzymatic modification of proteins. Arginyl tRNA is an intermediate in the transfer of arginine to the N-terminal end of certain proteins. Again this process does not require ribosomes and is not inhibited by puromycin(8,9). Although it appears that arginine is incorporated most actively, the system has been demonstrated for several other amino acids in eucaryotic cells(10).

Transfer RNA clearly functions in reactions other than protein synthesis. It may be expected that tRNA is associated with cell organelles which are not actively involved in the *de novo* synthesis of proteins. Since RNA has been reported to be associated with purified plasma membranes, this organelle is strongly implicated. To test the hypothesis that tRNA is associated with the plasma membrane, highly purified preparations are necessary.

Several methods have been employed for the isolation of plasma membranes from rat liver. Purity of the membranes has usually been monitored by various combinations of several criteria. These include microscopy, marker enzyme activity and chemical composition. Initial procedures called for the disruption of tissue by a Dounce homogenizer in water buffered to pH 7.5 by $NaHCO_3$ followed by low speed centrifugation and floatation of membrane material in a sucrose gradient(11). This procedure resulted in fragmentation of nuclei and mitochondria but was shown by electron microscopy to produce membranes void of contamination from other cell fractions. Many subsequent methods are modifications of this origional procedure.

Similar membrane preparations were subjected to extensive chemical and enzymatic analysis in addition to microscopic examination. A number of enzymes responsible for the splitting of phosphate esters were found to be associated with isolated membranes including Mg^{2+}- activated ATPase (E.C. 3.6.1.4), 5'-mononucleotidase(E.C. 3.1.3.5), acid and alkaline phosphatases(E.C. 3.1.3.1 and E.C. 3.1.3.2) and glucose-6-phosphatase(E.C. 3.1.3.9). Microsomal enzymes such as glucose-6-phosphatase were present in lower amounts than in microsomal preparations(12).

In order to minimize contamination, modifications such as the use of isotonic sucrose buffers were adopted to preserve the integrity of other cell components such as nuclei and mitochondria. Chemical analysis revealed that RNA is present in plasma membrane preparations in amounts up to 4% (13). Both RNA and DNA were detected in rat liver plasma membrane preparations displaying characteristic enzymatic and chemical composition. This observation was attributed to an inability to completely rid the membrane preparation of contaminating ribosomes and nuclear material(14). Significant amounts of RNA however, are also detectable in membranes prepared by methods designed to enhance membrane quality and yield(15, 16). Thus the purity of isolated preparations is critical to determining the association of RNA with the plasma membrane.

Studies on the enzyme markers of the plasma membrane show that there is considerable variation in reported specific activities for a given marker enzyme. These often depend on the method of preparation. As a result, a particular set of values for marker enzyme activities does not define the degree of purity of a rat liver plasma membrane preparation (17). Because of the heterogeneity of the hepatocyte membrane, a particular marker may be representative of only a certain area of the membrane. For example alkaline p-nitrophenyl phosphatase is localized in the bile canalicular regions indicating that a more uniformly distributed marker such as 5'-nucleotidase provides a better evaluation of total cell surface yield(16).

Morphological observation by electron microscopy may also lead to erroneous conclusions regarding membrane purity. Hepatocyte membrane morphological markers include junctional complexes, large bile canalicular membranes and microvilli. Considering the minute scale used in electron microscopy, quantification of such markers is hampered by the difficulty of examining samples. Therefore, morphological criteria are best reserved for detection of gross contamination(18).

Calculations have been made regarding the purity with respect to RNA content of plasma membranes isolated from rat liver in a buffered isotonic sucrose medium. On the basis of microsomal data from two different sources, 10 and 14.3% contamination with microsomes was needed to account for all of the RNA found to be associated with the purified plasma membranes. This is in close agreement with the 9% contamination calculated from the relative amounts of glucose-6-phosphatase activity in the plasma membrane preparation compared to a microsomal fraction(19). This analysis may be questioned since a value for contamination with RNA from the microsomal fraction upon which the enzymatic estimate was based, was not determined.

Other studies were performed to elucidate the origin and properties of the so-called plasma membrane associated RNA. After solubilization of 26-28% of the plasma membrane protein with 0.14 M NaCl none of the RNA was solubilized. Furthermore, the membrane RNA exhibited a relative resistance to RNAse. Using radioactive methods it was established that the rat liver plasma membrane RNA was maximally contaminated by 4-7% with other RNAs. In addition sucrose gradient centrifugation revealed two main peaks corresponding to 10-12 S and 3-3.5 S with a smaller heavy peak at 28-30 S. These peaks did not coincide with those of ribosomal RNA. With the exception of a 20-22 S component which was missing from plasma membrane RNA, the gradient profile resembled that of RNA from microsomal membranes which had been freed of ribosomes. This substantiates the claim that plasma membrane RNA is not derived from contaminating microsomal material(20, 21,22).

The presence of tRNA in the plasma membrane of eucaryotic cells has been implicated by studies of tRNA associated with RNA tumor viruses. Transfer RNA is a component of Avian Myeloblastosis Virus nucleic acid which is thought to be incorporated into the virus as it buds from the cell (23). The base ratios and the degree of methylation of the viral 4S RNA is similar to the host cell tRNA(24). The 4S RNA component of Rous Sarcoma Virus is similar to host 4S RNA with respect to electrophoretic mobility and degree of methylation. There are nevertheless, significant differences in base composition between the two 4S RNAs(25). There is evidence that the tRNA activity of the virion differs from that of the host indicating that the virus associated tRNA is not a random collection of host tRNA. Thus it has been strongly suggested that the tRNAs found in the virus may be tRNAs which are associated with the host cell membrane(26).

Evidence regarding the nature of the RNA associated with purified membranes is scarce. As a result questions remain as to the existence of a real association with the membrane and the subsequently implied biological function. This work is concerned with the detection of tRNA in highly purified rat liver plasma membrane preparations, that cannot be attributed to contamination with other cell fractions or adhering low molecular weight cytoplasmic RNA.

