*MEMBRANES OF ANIMAL CELLS, III. AMINO ACID INCORPORATION BY ISOLATED SURFACE MEMBRANES**

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Abstract.—Surface membranes isolated from mouse fibroblasts contain RNA organized in ribosome-like particles. These surface membranes can incorporate radioactive amino acids into material insoluble in hot trichloroacetic acid. Characterization of the radioactive products indicated that they are large polypeptides. Most requirements and inhibitors of the system were similar to those of the microsomal system of the endoplasmic reticulum. Further comparisons were made with microsomal and pH 5 fractions prepared from L-cells by standard procedures and by two methods attempting to simulate the conditions necessary for isolating surface membranes. The amount of L-leucine incorporated by the surface membranes per mg of RNA was comparable to the values obtained by the microsomal and pH 5 fractions prepared by standard procedures. In contrast, the activity of the two other microsomal fractions was less than 10 per cent of this amount. The most striking difference noted was in the products formed by the surface membranes and by the microsomal systems. These products were demonstrated to be quite different by electrophoresis on acrylamide gels. It is suggested that the surface membranes participate actively in protein synthesis *in vivo*, and that, because of this location, the protein-synthesizing mechanism may be able to perform special functions more efficiently.

Recently methods have been developed permitting the isolation of whole surface membranes from mammalian cells.[1] These preparations are essentially free of nuclei, mitochondria, and other cytoplasmic particulates, making it possible to investigate a cell membrane as a separate and unique cell organelle.

RNA is present in isolated surface membrane fractions regardless of the method of preparation.[2] Electron micrographs show the presence of ribosome-like particles on the inner surface of the isolated membranes.[3] The presence of the necessary components for synthesizing protein so closely associated with the surface of a cell could be effective for producing proteins which are exported or which contribute to the structure of the membrane. These protein products could also be agents by which cell metabolism and behavior are controlled from the surface of the cell. Examination of the surface membranes of a mouse fibroblast showed that they could incorporate amino acids into hot trichloroacetic acid-insoluble material. The incorporation was found to proceed without the addition of exogenous soluble or microsomal fractions from the whole cell. This paper describes the characteristics of this amino acid incorporation.[4]

Materials and Methods.—All materials were obtained from commercial sources unless otherwise specified.

Isolation of surface membranes: Mouse fibroblasts (L-cells) were grown in suspension culture as described previously,[1] with the exception that the cells were grown in the

presence of aureomycin 3 days per week. The cultures were examined for Mycoplasma each month and were found to be negative. Membranes were isolated by a modification[5] of the Zn ion method[1], which makes it possible to obtain surface membranes representing the complete covering of cells, in contrast to the original Zn ion method by which fragments were obtained. An aliquot of the final membrane preparation was removed for counting in the haemocytometer and for determination of the protein content,[6] using bovine serum albumin as standard. The membranes were resedimented at 6000 $\times$ g for 20 min, and solution A (0.35 M sucrose, 0.07 M KCl, 0.05 M Tris-HCl, pH 7.8, 6 mM mercaptoethanol) was added. Precautions were taken to minimize bacterial contaminations throughout the entire procedure. The final membrane preparations contained few or no nuclei and very little other particulate matter of the cytoplasm as observed in the phase contrast microscope.[3] The RNA-P of such membrane preparations was determined.[7, 8] The membranes contained 60–80 μg of RNA per mg of protein. The membrane fractions represented 8–11% of the total cell RNA and 10–12% of the total cell protein.

Preparation of microsomal and pH 5 fractions:[9] For comparison with the surface membranes, microsomal and pH 5 fractions were prepared from L-cells by three procedures. (*a*) The fractions were prepared by standard procedures[10] to compare a known system of protein synthesis. (*b*) The surface membranes were prepared without the addition of $MgCl_2$, so that microsomal and pH 5 fractions were therefore prepared by procedure (*a*) but without $MgCl_2$ added to any of the isolating solutions. These fractions are designated "$-MgCl_2$." (*c*) Microsomal and pH 5 fractions were obtained from the same L-cell homogenates that yielded the surface membranes in the presence of $ZnCl_2$. These fractions were processed as in procedure (*a*) but without $MgCl_2$, and the pellet was designated "Zn^{++} microsomes." Proteins were determined on all final fractions. The RNA-phosphorus of microsomes prepared by procedure (*a*) was 1 μmole per mg of protein. The Zn^{++} microsomes contained 0.53 μmole per mg of protein.

