Chapter 2

REVIEW OF LITERATURE

Electron microphotographs of rat hepatocytes reveal the ER to be present throughout the cytoplasm as an extensive network of tubules, vesicles and lamellae (28, 29). The cytoplasmic surface of 60 per cent ER is normally dotted with ribosomes. These regions, called the RER, are generally arranged in parallel arrays of broad flattened bags (cisternae). The smooth endoplasmic reticulum (SER), which is often found in the region of the Golgi apparatus, consists of widely dispersed tubules and vesicles not infrequently associated with glycogen deposits (28, 29). The membranes of the ER are seen in the electron microscope 50-80Å thick (30-32). The lumen of RER is 200-300Å wide and the lumen of SER is 300-600Å wide (29). The morphological and morphometric characteristics of ER vary from region to region (peripheral, midzonal and central) of the liver lobe (28).

Unlike other organelles, such as mitochondria and lysosomes, the ER is extensively disrupted even by gentle homogenization of the liver. This breakage is not a purely mechanical process, but seems to involve an active "pinching off" (33). Thus, fragments of ER, which can be isolated from tissue homogenates, are in the form of closed vesicles, the so called microsomes (34). These vesicles have ribosomes on their outer surface and contain secretory proteins such as albumin known to be present in the lumen of the ER in situ. The size of these vesicles of rough and smooth microsomes varies in the range of 150-300 nm. The rough
microsomal pellets contain vesicles with varying number of ribosomes, often disposed in groups of two or three tightly packed ribosomes or polysomes at small intervals (28).

2.1. Isolation of microsomes:

For the separation of this subcellular organelle three fundamental points should be borne in mind: first, the morphological, chemical and enzymatic properties of the organelle should be altered as little as possible during the isolation procedure; secondly, recovery of the organelle should be as high as possible; and thirdly the contamination by other organelles should be minimum as far as possible. For the isolation of the ER it is very difficult (rather impossible) to achieve all these goals together. The investigators must often choose between high recovery and lower purity or lower recovery and higher purity. Most of the studies aim at higher purity of microsomal preparations.

The major first problem involved during the isolation of microsomes from rat liver is due to the presence of large quantities of blood in this organ. As the fragments of ER are highly charged (35), they readily adsorb the constituents of broken erythrocytes (36), specially the hemoglobin, which is released when erythrocytes are disrupted during normal homogenization. Hemoglobin contamination may be especially troublesome in studies on the microsomal cytochromes. Serum components may also be adsorbed by microsomes and interfere with various investigations. For example, adsorption of serum glycoproteins can give a value for protein bound microsomal sialic acid which is twice the true value (37). These problems caused due to the presence
of blood, can be solved either by perfusing the liver before
homogenization or by washing the microsomes with an appropriate
medium.

2.1.1 Homogenization:

Homogenization is the first step in the isolation of the
microsomes. The liver is cut into small pieces with a pair of
scissors. In some cases the liver has been further disrupted
by forcing these pieces through a tissue press, but this procedure
should be avoided, since it damages organelles such as mitochondria
and peroxisomes (38). The liver is then homogenized in 0.25M
sucrose using a Potter Elvehjem homogenizer with a Teflon pestle.
Clearance between the pestle and the vessel is not critical, but
should not be too tight. The speed of the homogenizer and the
number of passes used for complete homogenization (often omitted
in published reports) have great importance. For maximum recovery
of microsomes extensive homogenization at high speed is required
to disrupt as many of the hepatocytes as possible; but such
homogenization also results in increased damage to, and fragmentation
of, cellular organelles and non-hepatocytes. Increased disruption
of the plasma membrane (39), Golgi cisternae (40), connective tissue
cells and cells of erythropoiesis (41), release of outer mitochon-
drial membranes (42) and rupture of lysosomes (43) can all cause a
serious increase in the contamination of the microsomal fraction.

Adelman et al. (44,45) reported that homogenization at
1000-2000 revs/min with 8-10 passes results in a microsomal
fraction containing about 10% of the total cytochrome oxidase
(a marker for the inner mitochondrial membrane) and 30 per cent
acid phosphatase (a lysosomal marker). On the other hand, when they homogenized at 400 rev/min with 4 passes, the microsomes contained only 0.7 per cent of the succinate-cytochrome C reductase (also a marker for inner mitochondrial membrane) and 11 per cent of the acid phosphatase. Amar-Costesc et al. (46) accomplished extensive disruption by first homogenizing the liver with a single pass, then sedimenting and rehomogenizing the 'nuclear' pellet, and once again collecting and homogenizing the 'nuclear' pellet. These homogenizations were performed at 1,300 rev/min and a relatively high recovery of microsomal marker enzymes was achieved from the combined supernatants. However, the total microsomal fraction contained twice as much protein and phospholipids as those obtained by Adelman et al. (44,45) but only about 10 per cent more of the microsomal marker enzymes.

Almost all procedures for the isolation of microsomes utilize the same medium during homogenization, namely, 0.25M sucrose. Siekevitz and Palade (33) obtained better morphological preservation of the elongated tube like structures of RER by homogenizing the liver in 1.46M sucrose and by carrying out centrifugation in 0.88M sucrose. In addition, higher concentrations of sucrose reduced microsomal aggregation (47).

However, it would seem desirable to use lower concentrations of sucrose whenever possible because of the probable inhibition of various enzymes by this solute. For example, the ATPase activity of rough microsomes is increasingly inhibited by increasing concentrations of sucrose, until at 1.2 M sucrose this enzyme is
75 per cent inhibited (48). It seems advisable to routinely test for such enzyme inhibitions by sucrose, especially after centrifugation in sucrose gradients. Another disadvantage of using high sucrose concentrations is the resulting high viscosity (dependent on temperature). More longer centrifugation times are required to harvest microsomes from solutions of high viscosity.

After homogenization the next step is the removal of unbroken cells and large particles, which are removed from the homogenate by differential centrifugation at 10,000 'g' for 20 min. This step removes unbroken cells, nuclei, mitochondria, lysosomes, and peroxisomes, as well as large fragments of plasma membrane and of cisternal Golgi. However, this centrifugation step also sediments a significant portion of the fragments of the E.R. The post 10,000 'g' supernatant is removed and further used for the preparation of microsomes.

2.1.2 Preparation of total microsomes:

The usual procedure for obtaining a total microsomal fraction from the 10,000 'g' supernatant is to centrifuge it at 105,000 x g for 60 min. When this centrifugation is performed in 0.25M sucrose in an angle-head rotor, a loose fluffy layer (shown electron microscopically to contain small smooth vesicles and free ribosomes) can be seen at the top of the pellet. In addition, a small amount of microsomal vesicles remain behind in the supernatant after this procedure. Thus, if one wishes to obtain a post-microsomal supernatant free of membrane fragments, centrifugation should be carried out for a longer time, not less than two hours, and the supernatant should be drawn off, not
decanted.

Because of the small size of microsomal vesicles, it is very tempting to use higher centrifugal forces to sediment them in order to decrease the time of centrifugation required. However, this temptation should be resisted. Centrifugation of microsomes in 0.25M sucrose in an angle-head rotor at forces greater than 105,000 'g' damages the vesicles; their permeability is increased and they are seen in electron micrographs to be oval in shape rather than round.

Apart from this method, new rapid methods were developed to isolate the microsomes at 10,000 'g' by chemical treatment of the post 10,000 'g' supernatant. Kamath et al. (16) reported that if 10,000 'g' supernatant is adjusted to contain approximately 8 mM CaCl₂ and 5 mM MgCl₂ the aggregated microsomes can be sedimented at 10,000 'g' by centrifugation for 20 min. For some purposes such a preparation may be quite adequate. For instance, microsomes prepared in this manner exhibit normal levels of drug metabolizing activity and of cytochromes P-450 and b5 (49). However, these microsomes must certainly be too highly aggregated for further subfractionation. In addition the microsomal endogeneous phospholipases (50,51) may be activated by Mg²⁺ and/or Ca²⁺ and damage the membranes of the vesicles. These divalent cations may affect the properties of the membranes directly as well. In addition it has been reported that in plant microsomes the Ca²⁺ ions activate ribonuclease which might damage the free or bound polyribosomes (19). Apart from these, such preparations are not readily solubilized with sodium cholate or Renex 690, a nonionic detergent, and therefore, may not be a suitable starting material for the purification of microsomal proteins (52).
Tangen et al. (13) prepared the microsomes from post-mitochondrial supernatant by gel filtration technique. Sepharose 2B columns having the capacity to elute the solute with molecular weight less than $2-4\times10^7$ (which is the range of the exclusion limit for Sepharose 2B) were used. The size of the microsomes ranges from 150-300 nm in diameter. Consequently, they pass readily through the spaces between the gel beads. The hydrophilic nature of the agarose gel and its almost total lack of electrical charges serve to minimize tailing and damage of the microsomes during gel filtration. The contamination of the microsomes (prepared by gel filtration) with other subcellular organelles was similar to that found by other conventional methods.