MATERIALS AND METHODS

Preparation of Plasma Membranes

Plasma membranes were isolated by a modification of the method of Ray(15). All operations were performed at 0-5° C. The homogenizing buffer consisted of 1.0 mM $NaHCO_3$ and 0.5 mM $CaCl_2$ at pH 7.5. Twelve g of fresh rat liver were washed in buffer several times and minced with a scissors. The minced liver was homogenized in 150 ml buffer using 25 gentle strokes in a Dounce homogenizer equipped with a loose pistle(Blaesig Glass Co., Rochester, New York).

The homogenate was diluted to 1200 ml with buffer and left for 5 min with occasional mixing. The diluted homogenate was filtered through four layers of cheese cloth and centrifuged at 1600 X g for 30 min in the GSA rotor of a Sorvall RC2-B refrigerated centrifuge. The supernate was saved for the isolation of mitochondrial and microsomal fractions. Pellet I was suspended in 50 ml buffer by gentle homogenization(2-3 strokes), diluted to 600 ml, and centrifuged at 1600 X g for 15 min. The supernate was disgarded and Pellet II was suspended in 300 ml buffer and centrifuged as above. The supernate was disgarded and Pellet III was suspended in buffer and brought to a final volume of 30 ml.

Pellet III suspension was mixed with 60 ml of 70% sucrose(W/W) in homogenizing buffer to a final concentration of 48% sucrose and approximately 10 ml were placed in each of 9 Spinco SW 25.1 centrifuge tubes. Over each was layered 8 ml 45% sucrose(W/W), 10 ml 41% sucrose(W/W) and 37% sucrose

(W/W) to the top of the tube. The tubes were centrifuged for 1 hr at 64,700 X g (25,000 rev min^{-1}) in a Beckman L3-50 ultracentrifuge. The particulate layer at the interface between 37 and 41% sucrose was collected with a Pasteur pipette. The pooled membrane fractions, designated membrane fraction #1, were washed by centrifugation in buffer and suspended in a volume of 20 ml. This membrane suspension was mixed with 40 ml of 70% sucrose to a final volume of 48% sucrose and divided among 6 Spinco SW 25.1 tubes. Discontinuous gradients were again constructed and centrifuged as above.

The washed plasma membrane fraction from the second discontinuous gradient centrifugation, designated membrane fraction 1_2, was split and layered on top of two continuous sucrose gradients of 25-50% sucrose(W/W) in Spinco SW 25.1 tubes. These tubes were centrifuged at 64,700 X g for 12-14 hr. Fractions were collected dropwise from the punctured tubes and those that banded in the density region of 1.16 g ml^{-1} were washed and suspended in approximately 6 ml of homogenizing buffer. This fraction was designated membrane fraction 1-G.

Isolation of Microsomes and Mitochondria

A mitochondrial fraction was isolated from the supernate of Pellet I by centrifugation at 10,800 X g for 10 min (Sorvall RC2-B, SS-34 rotor, 9500 rev min^{-1}). The pellet was suspended in homogenizing buffer and recentrifuged as above. The washed mitochondrial pellet was suspended in 2 ml buffer.

A microsomal fraction was isolated from the crude mitochondrial supernate by centrifugation at 134,800 X g for 1 hr (Beckman L3-50 ultracentrifuge, Ti-50 rotor, 45,000 rev min^{-1}). This fraction was washed by recentrifugation as above and suspended in 2 ml homogenizing buffer.

The remainder of the crude mitochondrial supernate was retained for the isolation of low molecular weight cytoplasmic RNA.

Chemical and Enzymic Determinations

Protein was determined by the method of Lowry et al (27) using bovine serum albumin as standard. RNA was determined by the Dische Orcinol technique(28). Inorganic phosphorous was assayed by the method of Fiske and SubbaRow(29).

5'-Nucleotidase(E.C. 3.1.3.5) and Mg^{2+} activated ATPase(E.C. 3.6.1.4) were assayed at pH 7.5 and 37° C under the conditions given by Ray(15). The incubation mixture for Mg^{2} activated ATPase consisted of 10 umoles Tris-Cl, 10 umoles $MgCl_2$, 10 umoles ATP and 10-50 ug membrane protein in a total volume of 1 ml. 5'-Nucleotidase was assayed in an incubation mixture consisting of 50 umoles Tris-Cl, 10 umoles $MgCl_2$, 10 umoles 5'-AMP and 10-50 ug of membrane protein in a total volume of 1 ml. The reactions were stopped by the addition of 10% trichloroacetic acid(TCA) after 10 min incubation. Insoluble material was removed by centrifugation and inorganic phosphorous was determined in the supernate.

Alkaline phosphatase was assayed by the method of Ray (15) at pH 9.0 and 37° C with p-nitrophenyl phosphate as substrate. The incubation mixture consisted of 100 umoles Tris-Cl, 2.5 umoles $MgCl_2$, 1.0 umole p-nitrophenyl phosphate, 0.1% Triton X-100 and 25-100 ug of membrane protein in a total volume of 1.0 ml. The reaction was stopped after 20 min incubation with the addition of 0.5 ml of 0.75 M Na_2CO_3 and the change in absorbance was measured at 420 nm.

Glucose-6-phosphatase(E.C. 3.1.3.9) activity was assayed by the method of Nordlie and Arion(30) at pH 6.5 and 30^{o} C in 60 umoles Sodium Cacodylate, 30 umoles Glucose-6-phosphate and 20-50 ug of membrane protein in a final volume of 1.5 ml. The reaction was stopped after 10 min incubation with 0.5 ml of 20% TCA and inorganic phosphorous was determined.

All enzyme assays were determined at two protein concentrations (usually a doubling of protein concentration) to ensure linearity of enzyme activity with concentration.

