THE EFFECT OF TRYPSIN ON ROUGH ENDOPLASMIC MEMBRANES

W. Chefurka and Y. Hayashi*

Research Institute, Canada Department of Agriculture

University Sub P.O., London, Ontario, Canada

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Electron microscopic studies of liver cells have shown that the endoplasmic reticulum (ER[1]) is a system of channels limited by membranes some regions of which are smooth while others are studded by ribosomes (Porter, 1960). When the ER is disrupted, the resulting fragments may be isolated as microsomes which have long been recognized to be active in protein biosynthesis (Siekevitz, 1963). However, the details of attachment of the ribosomes to the membrane are still obscure and have to date received little attention. Several reports have appeared recently which suggest that protein may be an integral binding component of large ribosomal assemblages of certain tissues (Rabinowitz et al., 1964; Manner and Gould, 1965). It is the purpose of this communication to describe experiments which suggest that nascent protein probably also anchors the polysomes and monoribosomes to the membrane of the ER in liver cells.

Materials and Methods

Livers (approximately 2 g) were removed from female white mice (about 20 g) and homogenized in 10 ml of cold 0.30 M sucrose in TMK buffer (Tris-HCl pH 7.6, 0.03 M; $MgSO_4$, 0.005 M; KCl, 0.025 M) with 5-6 strokes at about

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* N.R.C. Postdoctorate Fellow

[1] Abbreviations used: ER, endoplasmic reticulum; TMK, Tris-HCl, $MgSO_4$, KCl buffer; RNase, ribonuclease; t-RNA, transfer ribonucleic acid; m-RNA, messenger ribonucleic acid.

500 r.p.m. in a pre-chilled Teflon-glass homogenizer. The cell debris, nuclei and mitochondria were sedimented by centrifugation at 10,000 x g for 10 minutes in a Servall RC-2 at approximately 2°. The supernatant was layered over a discontinuous gradient (Wettstein et al., 1963) which consisted of 1.5 ml 0.5 M sucrose layered over 2.5 ml of 1.8 sucrose, both being buffered with the TMK solution. After centrifugation at 105,000 x g for 4 hours at 2-4° in a No. 50 rotor in a Spinco Model L or L-2 centrifuge, a translucent microsomal pellet was obtained. The fluid was carefully decanted and the walls of the centrifuge tube wiped with tissue paper. The microsomal pellet was suspended in 2 ml of TMK buffer and layered over the TMK buffered linear 10-34% sucrose gradient (total 23 ml) prepared according to Britten and Roberts (1960). Five ml of 50% sucrose solution were used as a cushion at the bottom of this gradient. As indicated later, the resuspended pellet was treated with trypsin at 2.5 and 25.0 μg/ml at about 2° for 60 minutes before loading onto the sucrose gradient. Sucrose gradient centrifugation was for 2.5-3.0 hours at 25,000 r.p.m. (2-4°) in a SW25 rotor in a Spinco Model L centrifuge. After centrifugation the bottom of the tube was pierced and fractions of twenty-nine drops (approximately 1/2 ml) were collected. Each fraction was analyzed for optical density at 260 mμ and for trichloroacetic acid (TCA) precipitable radioactivity. In the latter operation, 500 μg serum albumin was added as carrier, the precipitate collected on Millipore filters and washed extensively with 5% TCA containing 0.01 M DL-leucine. The filters were placed on the bottom of counting vials, dried, scintillation fluid added and the radioactivity counted in a Nuclear-Chicago liquid scintillation spectrometer.

In vivo incorporation of DL-leucine-1-C^{14} into protein was determined by injecting 10 μc intraperitoneally 10 minutes before killing.

The trypsin was purchased from Worthington Biochemical Corporation and under our conditions it was free of RNase activity. DL-leucine-1-C^{14} (S.A. 3-4 mC/m mole) was obtained from New England Nuclear Corporation.