Ibbetson and Freedman (14) used microsomes prepared by gel filtration technique for membrane-ribosome interaction studies using EDTA as the degranulating agent. They calculated from the peaks drawn after collection of the individual fractions that the RNA/protein ratio of rough microsomes decreased after degranulation from 0.2 to 0.05. The authors have pointed out several difficulties to use this technique for routine work. The isolation of microsomal fractions involves fractionation of discontinuous gradients followed by further centrifugation to concentrate the sample. Isolated microsomal fractions have a tendency to aggregate and this is apparently affected by slight variations in the history of samples. Further manipulations of a sample, such as EDTA treatment, exacerbate this tendency. As a result, microsomal samples are frequently eluted very slowly, or not at all, from Sepharose 2B columns, in which the spaces between beads should ideally range
from 9 to 30 \( \mu \)m in diameter (13). A further limitation of this technique is that 10 mg of protein is the maximum loading limit for the column, and the elution then produces fractions in which protein and RNA concentrations are at the limit of what can be determined chemically.

Hoeven (52) recently prepared microsomes at 8000 'g' by the treatment of PMS with polyethylene glycol (PEG) 6000 at a concentration of 5 per cent (w/v). For the preparation of degranulated microsomes, the PMS was treated with an equal volume of 0.2M Sodium pyrophosphate (pH 7.4) and sonicated for 20S on ice. Polyethylene glycol 6000 was then added to the above PMS to a final concentration of 5 per cent (w/v). These preparations have peptide patterns and levels of drug metabolic and other enzymatic activities similar to those of the microsomal fraction isolated by high-speed centrifugation. The PEG preparation can be solubilized by treatment with sodium cholate and Renex 690 and can be used as starting material for the purification of microsomal proteins.

2.1.3 Preparation of RER and SER:

Subfractionation of the total microsomal fraction into rough and smooth microsomes has also been achieved. Initial attempts to isolate rough and smooth microsomes were carried out using rate differential centrifugation (53). The difference in the average sedimentation rates of these two subfractions (which are influenced by a number of properties of which size is the most important) is sufficient to allow the preparation
of fractions enriched in rough or smooth microsomes. However, these fractions are so highly cross contaminated that the method is not useful.

Rothschild (54) therefore attempted to prepare rough and smooth microsomes using discontinuous gradient centrifugation. Smooth microsomal vesicles have an equilibrium density in sucrose ranging from 1.06 to 1.18; while the equilibrium density of most of the rough microsomes in sucrose is greater than 1.20. The 10,000 'g' supernatant in 0.15 M sucrose was layered over 1.31 M sucrose and then centrifuged at 105,000 'g'. If this centrifugation was continued until all particles had reached their final position in the pellet or at the interface, i.e., for about 10 h, too many smooth vesicles sedimented together with the rough and the separation of rough and smooth microsomes was not achieved. However, when centrifugation was terminated after 6-8 h, a lot of sedimented particles (SER) remained in the 1.13 M sucrose layer and the rough microsomes in the pellet showed little cross-contamination. Free ribosomes were found partly in the smooth microsomal fraction, from which they could be removed by centrifugation.

The time of centrifugation at 105,000 'g' is critical in this approach. Using a Spinco 40 rotor 7 h 40 min was optimal (55). Rothschild reported that the concentration of sucrose in the upper layer of this discontinuous gradient is also critical: that if concentrations of 0.25 M or higher are used, separation of rough and smooth microsomes is less effective (54).
However, 0.44 M sucrose can be used in the upper layer and an effective separation of the two subfractions is still achieved (55). Use of higher sucrose concentration helps to minimize microsomal aggregation which would interfere with the subfractionation.

Rothschild (54) also tried to separate rough and smooth microsomes by isopycnic gradient centrifugation on a continuous sucrose gradient, but there was considerable cross contamination between the subfractions obtained. The discontinuous gradient procedure, although somewhat time consuming, is the best available method for isolating rough and smooth microsomal fractions suitable for further subfractionation. However, it should be remembered that this procedure is not quantitative. Particles present in the 1.3 M sucrose layer after centrifugation are discarded. These particles represent chiefly smooth microsomes of high-than-average density and rough microsomes with few attached ribosomes and thus of lower density than the other rough vesicles. These fractions may originate from regions of the ER that are biochemically and functionally specialized.

Discontinuous sucrose gradients have also been used by Adelman and his coworkers (44,45) in a procedure designed to achieve a good recovery of rough microsomes. The RNA in the ribosomes bound to the ER represents about 60 per cent of the total rat liver RNA (56). In most procedures for isolating microsomes a large fraction of the total RNA sediments with the 'nuclear' and mitochondrial pellets, and much of this represents loss of rough microsomes (56-58). The object of
their method is to decrease this loss without worrying about possible increased contamination of the smooth microsomal fraction.

These investigators disrupt the liver with a tissue press and then homogenize it vigorously in 1.0 M sucrose. An equal volume of 2.5 M sucrose is added to the homogenate and this preparation is centrifuged at about 10,000 'g' for 45 min. Only the nuclei, relatively uncontaminated by rough microsomes, are sedimented by this procedure.

The supernatant from this first step is diluted with water and centrifuged to obtain the mitochondrial pellet. This pellet is then washed twice with relatively ion-free sucrose solutions in order to remove trapped rough microsomes. Washing with medium containing ions is much less effective, and washing more than twice does not remove any additional RNA from the mitochondria.

The combined post-mitochondrial supernatants are then placed over a discontinuous sucrose gradient consisting of two layers. The upper layer contains 1.6 M sucrose and low concentrations of Tris-buffer, KCl and MgCl₂; while the lower layer contains 2.0 M sucrose and the same ions in concentrations that are five times higher. This gradient is then centrifuged for 20 h at about 200,000 'g' after which smooth microsomes are recovered at the interface between the supernatant and the layer of 1.6 M sucrose; rough microsomes are found at the interface between the layers of 1.6 M and 2.0 M sucrose; while free ribosomes are found in the pellet. Considerable amounts of free
ribosomes are still present in the rough microsomal fraction as well, but these can be removed simply by diluting the rough microsomes with an ionic medium and sedimenting them at a centrifugal force that leaves free ribosomes behind in the supernatant. Also, there is an appreciable loss of rough microsomes to the smooth microsomal fraction.

This procedure developed by Adelman and his co-workers is a modification of previous subfractionation scheme and yields a fraction containing 50 per cent of the total rough microsomes. Actually, this yield does not seem to be much better than the yields of rough microsomes obtained by other methods. This rough microsomal fraction has not been systematically examined for possible contamination by other organelles.

The advantage of this subfractionation scheme is that it yields rough microsomes suitable for studies for in vitro protein synthesis (44,45). However, this approach also has certain disadvantages. The vigorous homogenization and the long centrifugation time probably results in a considerable loss of the activities of various microsomal enzymes. Furthermore, the vigorous homogenization and washing of the mitochondrial pellet almost certainly increases contamination of the smooth microsomes with mitochondrial membranes, pieces of the plasma membranes, and fragments of other organelles. Adelman et al. (44,45) themselves point out that their smooth fraction is also contaminated with rough vesicles. Thus, the smooth microsomal fraction obtained by this procedure can not be used for most purposes.
And finally, the presence of ions in the discontinuous gradient results in an aggregated rough microsomal fraction which is not suitable for further subfractionation.

The most widely used method for preparing rough and smooth microsomes is based on the finding that monovalent cations, and in particular Cs⁺, can selectively aggregate rough microsomes (59,60). The rough microsomes have an appreciably high affinity for Cs⁺ ions than do smooth microsomes, free ribosomes or rough membranes stripped off their ribosomes (61). Apparently binding of limited amounts of Cs⁺ by the rough microsomes decreases their negative surface charges, thereby diminishing the repelling forces between the vesicles and causing them to aggregate. This aggregation increases the mean radius of rough microsomal particles at least two to three folds and thus increases their sedimentation velocity four to nine folds (62).

In the procedure 10,000 'g' supernatant is treated with CsCl solution to give a final concentration of 15 mM CsCl. This preparation is layered over a solution containing 1.3 M sucrose and 15 mM CsCl and centrifuged for 90 min at 102,000 'g' in a Spinco 40.2 rotor. The smooth microsomal fraction is recovered from the boundary between the two layers, while rough microsomes are found in the pellet. Free ribosomes and some soluble cytoplasmic proteins are present in the smooth microsomal fraction obtained, but these contaminants can be removed by appropriate procedures. Centrifugation of the discontinuous gradient for longer than 2 h results in increased aggregation of smooth microsomes by Cs⁺ and, consequently, increased amounts of smooth
vesicles in the pellet.

In order to improve this situation a modification has been made (63). In this modification no CsCl is added to the 10,000 'g' supernatant. Instead, this supernatant is layered over a solution containing 0.6 M sucrose and 15 mM CsCl, which is in turn layered over the usual solution of 1.3 M sucrose-15 mM CsCl. Centrifugation is then performed in the normal manner. Use of this middle layer in the discontinuous gradients results in removal of most of the non-particulate components of the supernatant from the microsomes before they are exposed to Cs⁺ and apparently results in decreased aggregation of the smooth microsomes by Cs⁺. This modification has effectively separated rough and smooth microsomes. This subfractionation on a Cs⁺ containing discontinuous sucrose gradient is widely used because of its simplicity, the relatively short time it requires, and rather limited cross contamination of the rough and smooth microsomal fractions obtained. However, in certain types of investigations for instance, where further subfractionation is to be attempted aggregation of the rough microsomes is undesirable. In such cases it may be advisable to use the subfractionation scheme, developed by Rothschild. However, the aggregation caused by Cs⁺ can be reversed by EDTA (61).