Preparation for Electron Microscopy

Cell fractions were fixed at 4^{o} C in 2% glutaraldehyde in 0.1 M phosphate(pH 7.2) containing 0.25 M sucrose and 2 mM $CaCl_2$ for 2 hr. Specimens were washed overnight in buffer. After post fixation with 1.0% OsO_4 in the same buffer, specimens were dehydrated and embedded in Spurr low viscosity embeddment(31). Thin sections were stained with uranyl acetate and lead citrate and examined in a Hitachi HS-8 electron microscope.

Isolation of RNA

RNA was isolated from the cell fractions by extraction with phenol-dodecyl sulfate. Suspensions of the cell fractions were brought to 1% with sodium dodecyl sulfate and one-half volume of phenol was added. After thorough mixing and centrifugation to separate the phases, the aqueous phase was removed and the phenol layer was again extracted with an equal volume of homogenizing buffer. The RNA in the combined aqueous phases was precipitated with 0.1 volume of 20% sodium acetate(pH 5.4) and two volumes of 95% ethanol, centrifuged, and redisolved in a small volume of distilled water.

Total cytoplasmic RNA was isolated from a post mitochondrial cell supernate by the above procedure. Transfer RNA was prepared from total RNA by chromatography on DEAE-cellulose(32).

All RNA samples were de-esterified of amino acids by incubation in 1.0 M Tris-Cl (pH 9.0) at 37° C for 90 min(33). The RNAs were recovered by ethanol precipitation as above and redissolved in distilled water.

Preparation of Aminoacyl Synthetases and Aminoacylation of Transfer RNA

Transfer RNA synthetases were prepared as follows: Five g rat liver were homogenized in 25 ml buffer containing 10 mM Tris-Cl (pH 7.5), 1.0 mM $MgCl_2$, 20 mM 2-mercaptoethanol, 10 mM NH_4Cl and 10% glycerol (V/V). The homogenate was centrifuged at 27,000 X g (15,000 rev min^{-1}, Sorvall SS-34 rotor) for 5 min. The supernate was chromatographed on a DEAE-cellulose column (1X3 cm) previously equilibrated with the same buffer. The column was washed with buffer until the absorbance at 280 nm was less than 0.05. Protein fractions were than eluted with buffer containing 0.25 M NH_4Cl, combined, concentrated by dialysis against 20% Carbowax 5000 in buffer (W/V) and finally dialyzed against buffer containing 0.01 M NH_4Cl. The final solution was adjusted to 50% glycerol (V/V) and stored at -20° C. This preparation served as the source of aminoacyl synthetases.

RNA was aminoacylated in a 0.1 ml reaction mixture containing 10 umoles sodium cacodylate (pH 7.2), 0.2 umoles ATP, 1.0 umole KCl, 1.0 umole magnesium acetate, 1.0 uC ^3H labelled amino acid, 2-10 ug RNA and 10 ul synthetase preparation. Reaction mixtures were incubated at 37° C and 20 ul aliquots were precipitated on Whatman 3MM filter discs in cold 5% TCA at various times after incubation. Discs were washed 3 times in 5% TCA for 15 min, once in ethanol-ether mixture (1:1), and finally in ether for 10 min. The dried filter discs were counted in Omniflour (New England Nuclear) in a Packard TriCarb liquid scintillation spectrometer. Counting efficiency for tritium was about 7%.

RESULTS

Properties of the Plasma Membranes

The modifications of the Ray procedure(15) for the isolation of rat liver plasma membranes used in this work are outlined as follows: (1) Centrifugation times for the discontinuous sucrose gradients were reduced to 1 hr. (2) A second discontinuous gradient centrifugation was performed on the initial membrane fraction. (3) Membranes at the 37-41% sucrose interface after the second discontinuous centrifugation were layered on a continuous sucrose gradient and centrifuged for 12-14 hr at 64,700 X g.

These modifications resulted in a lower yield of plasma membranes but significantly increased their purity. It can be calculated from Table I that membrane fraction 1-G contains 35% of the protein, 34-39% of the activity of the plasma membrane marker enzymes(5'-nucleotidase, Mg^{2+} activated ATPase and alkaline phosphatase) but only 25% of the glucose-6-phosphatase activity found in membrane fraction #1. Also, in comparison to membrane fraction #1, the specific activities of the plasma membrane marker enzymes remained approximately the same while the specific activity of glucose-6-phosphatase decreased by 50% in membrane fraction 1-G. An increase in membrane purity as observed by electron microscopy supports these results.

The properties of rat liver plasma membranes isolated by the methods noted above are summarized in Table II. Values for the specific activities of 5'-nucleotidase and Mg^{2+}

TABLE I

PROTEIN CONTENT AND ENZYMATIC ACTIVITY OF VARIOUS PLASMA MEMBRANE FRACTIONS

Enzyme activities in umoles substrate utilized or product liberated per hr per mg protein. Average values and range values (parentheses) are the results of 4 different preparations. Percent recovery is based on total enzyme activity or protein content in the homogenate equal to 100%.

	Membrane Fraction 1	Membrane Fraction 1_2	Membrane Fraction 1-G
% Protein Recovery	1.7	0.8	0.6
5'-Nucleotidase			
% Recovery	17.2	10.6	6.4
Specific Activity	40(29-48)	56(36-98)	36(18-49)
Alkaline Phosphatase			
% Recovery	5.7	3.4	2.2
Specific Activity	0.9(0.8-1.4)	1.3(0.9-1.9)	1.1(0.7-1.7)
Mg^{2+} ATPase			
% Recovery	13.7	6.4	4.6
Specific Activity	84(68-105)	87(70-116)	103(54-162)
Glucose-6-Phosphatase			
% Recovery	1.6	0.7	0.4
Specific Activity	3.8(3.0-4.8)	3.1(2.0-4.3)	1.8(0-2.9)

TABLE II

ENZYMATIC AND CHEMICAL CONTENT OF PLASMA MEMBRANES AND MICROSOMES

Enzyme activities in umoles substrate utilized or product liberated per hr per mg protein. Average values and range values are the results of 4 different preparations. Percent recovery is based on total enzyme activity in homogenate equal to 100%.