Results and Discussion

A typical sedimentation profile of the microsomal suspension is seen in Fig. 1. Free monoribosomes (tube 30) and polysomes of various sizes are clearly discernible in the control preparation. A considerable quantity of rapidly sedimenting material was also trapped at the interface (tube 7) of the 50% cushion in agreement with Henshaw et al., (1963). This material showed a distinct absorption maximum at 260 mμ, had an RNA/protein ratio of 0.35 and phospholipid/RNA ratio of 0.29.

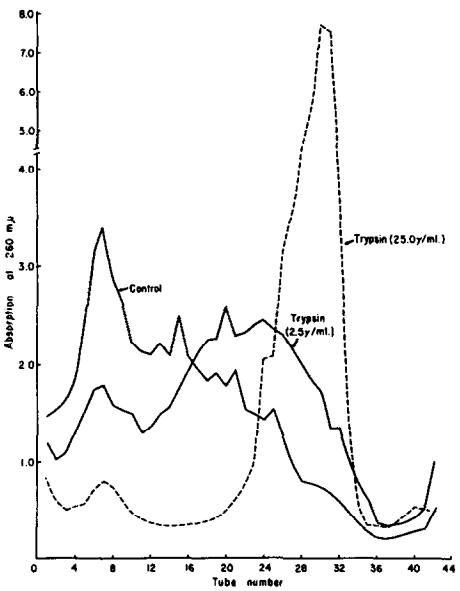

Fig. 1

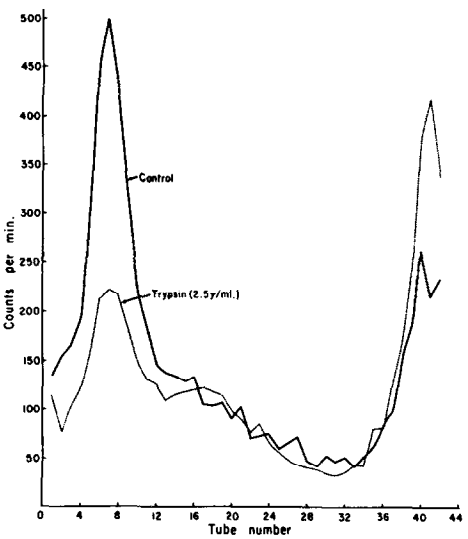

Fig. 2

Fig. 1. Sucrose density profiles of microsomes before and after treatment with trypsin.
Fig. 2. Sucrose density profiles of labeled microsomes before and after treatment with trypsin.

Mild treatment with trypsin (2.5 γ/ml) resulted in an increase of free ribosomes and polysomes at the expense of the heavy fraction. Greater amounts of trypsin (25 γ/ml) caused not only a further decrease in the recovery of the heavy material but a preponderance of monomers and Mg^{2+}-stabilized dimers. Presumably these resulted from a breakdown of polysomes due to an effect of trypsin on the binding site for m-RNA (Zak et al., 1966). It is therefore apparent that the heavy fraction trapped in the cushion consists of membrane-

associated polysomes. It will be shown elsewhere, that this fraction is virtually free of smooth membranes. Occasionally, as shown here, a subunit (tube 32) was detected after even mild treatment with trypsin.

The data in Fig. 2 show the results of a typical C^{14}-leucine incorporation profile. Most of the acid-precipitable counts sediment with rough membranes. This is in agreement with the results of Henshaw et al., (1963). After treatment with trypsin (2.5 γ/ml) the radioactivity of the rough membranes decreased. But whereas this treatment released polysomes and monomers the level of radioactivity of this region of the gradient did not change. Indeed, the specific activity of the polysomes decreased. Instead, the marked decrease in radioactivity of the rough membranes seems to be accounted for by that found in a light fraction located at the top of the gradient. An analysis of the acid washings of the TCA precipitates from fractions 36-42 showed at most 15% acid-soluble radioactivity. Routinely, about 85-100% of the radioactivity of the controls was recovered from the acid-insoluble fractions after trypsin treatment. The occurrence of this peak in the controls may represent endogenous protease activity (Siekevitz and Palade, 1958). However attempts to date to inhibit this endogenous activity with an excess of soybean trypsin inhibitor gave variable and hence inconclusive results.