Intense hydrostatic pressure is generated during ultracentrifugation and this could lead to membrane 'damage' and also detachment of membrane-bound ribosomes. Bronfman and Beaufy (64) have shown that some proteins are released from various isolated
cell organelles by pressures similar to those generated in the ultracentrifuge. This is a thermodynamic effect, and is not just due to a physical rupture of the organelles by the intense pressure. Ribosome detachment from rough vesicles could occur in an analogous fashion in separation procedures which involve prolonged exposure of the vesicles to high hydrostatic pressures viz. the discontinuous gradient and flotation methods. Ribosomes could also be detached by the 'shearing' forces which result when rough vesicles are subjected to ultracentrifugation in a medium of density intermediate between that of the ribosomes and of the membranes. In this case, since the ribosomes on rough vesicles would tend to float, a net force would be generated tending to tear the ribosomes off the vesicles.

There appears to be little unequivocal information on the possible ribosome detachment during the isolation of membrane-bound ribosomes. However, Sarma et al. (65) found that when a carefully purified membrane-bound ribosome fraction from mouse liver was resubjected to the discontinuous gradient procedure, about 20 per cent of the ribosomes were again recovered in the 'free ribosome' regions of the gradient. This implied that some ribosome detachment had occurred.

The separation procedures could also cause other changes in rough vesicles. Thus Bont et al found that the response of membrane-bound ribosomes synthesizing protein in vitro to exogenous amino acids depended on whether they were isolated in a dilute (<1 M) or more concentrated (>1 M) sucrose medium (11).
Ragnotti found that membrane-bound ribosomes were more active in \textit{in vitro} protein synthesis and their mRNA less degraded if they were sedimented on to a sucrose cushion rather than into a pellet (66). Long centrifugation times may also increase lipid peroxidation which would have a harmful effect on microsomal properties. Arstila et al. (10) demonstrated a correlation between the extent of lipid peroxidation of microsomal membranes and the breakdown and disappearance of membrane-bound ribosomes. The deleterious effects of lipid peroxidation are also manifested by an attack on the haem group of cytochrome P-450 (67). This would be expected to interfere with the metabolism of drugs and steroids during \textit{in vitro} incubations of microsomes. The protein synthetic activity of rough microsomes may also be inhibited (68). Palmer et al. (6), however, observed that lipid peroxidation occurring during the isolation of membrane-bound ribosomes from liver can be completely prevented if the liver is perfused with and all preparative buffers contain 1 mM Mg-EDTA.

Degradation of the mRNA of polyribosomes (free or membrane-bound) by endogeneous nucleases can also occur, particularly in those separation procedures involving prolonged ultracentrifugation. Mammalian liver cells (and some other mammalian cells also) are known to contain a natural ribonuclease inhibitor which is capable of almost completely (90–99 per cent) inhibiting the main neutral ribonuclease of microsome fractions (69). To prevent endonucleolytic breakdown of polyribosomes during isolation,
some authors have therefore included rat or mouse liver high-speed supernatant, which contains the inhibitor, in all preparative media, even when the tissue under study is not mammalian liver. This would seem to be an unwise practice because other degradative enzymes (such as proteases) are undoubtedly present in cell sap preparations. Moreover, rat liver cell sap contains an exonuclease (70). This would not cause breakdown of polyribosomes to smaller aggregates but could still degrade the associated mRNA or rRNA significantly. In fact, although the addition of small quantities of cell sap may generally be beneficial towards reducing effective ribonuclease activity, the addition of large quantities of cell sap can actually enhance the rate at which the ribosomal RNA of rat liver microsomes incubated at 37°C is degraded to acid-soluble fragments (71). The ribonuclease responsible appears to be insensitive to the natural inhibitor. Furthermore, it is not sure that a rodent liver inhibitor will be completely effective in inhibiting the neutral ribonuclease from other species. For example, Kraft and Shortman (72) found that the ribonuclease of chick liver cell sap was only partially inhibited by rat liver cell sap.

2.2 Studies on membrane degranulation:

The most widely used methods to achieve degranulation by chemical agents are the treatments with, KCl alone (73), puromycin plus KCl (74), citrate plus pyrophosphate (75), EDTA alone (76), or plus ribonuclease (77) and lithium chloride (78).

The earliest studies of fractionation of membrane-bound ribosomes showed that the 60s subunit was attached to the membrane
Increasing concentrations of EDTA removed 40s subunits from the membrane before 60s subunits, and electron microscopy showed the two subunits arranged on top of each other rather than side by side on the membrane. Direct electron microscopy of intact cells (80) and of RER (81) also showed 60s subunits next to the membrane. Treatment of rough membranes with EDTA chelates divalent cations (Mg$^{++}$) and causes the removal of the ribosomes from the surface of the vesicles. This suggests that the principle energy of interaction between the ribosomes and the membrane is ionic in nature. Thus treatments with LiCl (78) and citrate plus pyrophosphate (75) were used to disrupt ionic forces between ribosomes and membranes as extensions of EDTA treatment alone. Results from various laboratories showed that EDTA alone could not completely strip rough membrane (72,82-85). It is quite possible that EDTA does not completely strip the large subunit and RNA/protein ratios of non-purified degranulated membranes are higher than those of purified ones. Almost 90 per cent of rough membranes shift to 1,35 M STKM-2,0 M STKM interface after treatment with EDTA showing that its sedimentation behaviour is identical with smooth membranes. These observations have been confirmed by Hochberg et al. (86) who have reported that the binding of polysomes to rough membranes degranulated by EDTA is more efficient in comparison to those degranulated by citrate plus pyrophosphate. Czosnek et al. (83) have reported that the protein synthesizing activity of rough
membranes degranulated with EDTA is the same as of smooth membranes (10 per cent of native rough membranes). However, from electrophoretic patterns of the proteins of stripped membranes they have concluded that the EDTA treatment leaves most of the basic ribosomal proteins bound to the membrane non-specifically, which was not found in membranes degranulated by puromycin plus KCl.

High concentrations of monovalent ions have also been used to detach bound ribosomes and are presumably able to do so by interfering with the integrity of divalent ions serving as bridges between the interactants. Blobel and Sabatini (73) reported that higher concentrations of KCl in the presence of Mg$^{++}$ caused only a partial dissociation of large and small ribosomal subunits from microsomes, but these subunits retained their biological activity. High concentrations of lithium chloride (2.0 M) shatter the ribosomes with precipitation of ribosomal RNA. However, Scott-Burden and Hawtrey (78) have reported that the membranes degranulated by LiCl are intact and bind back fresh ribosomes (87). They have further reported that membranes degranulated by LiCl are not much damaged with respect to their enzymic and chemical composition. Membrane alteration is, however, indicated after the use of citrate plus pyrophosphate (88). The major handicap of all the above mentioned degranulating reagents used in various laboratories is that they lead to degradation or unfolding of the ribonucleoprotein particles. The nascent chains of rough membranes stripped by these methods
presumably remain associated with the membranes at the ribosome binding sites (89). The fresh polysome samples used for binding experiments will always be accompanied by their own nascent chains. The implications, if any, of the presence of nascent chains both on degranulated membranes and polysomes have not been considered so far.

The partial degranulation caused by high KCl in the presence of Mg²⁺ (resulting in biologically active subunits) was found to proceed to almost complete degranulation in the presence of 1 mM puromycin after a short incubation at room temperature (74). The release of ribosomes by this method resulted in translocating peptidyl-puromycin to the intravesicular space in membrane vesicles. At lower concentrations of KCl (100 mM) there was no detachment of ribosomes even in the presence of sufficient puromycin to react with 50 per cent of the nascent chains. These findings suggested that the association of ribosomes with the membrane is fundamentally mediated by ionic forces but the presence of nascent polypeptides does add substantially to the stability of the interaction. This concept has been corroborated by a number of laboratories on the basis of studies employing both direct (90-93) and indirect (9) methodologies for studying ribosomal binding to reticular membrane. Sarma et al. (91) showed that after the release of nascent chains in an in vitro protein synthesizing system of rough membranes, the subsequent degranulation of the microsomes was a function of the concentration of KCl used. Sarma et al. (65) and Blobel and Potter (94) found that puromycin treatment in vivo resulted in the disaggregation
of bound polysomes to bound monosomes in rat and mouse liver, but did not cause degranulation as a consequence of this. Blobel and Sabatini (95) treated free polysomes at 0°C with puromycin at high ionic strength and released most of the nascent polypeptide chains without dissociating the polysomes, which retained the mRNA and the tRNA moiety of peptidyl tRNA. But these polysomes were unable to continue the translation of mRNA. The polysomes were then heated to 37°C, when they dissociated completely into subunits. Similar treatments without puromycin resulted in only partial dissociation. Recently, nascent polypeptide chains have also been found to participate in ribosomal anchorage to reticular membranes in plant tissues, which has also been demonstrated by puromycin plus KCl treatments of plant microsomes (96).