Plasma Membranes	Average	Range	% Recovery
mg protein per g wet liver	1.17	0.65-2.17	--
ug RNA per mg protein	21.0	12.7 -28.9	--
5'-Nucleotidase	35.7	18.4-49.5	6.4
Mg^{2+} ATPase	102.5	54.0-162	4.6
Alkaline p-nitrophenyl phosphatase	1.08	0.50-1.71	2.2
Glucose-6-phosphatase	1.84	0.0-2.9	0.36
Microsomes			
ug RNA per mg protein	140	100 -242	--
Glucose-6-phosphatase	5.0	2.9-7.1	--

activated ATPase represent a 10-fold enrichment of enzyme activities relative to the initial homogenate, whereas that for alkaline phosphatase represented about a 5-fold enrichment. This is consistent with the observation that alkaline phosphatase yield is not representative of total cell surface yield(16). The specific activity of glucose-6-phosphatase in the microsomal fraction was enriched approximately 2-fold over the activity of this enzyme in the initial homogenate. In contrast, the glucose-6-phosphatase activity of membrane fraction 1-G decreased by one-half compared to the initial homogenate. The enzyme activities of the plasma membrane described compare favorably with previous studies(14,15,16). RNA and protein yields were also consistent with those reported by other investigators(14,15,16,20).

Electron micrographs of these preparations demonstrated that membrane fraction 1-G, except for the very occasional appearance of microsomal material, contained no visible contamination from other cell fractions. In addition, membrane fraction 1-G displayed the same morphological features such as junctional complexes and bile canaliculi, which are present in intact hepatocyte membranes. Figure 1 is a typical electron micrograph of plasma membrane fraction 1-G.

<u>Aminoacylation of RNA Extracted from Membranes</u>

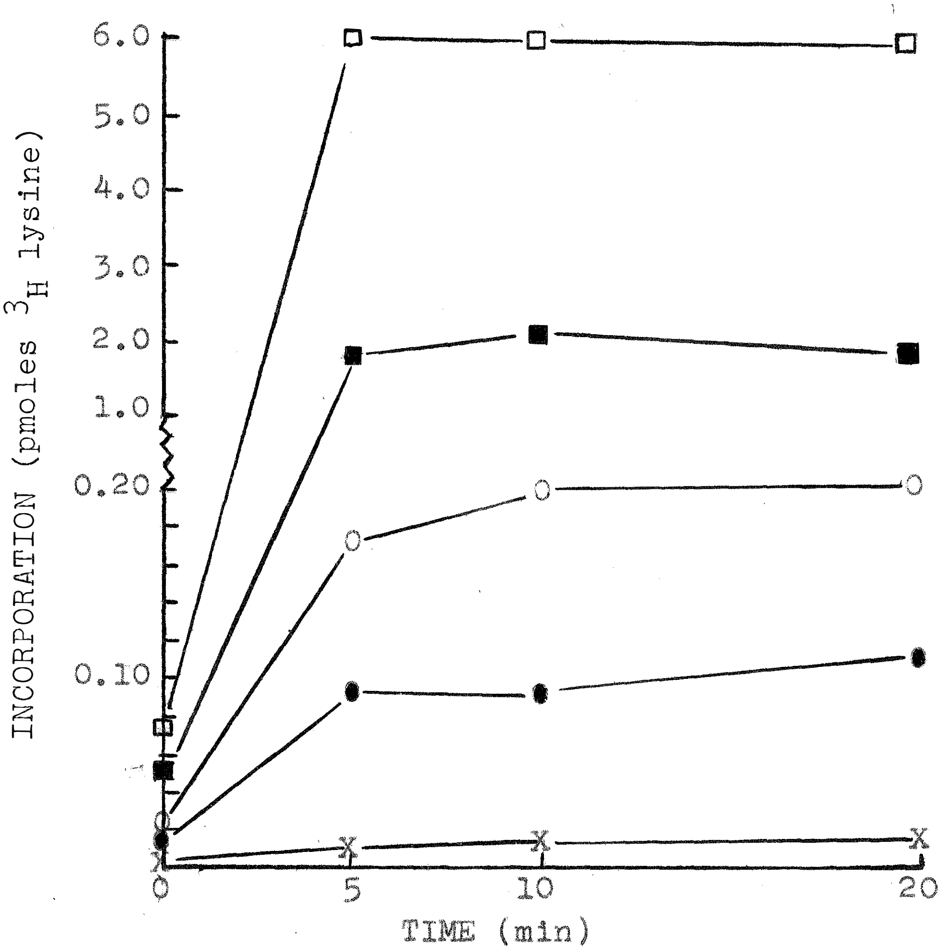
RNA extracted from the various cell fractions was assayed for transfer RNA acceptor activity and the results of a typical aminoacylation assay are given in Figure 2. The incorporation of lysine into membrane RNA was RNA dependent

Figure 1

TYPICAL ELECTRON MICROGRAPH OF MEMBRANE FRACTION 1-G

Figure 2

AMINOACYLATION ASSAY OF RNA FROM MEMBRANE FRACTION 1-G AND LOW MOLECULAR WEIGHT CYTOPLASMIC RNA



Specific activity of ^{3}H lysine was 55 Ci/mmole. Control with no RNA added (X——X). Incorporation of ^{3}H lysine by membrane fraction 1-G RNA 4.5 ug (●——●) and 9.0 ug (O——O) and by low molecular weight cytoplasmic RNA 1.7 ug (■——■) and 3.4 ug (□——□) was under assay conditions described in the text.

and not the result of a small amount of tRNA contamination in the synthetase preparation. Table III is a summary of the results of aminoacylation experiments with three different amino acids. It is clear from the results of Table III that RNA extracted from plasma membrane fraction 1-G accepted amino acids. Assuming that all 20 amino acid accepting tRNA species were present, at least at the levels detected for lysine, 0.02 pmoles/ug RNA, then the total tRNA content of plasma membrane RNA can be calculated as follows: 20 amino acids X 0.02 pmoles = 0.4 pmoles total tRNA per ug membrane RNA. Since the average molecular weight of tRNA 2.6 X 10^6 daltons, 0.4 pmoles tRNA is equivalent to 0.01 ug. Therefore the tRNA content of plasma membrane RNA is about one percent. This value is a minimal approximation since the charging reactions were kinetically limited by the amount of ^{3}H amino acid present. Because of limited quantities of membrane RNA, the specific activities of ^{3}H amino acids were somewhat maximized in the aminoacylation assays. This was useful for the detection of small amounts of tRNA but at the expense of accurate quantitative analysis.