Assay for amino acid incorporation: The incubation mixture unless otherwise specified consisted of ^{14}C-L-leucine (5.6×10^{-2} μc/ml), 20 amino acids (2 μM each), 0.1 M ATP, 0.018 M GTP, 8 mM $MgCl_2$, 0.048 M mercaptoethanol, 0.032 M phosphoenolpyruvate, and 30 IU of phosphoenolpyruvate kinase. The total volume of the reaction mixture was usually 0.25 ml with the enzyme fractions suspended in 0.15 ml of solution A to approximately 200 μg of membrane protein, or 150 μg of microsomal protein and 70 μg of pH 5 protein. The exact amounts were determined and are given with the particular experiment. Unless otherwise stated, the mixtures were incubated for 30 min at 37° and precipitated at 5° with 1 ml of 5% trichloroacetic acid after the addition of 250 μg of bovine serum albumin. The precipitated solutions were heated at 80–85° for 15 min, filtered on Millipore filters, washed, dried, and counted after the addition of Liquifluor-toluene scintillation fluid.

Examination of radioactive products: Fluorodinitrobenzene derivatives of the radioactive products were prepared.[11] Thin-layer chromatography of the hydrolyzed products (6 N HCl at 100° for 16 hr) was performed, using a two-dimensional system.[12]

Electrophoresis on acrylamide gels of doubly labeled mixtures was used to compare the products of amino acid incorporation by surface membranes and microsomes. Surface membranes were incubated with ^{14}C-L-leucine and the mixture for amino acid incorporation. Zn^{++} microsomes were similarly incubated with ^{3}H-L-leucine. After 40 min at 37° the two mixtures were brought to a final composition of 0.01 M EDTA, 0.29 M mercaptoethanol, and 0.5% sodium dodecyl sulfate. The two fractions were then combined, and the combined fractions were further treated as described by Sugiyama and Nakada.[13]

For sedimentation analysis, the surface membranes and Zn^{++} microsomes were prepared from L-cells labeled with ^{3}H-uridine (1 μc/ml of medium) for 24 hr. The ^{3}H-uridine-labeled membranes and Zn^{++} microsomes were each incubated under the conditions for amino acid incorporation with ^{14}C-L-leucine. After incubation, the mixtures were made 0.01 M with respect to Tris, 1.5 mM $MgCl_2$, 0.5% sodium deoxycholate, and Brij 58, and were placed on sucrose gradients from 15 to 30% (w/v) in 0.01 M Tris, 1.5

mM $MgCl_2$ and 0.01 *M* NaCl.[14] Centrifugation was for 2 hr at 21,000 rpm in the Spinco ultracentrifuge (25.1 rotor).

Results.—Properties of the amino acid incorporating system of surface membranes: The incorporation of ^{14}C-L-leucine was dependent on ATP, GTP, $MgCl_2$, and mercaptoethanol. The optimal concentration of $MgCl_2$ showed a relatively broad range of 8–16 mM. Other amino acids examined were incorporated. The highest incorporation was obtained with L-leucine, followed by L-proline and L-tyrosine. ^{14}C-D-leucine and ^{14}C-D-tyrosine were not incorporated under conditions in which ^{14}C-L-leucine was incorporated. Table 1 shows the effects of inhibitors on the incorporation of ^{14}C-L-leucine by the surface membranes. In general, the pattern of inhibition and other properties of the amino acid incorporation by the surface membranes were similar to properties that have been described for mammalian microsomal systems. The sensitivity to RNase further indicates that the incorporation of amino acids is dependent on RNA.

Activity of synthetic mRNA: The incorporation of ^{14}C-L-phenylalanine by the surface membranes (490 cpm per mg protein) was stimulated four- to sevenfold with poly U in the presence of a high concentration of $MgCl_2$ (16 mM). In the presence of either poly U or $MgCl_2$ alone, the incorporation was not significantly stimulated.

Effect of exogenous tRNA and microsomes: Purified tRNA of L-cells, when used at a concentration of 40 μg of RNA per assay, showed no stimulation of the amino acid incorporation by the surface membranes. The crude pH 5 fraction showed some stimulation when added to the membrane system, and the addition of tRNA with the pH 5 fraction showed no further increase. When microsomes were added to the surface membrane system, there was no stimulation of the amount of amino acid incorporated over that incorporated by either fraction alone.