The presence in some tissue culture cells of membrane bound ribosomes which can be detached by treatment with ribonuclease in vitro has lead to the suggestion that mRNA may be involved in ribosome binding (82,97,98). The finding that EDTA treatment in vitro and prolonged puromycin treatment in vivo also detached these "loose" ribosomes is consistent with this view because these agents are known to disrupt polyribosome structures. Evidence in favour of a direct or indirect involvement of mRNA in the binding of polysomes to reticular membranes has been accumulating. However, initial evidence came from Rosbash and Penman (82) who showed that all the above mentioned treatments removed about 50 per cent of membrane bound 60s ribosomal subunits. After EDTA treatment, only 60s subunits were left on the membrane,
however, intact monomeric ribosomes remained on the membrane after ribonuclease or puromycin treatment. The authors classified membrane attached polysomes as loose (detached by EDTA) and tight (EDTA resistant). It was further shown that loose polysomes were not attached to the membrane by polypeptide chains. The requirement of polyribosomal integrity for the attachment of loose polysomes and the ability of RNAase to strip them from membranes suggested that mRNA may be involved in their binding. EDTA is known to liberate mRNA from its attachment to ribosomal particles (99-101). The authors thus suggested that for tightly bound polysomes, the ribosomes must be bound to the membrane independent of their attachment to mRNA. On the basis of their results in two consecutive reports (82,102), they concluded that light ribosomes are tightly bound but the heavier ribosomes are loosely bound to the reticular membranes.

The reports that differential washing of RER separates ribosomes synthesizing secreted proteins from those making soluble tissue proteins suggest that membrane bound ribosomes are heterogeneous (12,103). The major studies of heterogeneity of membrane bound ribosomes have been carried out in HeLa cells (82,102). About 15 per cent of HeLa cell ribosomes are bound to membranes, but less than 2 per cent of the protein synthesized is secreted (104). Membrane bound ribosomes were separated into 'loose' and 'tight' classes on the basis of their ease of dissociation from membranes by EDTA or ribonuclease treatment of the membrane in vitro or by in vivo treatment of the cells with puromycin (82). About 50 per cent of membrane-bound 60s
ribosomal subunits were removed by all these treatments. Whereas EDTA treatment left only 60s subunits on the membrane, puromycin and ribonuclease treatments left monomeric ribosomes. If the two treatments are applied in succession there is no further removal of 60s subunits which indicated that all treatments removed the same population of ribosomes. Because all the treatments disrupted polyribosomes, it was concluded that binding of loose ribosomes needs intact polyribosomes but that of tight ribosomes did not. Moreover, nascent peptide chain released by puromycin did not correlate with loss of loose ribosomes and it was thus concluded that nascent chains were not involved in anchoring the loose polysomes to the membranes (82). Dani et al. (9) have recently demonstrated the involvement of nascent polypeptide chains in the binding of ribosomes to membranes employing protein disulphide isomerase assays rather than by direct measurements of RNA/protein ratios.

2.3 Effects of ascorbic acid deficiency on microsomal drug metabolism and other functions:

Most of the in vivo studies have shown decreased metabolism of a variety of pharmacological agents in vitamin C-deficient animals but there is little information to-date about the underlying biochemical basis for the action of this vitamin. The mechanism involved in hepatic drug metabolism is complex, involving an electron transport system. The microsomal pathway responsible for the detoxification of many pharmacological agents is shown in Fig.1. The type of reactions which utilize
FIG 1. LIVER MICROSOMAL ELECTRON TRANSPORT SYSTEM RESPONSIBLE FOR THE METABOLISM OF DRUGS, STEROIDS AND FOREIGN CHEMICALS.
this system include O-demethylation, N-demethylation, hydroxylation, nitro reduction as well as the hydroxylation of steroids, including cholesterol. The transport system contains a heme protein, cytochrome P-450, which is reduced by NADPH via a flavoprotein, cytochrome P-450 reductase. For oxidative metabolic reactions, cytochrome P-450, in its reduced state (Fe^{+2}), incorporates one atom of oxygen into the drug substrate and another into water. Many metabolic reductive reactions also utilize this system. In addition, there is a lipid component, phosphatidylcholine, which is associated with electron transport and is an obligatory requirement for drug metabolism. It is obvious that in such complex series of events the vitamin could participate at a variety of levels.

An effect of ascorbic acid on in vitro drug oxidation has been demonstrated. Laber et al. (105) reported that liver microsomes from scorbutic animals had significant decreases in the demethylation of aminopyrene, hydroxylation of acetanilide, and cytochrome P-450 content, but not on cytochrome b5 content. They also showed that phenobarbital and 3-methylcholathrene (inducers of the microsomal electron transport system) caused an increase in the mixed function oxygenases in the scorbutic animals, which could be blocked by the prior administration of ethionine. Kato et al. (106) studied the metabolism of a variety of compounds such as aniline, hexobarbital, zoxazolamine, aminopyrine, diphenhydramine, meperidine, p-nitroanisole, p-dimethylaminobenzene in microsomes prepared from ascorbic acid deficient adult guinea pigs maintained on a deficient diet for
12 days. In contrast to some of the previous studies, these investigators showed that although the metabolism of aniline, hexobarbital, and zoxazolamine decreased, the metabolism of other drugs examined was unaltered. Furthermore, there was no significant decrease in microsomal electron transport components, such as cytochrome P-450 or cytochrome b5, or in the activity of NADPH cytochrome C reductase or NADPH oxidase. They concluded that the effect of vitamin C deficiency is rather specific on hydroxylation reactions only and involves the "terminal oxidase" component of the electron system. In contrast to the above findings, in vitro studies (107) using young guinea pigs weighing 200-250 g indicated a substantial decrease in drug oxidation reactions such as aniline hydroxylation, aminopyrine N-demethylation and p-nitroanisole-O-demethylation. In addition, there was a significant decrease in the quantity of cytochrome P-450 and NADPH cytochrome P-450 reductase. However, the decreased activities occurred only when the microsomal ascorbic acid had reached 30 per cent of normal values. Studies conducted by Sikic et al. (108) indicate that both cytochrome P-450 and aminopyrine N-demethylation decreased to 60 per cent of control values in liver microsomes at 21 and 25 days of deficiency, whereas there were no significant changes in microsomes of other organs such as lungs and kidneys. Glutathione S-aryltransferase activity was decreased in scorbutic liver and remained unchanged in lungs. On the other hand, N-acetylation of p-aminobenzoic acid, a non-microsomal drug detoxification
reaction, was increased in kidneys of deficient animals with no changes in the other two organs. Repletion with ascorbic acid resulted in complete recovery of liver microsomal metabolism by day 7.

Zonnoni et al. (107) studied the effect of Vitamin C deficiency on drug enzymes and electron transport components in young male guinea pigs (200-250 g) maintained on a deficient diet for 10-21 days. They observed that the aniline hydroxylation, aminopyrine N-demethylation, and p-nitroanisole-O-demethylation were not altered after 10 days but were significantly decreased in animals on the deficient diet for 21 days. Similar results were found with the electron transport components in that the quantity of cytochrome P-450 and P-450 reductase were significantly depressed in 21 day-deficient animals. At this time the concentration of liver ascorbic acid was approximately 30 per cent of normal and the animals had lost at the most 5 per cent of their body weight and were not scorbutic. In addition fasted animals which were supplemented with the vitamin and had lost between 25 percent to 30 per cent of their body weight had either normal or higher drug oxidation activity, indicating that the decrease observed in the ascorbic acid-deficient animals was not due to reduced caloric intake.

Sato and Zonnoni (109) conducted in vivo studies on the requirement of ascorbic acid in animals undergoing rapid growth such as weanling guinea pigs (90-100 g, 1-2 weeks old). The animals were placed on a vitamin C-deficient diet for a relatively
short period of time, 8 or 15 days. In addition, an increased dietary intake of ascorbic acid in these animals resulted in a concomitant increase in a variety of drug oxidative reactions and electron transport components. Microsomal NADPH cytochrome P-450 reductase, N-demethylase and O-demethylase increased as much as 200, 60 and 300 per cent, respectively, compared to the activities in microsomes isolated from 15 days deficient animals. Under these conditions the increase in the quantity of cytochrome P-450 was less than 45 per cent.

Studies with fetal guinea pig livers also showed a correlation between drug metabolism activities, the quantity of electron transport components, and liver ascorbic acid concentration. Fetal livers with ascorbic acid concentrations of 4.5 mg/100 gm liver had no detectable cytochrome P-450 or O-demethylase activity. On the other hand, fetal livers with an ascorbic acid concentration of 17.5 mg/100 g liver, a level approaching that of the dams, had comparable cytochrome P-450 and O-demethylase activities, while livers with intermediate levels of ascorbic acid had intermediate levels of P-450 and O-demethylase activity (25).