<u>Criteria of Purity of Plasma Membrane Preparations</u>

The results of Figure 2 and Table III demonstrate that RNA associated with purified plasma membranes has amino acid acceptor activity. This result is valid only if the plasma membrane preparations are not contaminated with other tRNA containing cell fractions. Several experiments were therefore designed to check this possibility.

TABLE III

AMINO ACID ACCEPTOR ACTIVITY OF RNA ISOLATED FROM CELL FRACTIONS

Values in pmoles amino acid acylated per ug RNA

Cell Fraction	lysine	arginine	phenylalanine
Membrane fraction 1-G	0.020	0.050	0.053
Microsomes	0.033	0.057	0.079
Mitochondria	0.050	0.107	0.152
Cytoplasm	1.21	4.75	3.33

Assay conditions as described in the text. Specific activities of amino acids were as follows: (4,5-^{3}H) lysine, 55 Ci/mmole; (3-^{3}H) arginine, 27.3 Ci/mmole; (G-^{3}H) phenylalanine, 7.79 Ci/mmole.

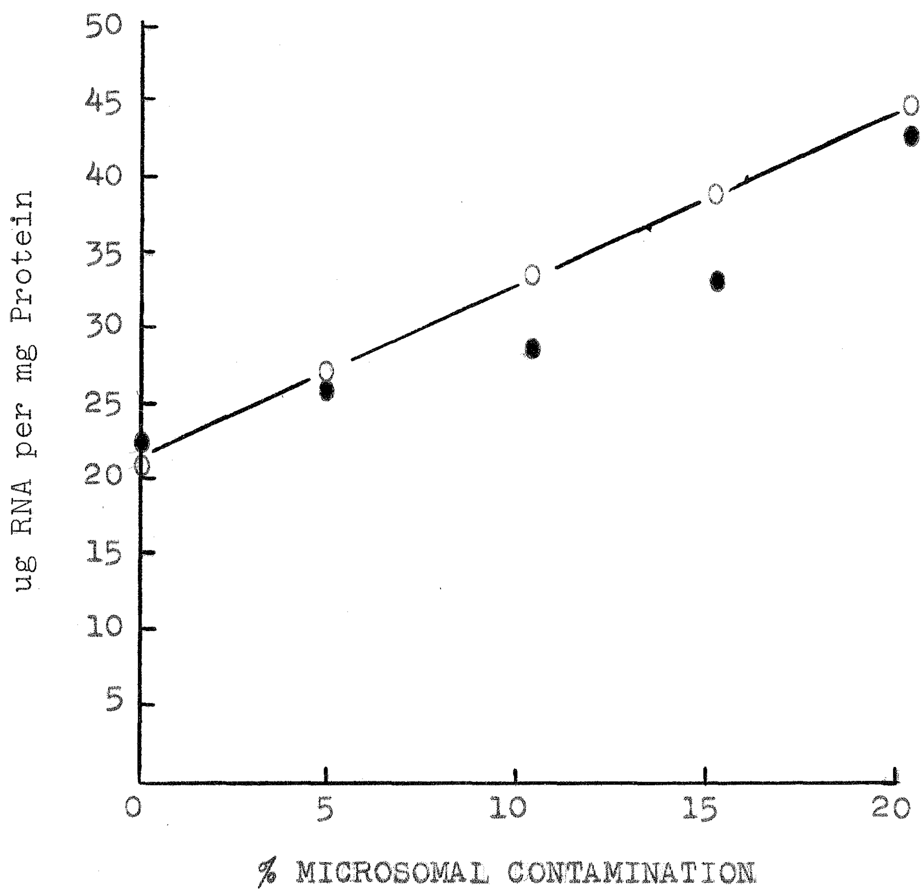
Inspection of Table III shows that membrane fraction 1-G contains about two-thirds as much tRNA as the microsomal fraction based on the number of pmoles of amino acid acylated per ug of RNA. However, since the microsomal fraction contains approximately seven times as much RNA per mg of protein as the membrane fraction(see Table II), a contamination level of about 10-15% microsomal material in the plasma membranes could account for the tRNA detected.

In order to check the above possibility, plasma membranes were mixed with microsomes to provide samples containing various levels of microsomal contamination. The synthetic mixtures were then subjected to the same RNA isolation and tRNA aminoacylation assays as membrane fraction 1-G. All samples contained approximately the same amount of protein and were processed at the same time. The results of these experiments are shown in Figures 3 and 4.

The data in Figure 3 demonstrates that the RNA yield of plasma membranes cannot be due to microsomal contamination since the extrapolation of the RNA yield to zero percent contamination results in approximately 21 ug RNA. In addition, the slope of the line in Figure 3 represents a value of about 6 ug RNA per every 5% increment in microsomal contamination. This value would require that the plasma membranes be 15-20% contaminated with microsomes, an unrealisticaly high value. Similar considerations apply to the data of Figure 4. In these experiments the RNA isolated from the synthetic mixtures was assayed for amino acid acceptor activity.