Characterization of the products: The radioactive products formed by the sur-

TABLE 1. *Effect of inhibitors on the incorporation of ^{14}C-L-leucine by surface membranes.*

Additions	L-Leucine incorporated ($\mu\mu$moles/mg protein)	Inhibition (%)
Experiment 1		
None	10.1	0
Chloramphenicol (42 μg)	10.8	0
Actinomycin D (12 μg)	9.5	0–10
Puromycin (75 μg)	1.3	87
Acti-dione (20 μg)	2.8	72
RNase (20 μg)	0	100
DNase (20 μg)	8.7	14
Experiment 2		
None	8.5	0
Trypsin (50 μg) after amino acid incorporation	2.3	73

Experiment 1: Surface membranes (152 μg of protein) in a volume of 0.15 were incubated for 10 min at 5° with the stated concentration of inhibitor. Subsequently, the reaction mixture was added in a volume of 0.1 ml and the incubation continued for 30 min.

Experiment 2: Surface membranes (304 μg of protein) in a volume of 0.5 ml were incubated for 30 min. Fifty μg of trypsin was added to one half of the incubate, and the incubation of both assays was continued for 10 min.

face membranes were degraded by trypsin (Table 1). Acid hydrolysis degraded the radioactive products, allowing the recovery of the radioactivity at the leucine position when it was cochromatographed with cold carrier leucine by two-dimensional thin-layer chromatography.

The incorporation of some amino acids by fractions of *E. coli* has been reported to represent amino-terminal addition rather than incorporation at the COOH-terminal.[15] To rule out this type of reaction the fluorodinitrobenzene derivatives were prepared from the radioactive material insoluble in hot trichloroacetic acid. Only 9 per cent of the radioactivity was soluble in ether, representing the fluorodinitrobenzene derivatives. Thus most of the leucine was incorporated within the polypeptide chain.

Patterns obtained after electrophoresis on acrylamide gel of the products formed by the surface membranes after incubation under conditions for the incorporation of ^{14}C-L-leucine showed that 74 per cent of the ^{14}C-radioactivity was located near the cathode in three major bands (Fig. 1). The products formed by the Zn^{++} microsomes treated similarly, labeled with ^{3}H-L-leucine, showed only 6 per cent of the ^{3}H-radioactivity located in this same area. The bulk of the ^{3}H-radioactivity (88%) comprised three major bands and migrated a considerable distance from the ^{14}C-radioactivity. The products formed by the microsomal and pH 5 fractions prepared by standard procedures showed a gel pattern similar to that produced by the Zn^{++} microsomes when examined under these conditions.

Association of the product with RNA-containing structures: To determine the percentage of the radioactive products that remained with the RNA, sucrose gradients were set up under conditions for the demonstration of polysomes.[14] Radioactive membranes and Zn^{++} microsomes were isolated from cells labeled with ^{3}H-uridine, and each fraction was incubated with ^{14}C-leucine under condi-

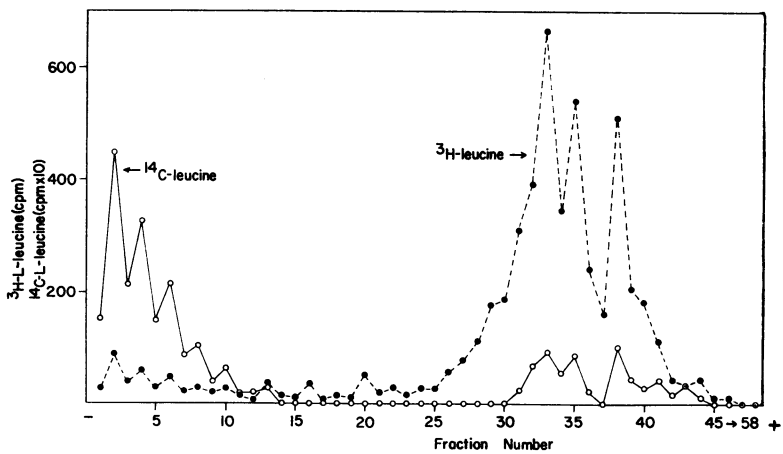

FIG. 1.—Electrophoresis on 10% acrylamide gel of the products formed by the surface membranes (O—O) and the Zn^{++} microsomes (●—●) after incubation under conditions of amino acid incorporation. Electrophoresis was from the cathode to the anode for 21 hr at 3 ma per gel.

tions of amino acid incorporation. The pattern obtained with the surface membranes after centrifugation through sucrose gradients (Fig. 2) showed that 90 per cent of the RNA was in the 60–80*S* region of the gradient. Two distinct peaks of ^{14}C-leucine were also in this area, representing 27 and 20 per cent of the amino acid on the gradient. Most of the remaining 50 per cent of the ^{14}C-radioactivity was found in fractions at the top of the gradient and was not associated with the RNA. Sucrose gradients of the Zn^{++} microsomes showed a pattern similar to the surface membranes.