Drug enzyme activities and the microsomal transport components required from 6 to 10 days to return to normal levels in 21 day vitamin C-deficient animals replenished with ascorbic acid inspite of the fact that the quantity of liver ascorbic acid reached normal levels within 3 days (107). These findings are in line with the in vivo studies of Axelrod et al. (110) who found that the plasma half life of such compounds as aniline,
Antipyrine, and acetanilide in vitamin C-deficient guinea pigs was increased by 60 per cent and took from 5 to 8 days to return to normal when the animals were given ascorbic acid. Sikic et al. (108) showed that although partial recovery from decreases in drug metabolism occurred in 3 days, complete recovery required 7 days. Degkwitz and Kim (111) found that cytochrome P-450 and cytochrome b5 returned to normal levels in a shorter period of time, from 1 to 1.5 days, after the liver ascorbic acid concentration had reached normal levels. They gave the vitamin intraperitoneally 5 times per day and to animals on a deficient diet for shorter periods (14 days). However, it required upto 3 days, after the liver ascorbic acid concentration had reached normal levels for cytochrome P-450 and cytochrome b5 to return to normal levels when the vitamin was given orally on the repeated dosage schedule.

The time required for the re-establishment of adequate drug metabolism activity in deficient animals could be due to the time needed for the resynthesis of specific proteins associated with drug enzymes. To test this, several laboratories using drug enzyme inducers such as phenobarbital or 3-methylcholanthrene have shown that the protein synthesizing machinery involved in the microsomal electron transport is not substantially jeopardized and is operative in vitamin C-deficiency (106,107). For example, in animals pretreated with phenobarbital, it was found that aniline hydroxylase, aminopyrine N-demethylase and p-nitroanisole O-demethylase, as well as cytochrome P-450, NADPH P-450 reductase, and NADPH cytochrome C reductase were induced to the same extent.
in ascorbic acid-deficient compared to normal animals; the fold increase in specific activity was of the same order of magnitude in both groups. It appears from these studies that ascorbic acid is not involved in general protein synthesis, but the possibility that it could participate more specifically in heme synthesis was also investigated. Luft et al. (112) reported that the administration of a precursor of heme, d-aminolevulinic acid (ALA), to vitamin C-deficient guinea pigs caused an increase in the quantity of cytochrome P-450 and suggested that ascorbic acid may be involved in the formation of this essential metabolite of heme synthesis.

In view of these findings, it was important to determine if vitamin C deficiency affected the activity of ALA synthetase, the rate limiting enzyme in heme synthesis (113). Studies conducted by Rikans et al. (114) indicate that although the cytochrome P-450 content in livers from guinea pigs on a vitamin C-deficient diet was markedly decreased compared to control livers (10.3 compared with 17.9 nmole/100 mg supernatant protein), there were no significant differences in ALA synthetase activity, either in whole cell homogenate or in mitochondria. Thus it appears that ascorbic acid deficiency does not affect the initial step in heme biosynthesis. However, the possibility that one of the other enzymes in heme synthesis may be affected in vitamin C-depletion should be considered. For example, there could be differences in ferrochelatase activity, since iron must be maintained in its ferrous state for incorporation into
protoporphin IX (115), and ascorbic acid might be involved at this level, Rikans et al. (26) demonstrated that the activities of key enzymes involved in heme synthesis, ALA synthetase, ALA dehydratase and ferrochelatase, were not significantly reduced in livers from ascorbic acid deficient animals. In addition there was no significant difference in the amount of mitochondrial heme in normal and ascorbic acid deficient livers. However, ascorbic acid deficiency did affect induction with diethyl-1, 4-dihydro-2, 4, 6-trimethylpyridine-3, 5 dicarboxylate; a 6 fold increase in ALA synthetase activity occurred in liver homogenate prepared from normal animals in contrast to no significant increase in homogenates prepared from ascorbic acid deficient animals. These investigators further studied the effect on the multiple forms of cyt P-450. Separation of 44,000 to 60,000 dalton polypeptides (molecular weight region for the various forms of cyt P-450) by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate revealed quantitative differences in the polypeptides from normal and ascorbic acid deficient microsomes. Ascorbic acid deficient microsomes consistently demonstrated reduction in three polypeptide bands (molecular weights 44,000, 52,000 and 57,000) and increases in two polypeptide bands (54,000 and 55,000) compared with normal microsomes. Evidence that these polypeptides are cytochrome P-450 was obtained from heme staining with tetramethylbenzidine and from induction studies with phenobarbital and 3-methylcholanthrene. These results indicate that ascorbic acid deficiency
does not affect the availability of heme for cytochrome P-450 synthesis and the effect of ascorbic acid may be on the apoprotein moiety of cytochrome P-450.

A qualitative difference in drug cytochrome P-450 binding spectra occurs in vitamin C-deficient guinea pig microsomes with type II substrates such as aniline (107). The spectrum is atypical in that the trough is at 405 nm instead of 390 nm and the peak at 440 nm instead of 430 nm. There is also a decrease in the adsorption intensity, which correlated with the decrease in the quantity of cytochrome P-450 in the deficient animals. The addition of ascorbic acid or other reducing agents such as glutathione or reduced 2,6-dichlorophenolindophenol did not reverse the altered aniline-cytochrome P-450 binding. However, ascorbyl palmitate, a more lipophilic analogue of ascorbic acid, did restore atypical aniline-cytochrome P-450 binding spectra but the adsorption intensity at 450 nm was still depressed. Gunderman et al. (116) also found differences in drug cytochrome P-450 binding in that the Type I hexobarbital binding spectra required higher concentration of the barbiturate to achieve half maximal spectral changes.

Kinetic studies indicate no significant changes in the apparent Km of overall drug metabolism reactions such as aminopyrine N-demethylation, p-nitroanisole O-demethylation, or hexobarbital hydroxylation in normal and ascorbic acid deficient microsomes (107,25,116). Gundermann et al. (116) also found no apparent change in the Km of hexobarbital under similar conditions.
It is known that lipid peroxidation is detrimental to drug metabolism and antioxidants which inhibit peroxidation, such as N,N'-diphenylenediamine, protect drug enzyme activities (117). The possibility existed that ascorbic acid through its property as an antioxidant could be functioning by inhibiting lipid peroxidation in normal and deficient guinea pig microsomes. Using three methods of assay, oxygen consumption, NADPH disappearance and malonaldehyde formation, it has been shown that the rate of lipid peroxidation was, in fact, somewhat lower in microsomes deficient of the vitamin compared to control microsomes and it is unlikely that the decrease in drug metabolism activities and quantity of electron transport components observed in vitamin C-deficient animals can be accounted for via this mechanism (109). Furthermore, analysis of the quantity of the essential lipid component involved in drug metabolism, phosphatidylcholine in normal, vitamin C-deficient, and starved animals indicates that the ascorbic acid-deficient microsomes showed only an 18 per cent decrease in the quantity of this phospholipid compared to nondeficient microsomes (109). However, in fasted animals given ascorbic acid, the quantity of phosphatidylcholine was even lower than in vitamin C-deficient animals, despite the fact that the concentration of ascorbic acid in livers of the starved animals was adequate (42.6 mg/100 g wet liver) and they had normal drug metabolism activities (118). In addition, there was no significant difference in the chromatographic migration of either phosphatidylcholine or its
precursor, phosphatidylethanolamine in normal or vitamin C-deficient animals (109).

Inspite of all the efforts to understand the effect of deficiency of vitamin C on drug metabolizing enzymes and other microsomal components, no work has been done to demonstrate whether ascorbic acid is a component of microsomes or not. Efforts in this direction have been made during the present investigations.

2.4 Requirements of glutathione for some microsomal based cellular functions:

Glutathione is a ubiquitous cellular constituent which is the most abundant thiol reducing agent in animals, plant cells and in microorganisms. It is found predominantly intracellularly in relatively higher concentrations (0.5-10 mM). Glutathione has two characteristic structural features: a sulfhydryl (SH) group and a γ-glutamyl linkage. That this is usually the most abundant γ-glutamyl compound (except perhaps for glutamine, which also occurs extracellularly) suggests that this tripeptide has important biological functions. It has long been known that glutathione can be reversibly oxidised to glutathione disulfide, and enzymes that catalyze this transformation have been purified and studied. That the intracellular concentration of glutathione is much greater than that of glutathione disulfide is consistent with the presence of the highly active and widely distributed enzyme glutathione
reductase. Glutathione peroxidase catalyses the interaction of glutathione with hydrogen peroxide and other peroxides to yield glutathione disulfide. A number of investigations support the view that glutathione protects cell membranes and proteins by maintaining -SH groups and interacting with peroxides and free radicals. Apart from these, glutathione plays a role in the removal of $\text{H}_2\text{O}_2$, transport of amino acids and detoxification of foreign compounds. Although a number of reviews have been published on its biological reactions (21,22), still its physiological significance needs elucidation.

Recently, the conjugation of glutathione, one of major detoxifying pathways in mammals ultimately leading to the formation of N-acetyl-S-derivatives (mercapturic acids), has been implied in the activation of foreign compounds to potentially carcinogenic electrophiles. Conjugation of glutathione with carcinogens has been extensively studied.

The presence of a series of enzymes in soluble fraction of rat liver preparations which catalyze the reaction of glutathione with organic substrates that possess reactive groups has been recognized for many years (119). With aromatic hydrocarbons such as naphthalene, however, the presence of the microsomal fraction together with appropriate cofactors, as well as soluble fraction is necessary before conjugation with glutathione can occur (120). This observation lent support to the theory that hydrocarbons were metabolized via epoxides, and the glutathione conjugates with these epoxides.
In the metabolism with rat liver homogenates of benz(a) anthracene and 7,12-dimethylbenzantracene (121) and its related hydroxymethyl derivatives (122) K-region glutathione conjugates were detected, but there was no direct evidence for the formation of non-K-region products (123). When a mixture of the non-K-region epoxide, benzantracene 8,9-oxide, and the K-region epoxide, benz (a) anthracene-5,6-oxide was incubated with rat liver microsomal and soluble fractions in the presence of glutathione, the 8,9-oxide was converted mainly into the related 8,9-dihydrodiol, whereas the 5,6-oxide yielded mainly the glutathione conjugate (122). These observations are in agreement with the results obtained in the metabolism of the hydrocarbon (Fig.2).