Figure 3

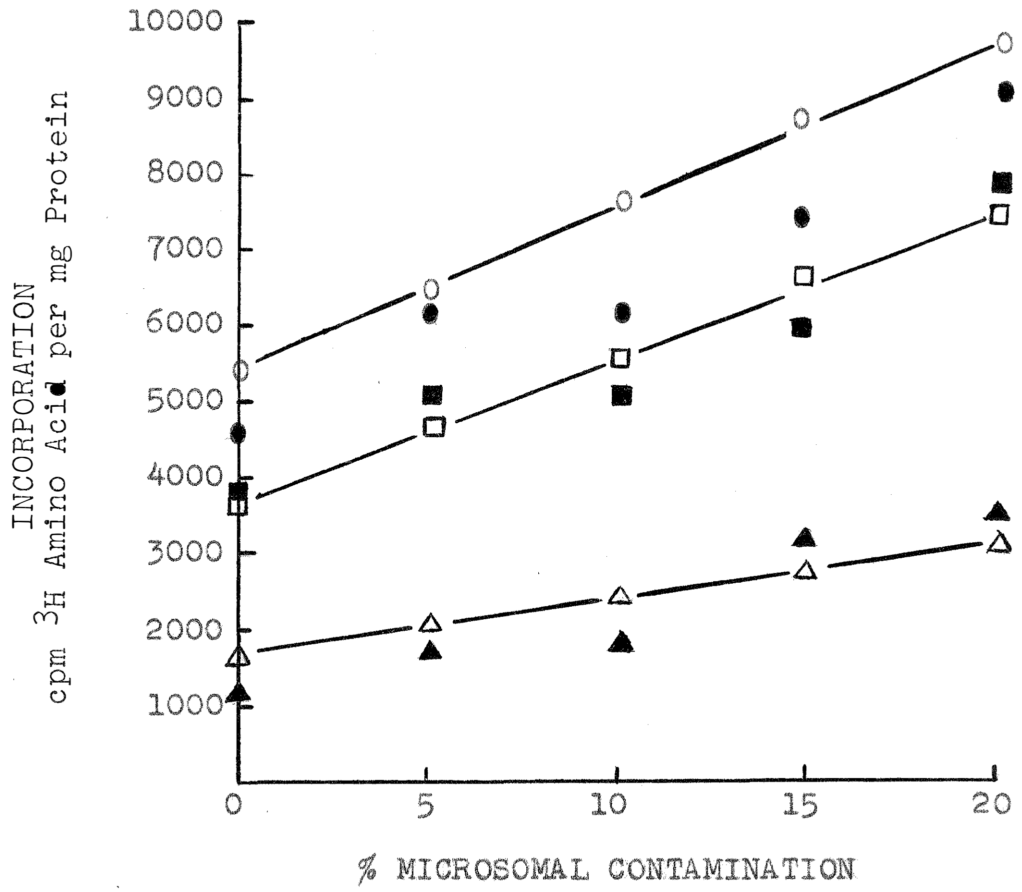
MICROSOMAL CONTAMINATION BASED ON RNA CONTENT



The curve (O——O) represents the expected values for ug of RNA per mg protein calculated from Table II for membrane fraction 1-G contaminated with various amounts of microsomal fraction. Points (●) represent actual values from prepared synthetic mixtures. Percent contamination is based on protein content of microsomal and plasma membrane fractions.

Figure 4

MICROSOMAL CONTAMINATION BASED ON AMINO ACID INCORPORATION



Specific activities of amino acids as given in Table III. The curves represent the expected cpm ^{3}H amino acid incorporated per mg of protein for various levels of microsomal contamination calculated from aminoacylation assays (described in text) with ^{3}H lysine (O——O), ^{3}H arginine (□——□), and ^{3}H phenylalanine (△——△). Points represent values obtained from aminoacylation of RNA from actual synthetic mixtures with ^{3}H lysine (●), ^{3}H arginine (■) and ^{3}H phenylalanine (▲). Percent contamination is based on protein content of microsomal and plasma membrane fractions.

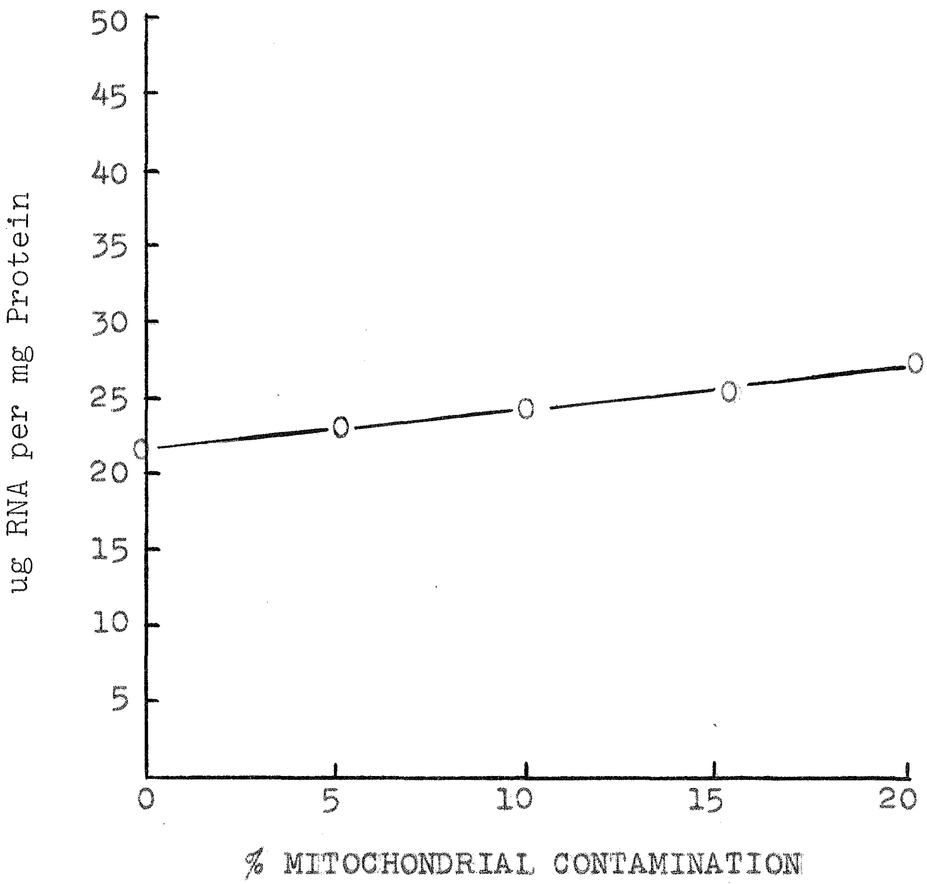
Extrapolation of the amounts of tRNA acceptor activity present in the membranes would require that they be of the order of 15-20% contaminated with microsomes in order to give the yields of amino acid incorporation into plasma membrane RNA for the three amino acids tested.