These data clearly show that the light fraction is the only constituent of the rough membranes that is preferentially labeled in short term experiments. The nature of this fraction is currently under investigation but its high specific activity and its acid precipatibility suggest that it may be the nascent protein which anchors the polysomes to the membrane. It is improbable that it represents fragments of the membrane resulting from trypsin digestion because electron microscope studies of Lust and Drochmans (1963) clearly show that trypsin under more drastic conditions of digestion, strips ribosomes off the supporting membrane with no apparent damage to the membrane.

Puromycin, which substitutes for t-RNA, releases the growing polypeptide from polysomes (Morris and Schweet, 1961; Allen and Zamecnik, 1962) presumably

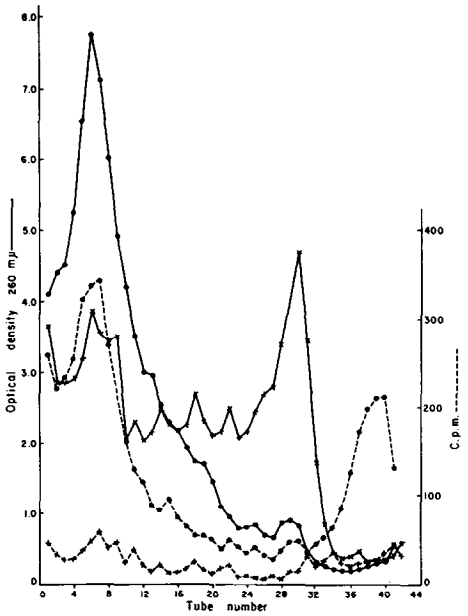

Fig. 3. Recovery of rough membranes, free polysomes and monoribosomes from mouse livers after injection of puromycin. Aqueous solution (0.2 ml) of puromycin (100 μg/gm live weight) was injected intraperitoneally. This was followed immediately by an injection of C^{14}-leucine as described in text. The animals were sacrificed 10 minutes later. (•) controls, no puromycin; (x) puromycin treatment.

because the polypeptide-charged puromycin is unable to hydrogen-bond with the m-RNA. However in our case, since the nascent protein is presumably attached to the membrane, free polysomes should be released. This is confirmed by the data in Fig. 3 which show that after in vivo treatment with puromycin, less rough membranes and more free polysomes and ribosomes were recovered. In other experiments, fewer polysomes but more monomers were recovered. The in vivo incorporation of C^{14}-leucine into the rough membranes was inhibited about 80% by puromycin.

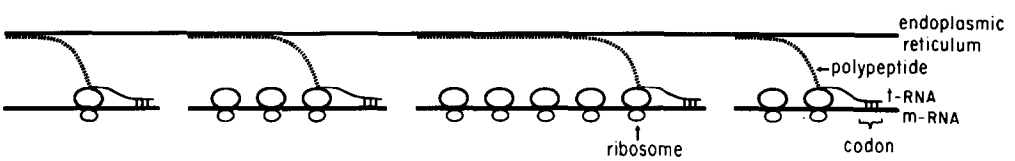

Fig. 4. Schematic representation of the role of nascent protein in the architecture of the rough membranes.

The envisaged model of the rough membranes is seen in Fig. 4. According to this tentative model, the ribosomes are anchored to the limiting membrane predominantely as polysomal arrays via the nascent protein. Furthermore only

the terminal monomer is connected to the membrane whereas the array is free. Recent electron microscopic studies are compatible with this model (Benedetti et al., 1966). Presumably, after completion of protein synthesis, the polysomal arrays slough off and migrate to other parts of the ER to resume protein synthesis. Smooth membranes thus result from rough membranes.

The mechanism by which trypsin selectively strips the polysomes off the membrane with a concomitant release of nascent protein is yet unclear. This and the nature of the nascent protein are currently under investigation.

Acknowledgment

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