The non-K-region dihydrodiols of 7,12-dimethylbenz(a) anthracene and its hydroxymethyl derivative are themselves further metabolized by rat liver homogenates to products that are probably glutathione conjugates and these are more polar than the similar conjugates discussed above (121). The structures of these metabolites have not yet been determined, although they could be epoxides produced by metabolism on other double bonds in the molecules.

The metabolism of a number of aromatic hydrocarbon epoxides has been investigated using either whole liver, homogenates or liver soluble fractions and glutathione.

The enzyme catalyzing the addition of glutathione to
FIG 2. FORMATION OF GLUTATHIONE CONJUGATES FROM BENZ(A)ANTHRACENE.
alkyl epoxides has been called glutathione-epoxide transferase (124) and presumably the same or a similar enzyme is involved in the formation of conjugates from epoxides formed on aromatic double bonds (125). The distribution of the epoxide transferase has not yet been studied in detail, although it is known to occur both in rat liver and kidney (124).

Apart from the carcinogens, the activation of mutagens is also glutathione dependent. In a report on the mutagenicity of 1,2-dichloroethane (126) it was shown that the mutagenic effects of this compound towards bacteria were greatly enhanced by the presence of cytosol (115,000 'g' supernatant), as a source of glutathione transferases, and added glutathione. In terms of chemical reactivity, this phenomenon is readily explainable (127) because substitution of one of the chlorine atoms by glutathione produces the structure of Β-halogen thioester or sulfur mustard, one of the oldest mutagen known (128). In fact S-(2-chloroethyl)-L-cysteine was tested in Drosophila as early as 1960 (129) and found to be mutagenic. Activation of vicinal dihalogen compounds to mutagens by glutathione involved the stereochemical factors (130). This hypothesis was put to test by comparing the mutagenic behaviour of cis and trans-1,2-dichlorocyclohexane with and without metabolic reactions. Assuming that glutathione transferases catalyze an SN2 reaction, cis-1,2-dichlorocyclohexane would be expected to give a trans-substituted conjugate whereas trans-1,2-dichlorocyclohexane should give the corresponding cis-conjugate, in which the sulfur.
atom is not in a favourable position to activate the halogen substitute. In the presence of 100,000 'g' supernatant and excess of glutathione however, cis-1,2-dichlorocyclohexane becomes a powerful mutagen.

In the course of investigating protein synthesis in regenerating rat liver, it was observed (131) that thiol compounds played a role in regulating the 'in vitro' capacity of microsomes to incorporate labelled amino acids in peptides. When microsomes prepared from normal liver were incubated with tRNA, GTP and a pH-5 supernatant containing transferases I and II, they displayed a low capacity to incorporate labeled amino acids until either 5 mM glutathione or other sulfhydryl donor was added to the incubated medium. Microsomes prepared from regenerating liver showed much less dependence on the presence of sulfhydryl groups, and it was concluded from this and other evidence that the redox conditions within the cell can regulate the rate of protein synthesis through sulfhydryl donors, and that regeneration of the liver may alter the intracellular redox potential. This effect of liver regeneration appears to be specific to the membrane-attached polysomes in the microsomal fraction, since it is known that polysomes detached from membranes by deoxycholate treatment show the same capacity for in vitro amino acid incorporation into peptide (132,133) and the same response to sulfhydryl groups (131) irrespective of whether they come from normal or regenerating liver. This suggests that the membranes of the endoplasmic reticulum modify the activity
of the attached ribosomes in their response to the sulfhydryl content of the medium and that regeneration alters this function of the membranes.

Nolan and Munro (27) studied the in vitro amino acid incorporation capacity of authentic rough endoplasmic reticulum and reconstituted rough membranes to various concentrations of sulfhydryl donors. The role of the membrane in influencing cell-free peptide bond formation shows parallel responses for authentic rough membranes to various concentrations of sulfhydryl donors. The membrane increases very considerably the requirement for -SH groups in order to promote peptide bond formation by the polysomes to which stripped rough endoplasmic reticular membrane has been added.

There does not seem to be any recorded observation about glutathione as the component of the endoplasmic reticulum. However, the interactions of glutathione with carcinogens, mutagens and toxic substances, the modifying effect of the SH donors (particularly glutathione) on reconstituted rough endoplasmic reticulum to incorporate amino acids for peptide bond synthesis, and the role of -SH groups for maintaining the stability of the membranes incited us to check whether glutathione is the component of the reticular membrane or not. Experiments on this aspect are reported in this thesis.

2.5 Molecular mechanism of chemical carcinogenesis:

For the studies on the possible mechanism of chemical
In the case of unsubstituted PAH Scribner (147) suggested the formation of sigma complex as a hypothetic rate determining step in the carcinogenic action. Pullman and Pullman (148) developed a theory that explains the special sites on the molecule which acts as a carcinogen. This theory is based on two fundamental qualitative assumptions.

a. A molecule which acts as a carcinogen must have an active K-region.
b. If the molecule also has an L-region, this region must be somewhat inactive.

According to this theory, a region is an assembly of two carbon atoms placed in such a way that they may undergo an addition reaction. A reactive centre is a single carbon atom with an affinity to undergo a substitution reaction. The reactive centre may or may not be on a reactive region. In the case of benzo (a) pyrene, there is a reactive centre on C5 but there is no L-region.

The K-region is defined as a bond of a conjugated molecule possessing a low ortholocalization energy. The presence of a bond, which is active for addition reaction (K-region), favours the appearance of carcinogenic activity. In case of L-region two atoms possess a low paralocalization energy. This presence of two reactive para-positions (L-region) is unfavourable for carcinogenic activity. The carcinogenic potency is decreased by substituents due to the decrease in the electronic charge of the K-region.

Three processes like charge transfer (149-151), chemical
carcinogenesis the major emphasis has been laid on physicochemical and biological properties of the carcinogens and their interaction with vital cell constituents (134-139). Arcos and Argus (140) proposed that there is a direct relationship between the molecular geometry of the compounds and their carcinogenic activity and on the basis of these observations these investigators reported the carcinogenic activity of the aromatic compounds. Certain carcinogens like polycyclic aromatic hydrocarbons (PAH) have been shown to interact with nucleic acids (141-143).

Miller (144) proposed a general theory to explain the chemical carcinogenesis, which has a significant importance on cancer research. This theory explains that most chemical carcinogens which are not chemically reactive must be converted metabolically into a chemically reactive form. For the initiation of carcinogenesis the activated metabolite which is electrophilic in nature reacts with nucleophilic groups in cellular macromolecules.

Miller and Miller (137) further extended this work and suggested that for the initiation of tumor formation these molecules (carcinogens or their metabolic products which act as electrophiles) interact covalently with nucleic acids or proteins of the cells. This hypothesis was further supported by recent investigations (145) and by experimental findings showing that the amines and other alkaline compounds can break the linkage of carcinogens to proteins, thus protecting the cells from carcinogenesis (146).
reactions (148) and energy transfer (152) have been suggested for the interaction between the bioreceptor and carcinogen at the molecular level.

Apart from these, Popp (153-155) developed the resonance hypothesis to explain carcinogenesis. For explaining the resonance hypothesis the same author compared the physicochemical properties of 1,2-benzopyrene (non-carcinogenic) with 3,4-benzopyrene (strongly carcinogenic). Optical enantiomers of benzo(a)pyrene derivatives also show differences in their carcinogenic activity (156). Tumor-initiating activity in CD-1 mice of the (-) enantiomer of trans-7, 8-dihydroxy-7, 8-dihydro benzo(a)pyrene was greater than the corresponding (+) enantiomer. Similarly among the 12 possible isomeric phenols of benzo(a)pyrene, only 2-hydroxybenzo(a)pyrene is weakly active (157). Kapitulnik et al. (158) observed the lack of carcinogenic activity of 4-, 5-, 6-, 7-, 8-, 9-, and 10-hydroxybenzo(a)pyrene on mouse skin.

The most important hypotheses for tumor induction with carcinogens are:

a. Direct interaction of the carcinogens with vital cell constituents causing the formation of precancerous and/or cancer cells.

b. Metabolic conversion of PAH into their carcinogenic forms.

c. Activation of a carcinogen, as well as enhancement of its carcinogenic potency, in the presence of a co-carcinogenic agent such as croton oil (159).

d. Conversion of viruses into tumorigenic agents through
carcinogens e.g. methylcholanthrene or benzo(a)pyrene, which catalyzes the induction of mammary tumors in mice.