With the exception of actual construction of synthetic mixtures, a similar estimation was made regarding mitochondrial contamination. The slope of the line in Figure 5 represents 5.7 ug of RNA for every 20% increment in mitochondrial contamination. This value would require that the plasma membranes be over 70% contaminated with mitochondria. Likewise, the slopes of the lines in Figure 6 show that 18-25% mitochondrial contamination would be necessary to account for the yields of amino acid incorporation into plasma membrane RNA. Since these levels of contamination are unrealistic in view of the fact that mitochondria were not observed in electron micrographs of the purified plasma membranes, mitochondria were not considered to be a viable source of contamination.

As previously noted, electron micrographs of membrane fraction 1-G revealed little or no contamination with microsomal material. However, in order to obtain some quantitative estimate of the amount of microsomal contamination that could be detected, synthetic mixtures of membranes and microsomes were prepared and centrifuged at 134,800 X g. The resulting pellets were prepared for electron microscopy. Plasma membrane samples containing 5, 10, 15 and 20% microsomal

Figure 5

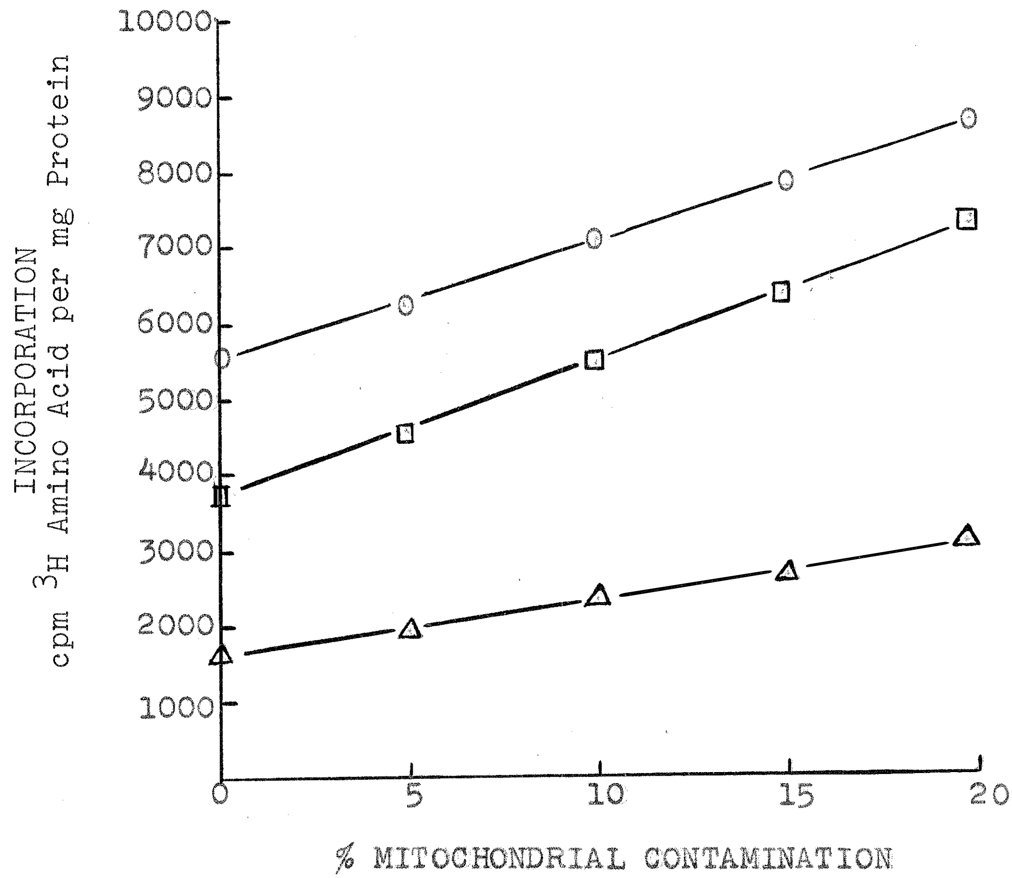
MITOCHONDRIAL CONTAMINATION BASED ON RNA CONTENT



This curve represents the expected values for ug of RNA per mg protein calculated for contamination of membrane fraction 1-G with varying amounts of mitochondrial fraction. The mitochondrial fraction contained 52 ug of RNA per mg protein. Actual synthetic mixtures were not constructed. Percent contamination is based on protein content of mitochondrial and plasma membrane fractions.

Figure 6

MITOCHONDRIAL CONTAMINATION BASED ON AMINO ACID INCORPORATION



Specific activities of amino acids as given in Table III. The curves represent the expected cpm ^{3}H amino acid incorporated per mg protein for various levels of mitochondrial contamination of membrane fraction 1-G calculated from aminoacylation assays (described in text) with ^{3}H lysine (O——O), ^{3}H arginine (□——□) and ^{3}H phenylalanine (△——△). Actual synthetic mixtures were not constructed. Percent contamination is based on protein content of mitochondrial and plasma membrane fractions.

contamination as well as a pure plasma membrane fraction 1-G were examined by Dr. Thomas P. Bonner of this Department without his prior knowledge of sample content. The plasma membrane fraction 1-G was clearly distinguished from the artificially prepared samples. This experiment demonstrated that 5% microsomal contamination is detectable and that the pure plasma membrane fraction must contain substantially less than this amount. The experiment supports the notion that the tRNA acceptor activity found in the membrane is not the result of microsomal contamination.