The polysome area (inset of Fig. 2) contained 1.5 per cent of the RNA with 4 per cent of the incorporated amino acids. In shorter incubations, most of the leucine was found in the polysome area. Thus most of the amino acid incorporated could leave the polysome area and form material no longer associated with RNA.

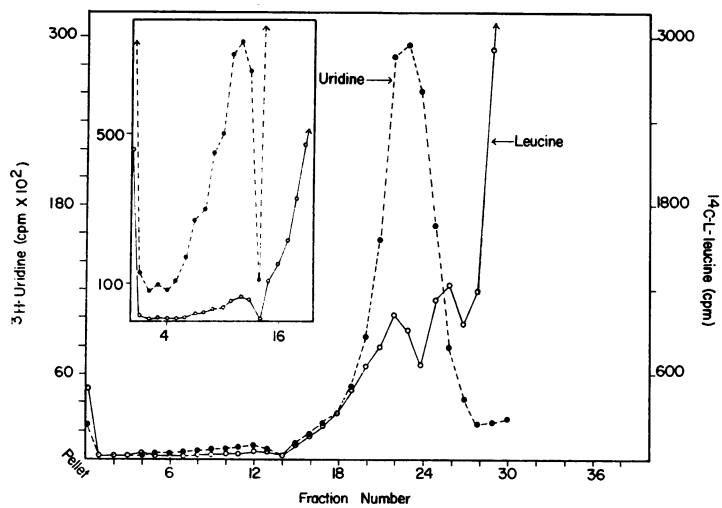

FIG. 2.—Sucrose density gradient of an incubation mixture as described in *Materials and Methods*. Fractions of 1 ml were precipitated with trichloroacetic acid, and the radioactivity was determined. The inset represents the polysomal area of the gradient. Radioactivity ^{14}C-L-leucine (O—O); ^{3}H-uridine (●—●).

Differences between the surface membrane and microsomal systems: Table 2 summarizes a comparison of the incorporation of ^{14}C-L-leucine by the surface membranes with its incorporation by microsomes. The latter were prepared by three different procedures. The comparisons were made in the presence or absence of cold amino acids and leucine and the presence or absence of pH 5 fractions. In all cases the maximum incorporation occurred in the presence of cold amino acids including ^{12}C-leucine. This would eliminate the possibility that the differences in the amounts of ^{14}C-L-leucine incorporated were due to varying amounts of endogenous amino acids in the preparations. With the exception of the Zn^{++} microsomes, increased incorporation was obtained with all fractions in the presence of the pH 5 fraction, indicating similar requirements. The Zn^{++} microsomes were not stimulated with any form of the pH 5 fraction when pre-

pared by standard procedures with or without $MgCl_2$. Microsomes prepared without $MgCl_2$ usually were active only in the presence of the pH 5 fraction.

Surface membranes could be frozen and still retain the ability to incorporate amino acids after one month of storage at −20°. However, after thawing and refreezing 90–100 per cent of the activity was lost. The presence of bentonite to inhibit RNase did not prevent this loss of activity, nor did the addition of pH 5 fraction restore the loss. Only the surface membranes showed this extensive loss of activity upon refreezing. Microsomal preparations could be frozen and thawed many times without significant loss of activity.

The membranes in the experiment reported in Table 2 incorporated 340 μμmoles of ^{14}C-L-leucine per mg RNA. The microsomal and pH 5 fractions prepared by standard procedures incorporated comparable amounts, i.e., 245 μμmoles/mg of RNA. Under similar conditions the activity of the Zn^{++} microsomes was considerably less (13 μμmoles/mg of RNA).