Of course, an extrapolation of tumor induction from animals to man must take several factors into consideration, particularly species differences and the host protective mechanism against carcinogenic pollution. Most of the carcinogenic agents react with the sulfhydryl group of the cells (160). This cellular interaction of the carcinogens can be inhibited by sulfhydryl blocking agents (161). Since -SH groups are important for cell division, relatively high intracellular concentrations of sulfhydryls are required in plant and animal cells for the mitosis to proceed (162,163). When oncogenic agents react with the -SH groups directly involved in the control of mitosis, they either temporarily or permanently block the cell division. When cell division is inhibited, an initiation process of carcinogenesis might start.

Theoretically, carcinogens can attack different cell constituents causing cytological alterations which might promote the formation of cancer cells. The possible cellular components which may be attacked by carcinogens are chromosomes, mitochondria, lysosomes and microsomes.

2.5.1 How degranulation assists promoters of carcinogenesis:

In experimental animals carcinogenesis is an extremely complex process (164). In many experimental systems it can be
divided into a two-stage process (165). The first stage is initiation, which causes the formation of dormant tumor cells. These tumor cells do not necessarily give rise to tumor clones in the absence of further set of events, called promotion. The most powerful tumor promoters known are the phorbol esters derived from croton oil (166) and these are pure promoters in the sense that alone they cannot accomplish the process of carcinogenesis, they simply complete the process already initiated by a prior event.

The biochemical lesions produced in initiation and promotion must clearly be very different. Initiation is for most practical purposes an irreversible process and many workers believe that it involves genetic damage. Some evidence for this is given by the existence of repair-deficient human disorders which predispose to cancer (167) and the indications that a failure to excise pyrimidine dimers in u.v. irradiated cells may cause the production of thyroid tumors (168). The formation of totipotent mouse teratocarcinoma cells by transplantation which can be reversed by insertion of these cells into a blastocyst (169) argues for an alternative additional mechanism of initiation which does not involve genetic damage.

Tumor promoters cause a pleiotropic response in target cells (165) with induction of the enzymes plasminogen activator and ornithine decarboxylase. They block terminal cell differentiation in rodents (170) but not human (171) cell lines. They clearly change the programme of expression of the cellular genetic information. They also appear to suppress a natural anti-tumor defence mechanism (172).
Tumor promoters are of immense importance in the etiology of cancer and it is desirable to have a method of detecting them which does not depend on laborious, time-consuming and expensive animal tests. Rabin et al. (173) recently used the degranulation method for the detection of the promoters and in order to make this in vitro method to fit in the model of chemical carcinogenesis, a new hypothesis, "the deletion-depletion hypothesis" was proposed. This simple model explains the total process of carcinogenesis incorporating the processes of both initiation and promotion. This hypothesis takes as its starting point the general ideas of Comings (174). He suggests that all cells contain multiple structural genes coding for transforming factors which release the cell from normal constraints in cell division. These genes, it is speculated, are suppressed in adult cells by regulatory genes. The extension of this scheme by Rabin et al. (173) is illustrated in Fig. 3 in what is called the deletion-depletion model. In this model the authors use deletion in the sense of deletion of function, which could also, of course, include genetic deletion.

The scheme is essentially simple. The products of gene 2 control entry into S-phase of the cell cycle. Products derived from gene 1 suppress the expression of gene 2 and also control the expression of some differentiated functions and possibly also the production of a nuclease specifically involved in the turnover of mRNA-1. Gene 1 is switched on by a signal in embryonic development. If cells are removed from the embryo prior to this gene switch, teratocarcinoma cells are produced. Returning teratocarcinoma cells to their normal environment in a blastocyst
FIG 3. DELETION - DEPLETION MODEL OF CARCINOGENESIS.

——→ PATHWAYS EXPRESSED IN PRESENCE OF ACTIVE PRODUCTS OF GENE 1. ———→ PATHWAYS EXPRESSED ON DEPLETION OF PRODUCTS OF GENE 1. ———— CONTROL CIRCUITS, INCLUDING PATHWAYS OF EXPRESSION OF GENE 1. (REPRODUCED FROM REF. 173).
mRNA-2 \longrightarrow protein-2 \longrightarrow entry to S-phase

Gene 2, gene 1, genes 3-n

Initiators delete

mRNA-1

Secondary control molecules?

Protein-1

Promoters deplete

Differentiated functions

mRNA-1 turnover?

Proteins (3-n)

mRNAs (3-n)
causes gene 1 to be switched on and malignancy reversed. To complete the model, the authors suggest that the control materials produced as a result of expression of gene 1, have a higher affinity for control sites in gene 2 than analogous sites in the rest of the gene bank.

In this model suggested by Rabin et al. (173) the act of deletion involves genetic damage to an allelic pair of genes so that these genes become incapable of producing functional proteins, or products derived from these proteins. These acts of initiation in themselves will not change the cell phenotype. This can only occur when all the products of expression of gene 1 have been depleted from the cell. The act of promotion enhances this depletion process, which would otherwise be slow for two possible, but wholly conjectural reasons: firstly, if a nuclease specific for the turnover of the mRNA for gene 1 is produced from a gene (3 to n) under positive control of products of gene 1, and the affinity of the control substances for the control element of the gene producing this nuclease is low compared to its affinity for the control elements of gene 2; and secondly, if the ultimate control ligands derived from gene 1 are molecules of small enough size to pass into the initiated cell from its neighbours via gap-junctions.

Thus, degranulation could function in promotion by causing mRNA-1 to be displaced from its normal membrane location. This displacement might reasonably be expected to enhance the susceptibility of the messenger to spontaneous or enzymic degradation. This would be a process of direct depletion within the initiated cells. However, there is another possible depletion process which could
occur by degranulation in adjacent normal cells in contact with the initiated cell via gap-junctions. Displacement of mRNA-1 from the membranes in these cells could block the correct functional intracellular locations of gene products. This could prevent the flow of control molecules through the gap-junctions suppressing expression of malignancy, if indeed this is an important process. This could result from the impaired biosynthesis of either gap-junction proteins or enzymes involved in the production of control molecules, or both.

In this model it is possible to explain the transient hyperblastic action of tumor promoters; by displacing mRNA-1 from the membrane, normal expression of this messenger is blocked and the cell enters S-phase as the ultimate translation products of gene 1 are depleted. Some cells can be naturally triggered into S-phase. This is suggested to be due to a signal which switches on another system (like the sarc gene?) which covalently modifies the control materials produced from gene 1 so that the suppression of expression of gene 2 is relieved. The switch is temporary, since gene 1 in uninitiated cells can still be expressed to give unmodified control products. In initiated cells, remaining mRNA-1 molecules will be available, following removal of the promoter, to produce the control products which suppress re-entry into S-phase.

A notable feature of this model is that cell division could function as a promotional influence by simple messenger depletion. A further consequence of the model is that specific, differentiated functions can progressively be switched off in
the final stages of the depletion process as the expression of each of the genes 3-n is successively and inevitably lost.

2.6 **Short-term tests for the screening of carcinogens:**

The assessment of the carcinogenic potential of food additives, drugs and environmental contaminants presently requires lengthy and expensive animal studies. If a chemical is consistently found to be carcinogenic in animals, regulatory agencies assume that it poses a carcinogenic hazard for man. Over the years, studies on carcinogenicity in animals have been modified to diminish the latent period for tumor formation. Thus, the experimental animals are generally treated with agents under test with high (maximally tolerated) dose levels. With such a protocol involving a few hundred or even fewer animals one may obtain a forecast of the possible effects of low doses of carcinogens acting for a long time in human populations consisting of millions of individuals. Despite their value such *in vivo* tests are cumbersome, lengthy and costly and are no longer adequate as the sole means of gauging the carcinogenic hazards in human environment.

There are two major aspects of chemical carcinogenesis that have a direct bearing on the development of a preliminary screening test for carcinogens: (a) the metabolic activation of most chemical carcinogens and (b) the formation of cancer in two or more distinct steps. In the last 20 years the metabolic activation of many carcinogenic agents to carbonium ions or electrophilic agents has been elucidated. Further, the identity
of several nucleophilic targets of carcinogenic electrophiles has been established.

Eight different parameters have been proposed to be applied in short-term tests to detect chemical carcinogens. Four of the end points are based upon an interaction of carcinogens or their metabolites with DNA: chromosomal aberrations, mutagenesis, DNA repair and DNA binding. These endpoints are a logical outgrowth of the somatic mutation hypothesis of cancer causation (175). Another endpoint, cell transformation, is directly related to the carcinogenic process itself. The proposed use of teratogenesis as a screen for chemical carcinogens is based on the hypothesis that cancer results from an abnormal phenotypic expression of an unchanged genotype. The seventh short-term test to be discussed consists of various cytological alterations that have become associated with carcinogen administration. The last test is based on microsomal degranulation caused by the carcinogens.

2.6.1 Chromosomal aberrations Test:

Although the role of chromosomal aberrations in the development of cancer is still debatable, the close association between the two has been well established by a number of observations: (a) numerical abnormalities of the sex chromosome and leukemia are familially aggregated (176); (b) several carcinogens can cause chromosomal changes in cells (177); (c) people with syndromes characterized by inherited
susceptibility to certain types of chromosomal damage often develop cancer (178), (d) many tumor cells have altered karyotypes, which in some cases can be correlated with malignancy (179), (e) cellular transformation in vitro in some cases appears to involve karyotypic changes (180). Further evidence for a close correlation of chromosomal abnormalities and carcinogenesis derives from studies by Kurita et al. (181) who demonstrated that frequencies of chromosomal aberrations induced by 7, 12-dimethylbenz (a) anthracene and urethan in mouse hematopoietic cells vary significantly with the age at exposure and the strain of mouse and vary directly with susceptibility to leukemogenesis. Possibly, the very factors which influence susceptibility to leukemia also influence the production of chromosomal aberrations.