The possibility exists that the plasma membrane associated tRNA is merely cytoplasmic tRNA which adheres to the membrane during isolation. To eliminate this possibility, 1.8×10^{6} counts min^{-1}(cpm) (90 ug) of ^{32}P-labelled low molecular weight RNA prepared from KB cells(32) was mixed with Pellet I. The plasma membranes were isolated and the RNA extracted from the membranes as described previously. Twenty-five ug of RNA from membrane fraction 1-G was precipitated on a 3MM filter disc in cold 5% TCA, dried and counted for radioactivity. The RNA contained less than 40 cpm. This represents 2×10^{-3} ug of the added RNA present in 25 ug of membrane RNA, or approximately 0.008% contamination. This level of cytoplasmic tRNA cannot account for the tRNA acceptor activity detected in the plasma membrane preparations.

DISCUSSION

The presence of RNA in purified rat liver plasma membranes is a generally observed phenomenon. It has been demonstrated in this work that tRNA is a component of the RNA extracted from highly purified rat liver plasma membranes. Primary consideration has been given to establishing the purity of this fraction with regard to contamination from other cell fractions. The evidence indicates that the plasma membrane preparations described here are in excess of 95% pure with respect to contaminating RNA. This level of contamination is not adequate to account for the amount of RNA or the amino acid acceptor activity present in the preparation. It has also been shown that this observation is not the result of adherence of RNA to the plasma membrane during the isolation.

Calculations made by Berman(19) suggest that there is a correlation between the amount of glucose-6-phosphatase activity in the plasma membrane fraction and contamination with microsomal RNA. The glucose-6-phosphatase activity was determined on a microsomal fraction prepared by centrifugation at 23,000 X g. This procedure fails to pellet free ribosomes and may result in a microsomal preparation having a high specific activity of glucose-6-phosphatase and a low RNA to protein ratio. Since the microsomes for which RNA contamination was calculated were isolated by centrifugation at 105,000 X g(34) under different conditions, the correlation may not be justified.

It is possible that the plasma membrane normally contains varying amounts of glucose-6-phosphatase. For this reason and because of the variability in values reported for the various marker enzyme activities(17), the values obtained here were used as a rough assessment of the quality of the preparations. Purity was estimated on the basis of RNA extracted from cell fractions isolated from a common homogenate and synthetic mixtures of these fractions. These data were correlated with electron microscopic observation.

There exists a possibility for contamination for which evidence has not been provided. Portions of the hepatocyte membrane are in the form of numerous microvilli. These form minute vesicles when the tissue is subjected to shear forces such as those produced by Dounce homogenization(35). The vesicles may be of sufficiently small size so as to be stable against disruption against further shearing. It is possible that a small amount of cytoplasmic tRNA is incorporated in these membrane vesicles. It can only be assumed that the method of isolation insures that the vesicles formed are rid of their contents.

It was not conclusively demonstrated in this work that tRNA is responsible for the amino acid acceptor activity of the RNA from purified plasma membranes. It has been demonstrated that higher molecular weight RNAs present in certain plant viruses are able to accept amino acids(36). This however, is an isolated case and no evidence has been found supporting this observation in a mammalian system. It is therefore reasonable to assume that the amino acid accepting species is tRNA. (Preliminary studies done in this laboratory using reverse phase chromatography indicate that plasma membrane RNA displays characteristic isoaccepting profiles for at least one amino acid.) Further molecular characterization of the tRNA associated with plasma membranes will serve to verify this assumption and provide information about its origin and function. For example plasma membrane associated tRNAs may be compared with respect to base composition and isoaccepting profiles to tRNAs found in the cytoplasm or in RNA tumor viruses.

Because of the drastic measures employed in the isolation of plasma membranes, the problems encountered in determining absolute purity and the very small amounts of tRNA detected, the possibility of artifact cannot be totally eliminated. Central to the problem of establishing a real association of tRNA and higher molecular weight RNAs with the plasma membrane is the elucidation of an RNA dependent process occurring at the site of the membrane. A functional association of RNA with the plasma membrane may serve to explain some of the activities of this organelle.

Some speculation may be considered regarding the function of membrane associated tRNA. It is reasonable that this tRNA may act in conjunction with higher molecular weight RNAs associated with the membrane. This implies plasma membrane associated protein synthesis. Incorporation of amino acids into polypeptides by a surface membrane preparation has been reported(37). The characteristics of this system are almost identical to microsomal protein synthesis with regard to ribosome dependence and drug sensitivities. Since the preparations contained relatively high RNA to protein ratios(60-80 ug RNA per mg protein) microsomal contamination may possibly account for this result.

The plasma membrane may be involved in ribosome independent polypeptide synthesis. RNA associated with the membrane could function as messenger RNA which may be translated at a site provided by certain membrane proteins. An environmental stimulus such as hormone binding or cell contact could activate this process. The resulting polypeptide product could conceivably facilitate the cell's response. No evidence exists for such a system.

Plasma membrane associated tRNA may function independently from the higher molecular weight RNAs found with the membrane. It is useful to consider two possibilities. First, as described above, tRNA is an intermediate in the ribosome independent addition of amino acids to both phospholipids and proteins. Since both of these are major components of the plasma membrane it is not unreasonable to suspect that tRNA associated with the membrane is in some way involved in such a reaction. These modifications may alter the properties of the plasma membrane in response to an external stimulus.

It has been well documented in the case of threonine deaminase that leucyl-tRNA can function as an allosteric inhibitor of enzyme activity(38). Therefore it is conceivable that aminoacyl-tRNA may function as an allosteric regulator of a membrane bound enzyme or transport protein. One example would be the inhibition of a plasma membrane bound amino acid transport protein by aminoacyl-tRNA. If this were the case one might expect that plasma membranes isolated from cells grown in an environment deficient in essential amino acids would have little or no tRNA associated with them. A relatively high yield of membrane associated tRNA in the aminoacyl form would also support this possibility.

In conclusion, the evidence presented here supports the hypothesis that RNA and specifically tRNA exists in a real association with the hepatocyte plasma membrane. The final proof rests in the elucidation of an RNA dependent function associated with the plasma membrane.

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