TABLE 2. *Comparison of surface membranes with microsomes prepared by three procedures.*

	Cold Amino Acid Mixture					
	None		Minus ^{12}C-leucine		Plus ^{12}C-leucine	
	pH 5 fraction					
	None	70–80 μg	None	70–80 μg	None	70–80 μg
Fraction of L-cells	μμmoles of L-leucine/mg protein					
Membranes (Zn^{++})	3.8	12.0	16.7	36.9	24.0	54.0
Microsomes (Zn^{++})	1.2	0.8	1.7	1.2	4.8	2.5
Microsomes ($-Mg^{++}$)	0	3.0	0	14.0	0	24.0
Microsomes ($+Mg^{++}$)	14.6	20.0	25.0	29.0	72.0	90.0

The protein concentrations were: surface membranes, 199 μg; Zn^{++} microsomes, 175 μg; microsomes $-Mg^{++}$ (prepared without $MgCl_2$), 143 μg; microsomes $+Mg^{++}$ (prepared by standard procedures), 162 μg. The pH 5 fraction added to the surface membrane incubate was prepared by standard procedures but without $MgCl_2$.

Discussion.—From these results it appears that isolated surface membranes of L-cells and Ehrlich ascites cells[3] can incorporate amino acids by a system that shows some requirements similar to those of the microsomal system of the endoplasmic reticulum. Some differences, however, were found. The most striking was in the nature of the products formed by the surface membranes. Gel electrophoresis showed that these products were different from those formed by the microsomal preparations. Comparisons of all these products with the proteins of the isolated membranes are currently under investigation with immunological procedures.

Among the other differences noted was the response to pH 5 fractions. Microsomes, prepared to simulate the conditions used in the preparation of the surface membranes, either responded not at all to the addition of pH 5 fractions (Zn^{++} microsomes) or were not active without pH 5 fractions (microsomes prepared without $MgCl_2$). Protein synthesis by surface membranes did not require pH 5 fractions for activity but was stimulated by the presence of such a fraction. In addition, the amount of amino acid incorporated by the Zn^{++} or Mg-deficient microsomal systems was less than 10 per cent of the amount incorporated by the surface membranes isolated under comparable conditions or by the microsomal

and pH 5 fractions prepared by standard procedures. The amino acid incorporation of the surface membranes exhibited extreme lability to freezing and thawing, as contrasted with the lability of the three microsomal preparations.

We have asked whether or not 10 per cent of the RNA of a cell is in some way associated with the surface membrane *in vivo* and whether or not the ribosomes on the isolated surface membrane are artifacts of the isolation procedures. Electron micrographs of thin sections of fibroblasts show ribosome-like particles near the surface, but they are not as closely associated there as in the isolated membranes. It is conceivable that the techniques used for electron microscopy pull the ribosomes away from the membranes or, conversely, that in the procedures for isolating the surface membranes the ribosomes may be pulled toward the membranes. The surface regions are known to be subject to extractions, shrinkage, and other artifacts after chemical fixation.[16] With the standard procedures for electron microscopy, structures are not visible that are seen by other techniques, for example, freeze etching. Recent studies[16, 17] have shown the presence of 150–200 Å particles closely associated with the inner and outer surfaces of cells. These particles, which could be ribosomes, are not seen in sections prepared for electron microscopy by standard procedures. More direct evidence to support the concept that the ribosomes associated with the surface membranes are not artifacts of the isolation procedures will be published.[3]

It is possible that some of the activity reported for the microsomes prepared by standard procedures from L-cells was due in some part to the presence of surface membranes. We have observed, by phase contrast microscopy, fragments of surface membranes contaminating preparations of microsomes. In contrast, significant contamination of the surface membrane preparations with other cell particulates as well as with bacteria has not been found.[3]

When the products formed from amino acid incorporation by the isolated surface membranes were examined on a gradient designed to demonstrate polysomal RNA, the patterns of the incorporation during the first 15 minutes showed that much of the radioactivity of the amino acid was in the polysomal region. Later, as shown in Figure 2, the radioactivity appeared in the area of the gradient containing very little RNA, suggesting that the incorporation *in vitro* proceeded through polysomes. However, no conclusions can be made concerning the release of the proteins from the polysomes because of the possible presence of degradative enzymes.

The association of the protein-synthesizing machinery with the surface membranes could facilitate production of proteins for membrane synthesis or for export. In addition, proteins synthesized at the surface could play a role in the process of viral infection and transformation. Such synthetic machinery could play a part in the rapid action of hormones, where many biochemical reactions appear to be simultaneously effected. Rodbell has reported on the action of insulin and lipolytic hormones on the plasma membranes of fat cells.[18] The possibility that the structural integrity of the surface membrane is affected by the hormone and thus influences the protein synthetic capabilities of the cell cannot be overlooked. Indeed, such an alteration in the surface has been proposed.[19, 20]

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