The short-term human lymphocyte culture is commonly used to assess the effects of chemicals upon chromosomes. However, to date cytogenetic procedures have not taken into account metabolic activation of potential mutagens and thus do not follow accepted toxicological principles (182). This is one reason for which in vivo cytogenetic procedures are more valid than their in vitro counterparts for predicting a mutagenic hazard to man.

Chromosome stains capable of differentiating specific regions along the chromosomes have been developed (183,184). These differential chromosome staining procedures utilizing
either quinacrine or Giemsa have yet to be applied for the identification of induced chromosome aberrations. The increased resolution of the new technologies may increase the potential of cytogenetics in mutagen and carcinogen screening.

Several carcinogens have given positive responses with this system. These include such alkylating agents as N-methyl-N'-nitroso-N-nitroguanidine (185), nitrogen mustard, ethylenediamine (186), β-propiolactone (187) and epoxides (188), as well as, arcidines (177), 7, 12-dimethylbenzanthracene (189), 4-nitro quinoline-N-oxide (185), N-Hydroxyurethane (190) and aflatoxin (191). However, few studies have been reported on chromosomal breakage by the aromatic amines, aminoazodyes, nitrosamines and hydrocarbons (192). Further, a very high proportion of drugs that apparently do not possess any carcinogenic activity similarly give positive responses in this test (193).

On the other hand, the antibiotics mitomycin C and actinomycin D, which lead to chromosome damage in mammalian cells (194,195), have also demonstrated carcinogenic activity.

2.6.2 Test-mutagenesis:

One of the more promising methods for screening chemical agents for their potential carcinogenic activity is their ability to cause mutations in microbial or in cell culture systems. Although, as discussed above, a mutation has long been proposed as a critical event in carcinogenesis (196,197), a close structural correlation between mutagens and carcinogens
has only been possible, since the discovery of carcinogens which require little or no metabolic activation to demonstrate their mutagenic activity (198, 199). Because the microbiological system normally used in mutation studies appeared to lack the enzymes necessary for metabolic activation of the carcinogens, mutations could only occasionally be affected by the carcinogens under investigation (198). To-date, the classes of chemical carcinogens shown to cause mutations include the direct acting alkylating agents such as the nitrosamides, the nitrogen mustards, epoxides, imines, and sterically strained lactones (177, 200, 201). When appropriately reactive derivatives or metabolic activating systems are employed, the list of carcinogens exhibiting mutagenic activity extends to the polycyclic hydrocarbons (202, 203), the aromatic amines (179, 204, 205), the amino acid dyes (206), the nitrosamines (198) and the carbamates (184). Aflatoxin demonstrated mutagenic activity both in vivo (207) and in vitro (208).

The major obstacle to be overcome in the use of mutagenesis as a screening procedure for chemical carcinogens is the metabolic activation of the carcinogen in the mutagenic system under test. The vast majority of compounds whose possible carcinogenic activity is to be investigated will not be direct-acting alkylating agents. If they are to be active either as mutagens or as carcinogens, they will almost certainly require some form of metabolic activation (209). Several
recent studies have successfully linked drug activation with cell or microbial mutation (207, 210, 211). According to the mode of drug activation employed, these studies have followed three main lines: chemical, microsomal and host mediated carcinogen activation.

Among the means used to study the mutagenicity of procarcinogens is the host mediated bioassay (210, 212, 213). By this procedure a suitably sensitive microbe is implanted into animals and the animals are subsequently treated with the prospective mutagens; the cells are then isolated and examined for the presence of mutant forms. This procedure relies on the host for both the formation and the transport of the active form of the agent to the critical sites of the target cells. The mutagenic activity of dimethylnitrosamine could be observed with *Salmonella typhimurium* injected intraperitoneally into rats which had subsequently received a dose of the compound intramuscularly. Dimethylnitrosamine had no effect on cultures of salmonella grown *in vitro* (210, 212). Cycasin, a glycoside of the carcinogen methylazomethanol, was similarly found to be mutagenic to *S. typhimurium* in the same host mediated bioassay (210, 212). In a similar system, dimethylnitrosamine was found to be mutagenic to *Neurospora crassa* located in the host's liver (214).

These early reports linking mutagenesis with carcinogen activation offer promising leads in the use of mutagenesis as an assay for carcinogenesis. However, one of the major limitations of any cell system using mutation as a parameter
is the relatively small number of mutant forms available. The nutritional mutants of Ames (200), of Kao and Puck (215), and of Capizzi et al., (216), the drug resistant mutants of Fischer (217) and of Chu and Malling (218) and the colored mutants of deSerres and Malling (219) all require a 'hit' of the carcinogen on a restricted number of 'target' genes to reveal the activity of the mutagen. However, if an attack occurs at an insensitive site, the event cannot be scored, due to the lack of a suitable detection system. Because of the prospect that a weak carcinogen or mutagen may pass unnoticed, it may be desirable to amplify the detectable number of mutants in a given system. Two approaches along those lines are the use of repair-deficient mutants and Drosophila. Mutagen detection by the use of repair-deficient mutants has been successfully employed by the group of Stater et al. (211).

One technique that has been used to overcome the problem of drug activation in mutagenesis in a mammalian host has been the dominant-lethal test (207, 220-222). This test is based on the pre-implantation loss of eggs or on the formation of dead embryonal implants following the injection of a mutagen into male or female mice at a specific time before mating. The dominant lethal test assumes that a single mutation has occurred in the egg or sperm and has killed the embryo heterozygous for it (220). Carcinogens that apparently require metabolic activation to exert their effects and that gave a
positive response in the dominant lethal test include aflatoxin and benzo(a) pyrene (222). However, a disturbingly high number of carcinogens have given a negative response with this test (207, 220).

2.6.3 DNA repair synthesis test:

The idea of utilizing DNA repair synthesis as an indicator for a carcinogenic activity of a compound is based on the assumption that chemical carcinogens interact with DNA, and that the induced molecular alterations will result in DNA repair which can be readily estimated as an unscheduled incorporation of several DNA precursors (223, 224). Although there are many pros and cons to this proposal the possibility of using DNA repair synthesis as an economic and rapid bioassay cannot be rejected outright. There is convincing link between highly and weakly oncogenic derivatives or isomers of 4-nitroquinoline-N-oxide or 4-nitro-pyridine-N-oxide and high and low levels of DNA repair synthesis (225). Furthermore, procarcinogens, including 2-acetylaminofluorene, 4-aminostilbene, 4-acetylamino-stilbene, 2-acetylanaminophananthrene, 4-acetylanaminobiophenyl and 4-aminobiphenyl do not have the capacity of evoking a DNA repair synthesis which follows the application of their active N-hydroxy or N-acetoxy metabolites (223, 224). Similarly the highly mutagenic (203) and cell transforming (226) K-region epoxides of benz(a) anthrene and 3-methylchlanthrene are highly active inducers of an unscheduled DNA synthesis.
whereas the parental benz(a) anthracene and 3-methylcholanthrene or their inactive dihydrodiols (227) do not provoke a detectable level of DNA repair (228).

Many of the techniques presently available to estimate DNA repair synthesis are cumbersome and time consuming. However, a few of them could be adapted for large scale prescreening or screening programs. The most direct evidence of a carcinogen induced DNA change is obtained by examining shifts in sedimentation profiles of DNA (229, 230). The return of broken DNA strands to the original molecular weight is interpreted as evidence for DNA repair (230, 231). A second approach for the estimation of DNA repair relies on the incorporation of precursors during resynthesis of short nucleotide sequences that were eliminated from the DNA following their damage by physical or chemical carcinogens (232, 233). The most promising procedure makes use of an unscheduled incorporation of tritiated thymidine into nondividing cells with subsequent detection by autoradiography (233). This technique is relatively sensitive, requires only few cells, can be applied to cultured mammalian cells, biopsy samples in vitro, in vivo systems (234), permits the visualization of DNA repair in individual nuclei or even chromosomes, and facilitates a quantitative evaluation of DNA repair synthesis in different cell types of a tissue or organ. The prerequisites of this procedure are: a normal uptake of DNA precursors into the nucleotide pool, a normal usage of these precursors, a normal diploid DNA content and unimpaired DNA repair system. Another promising approach makes use of the incorporation of bromodeoxyuridine into
DNA thus photosensitizing the position of incorporation to ultraviolet radiation of long wavelength (235). This procedure which was successfully applied to estimate the extent of DNA repair, may prove to be an extremely sensitive one (235).

The aforementioned results and recent studies on about 110 different compounds revealed several restrictions to the use of DNA repair synthesis in a prescreening or screening program for potential carcinogens. One of the key unsolved issues is the inability of cultured lymphocytes or fibroblasts to respond to several procarcinogens that required activation to potent metabolites. Thus, the proposed test system in its simplest form misses many of the widely spread and naturally occurring carcinogens belonging to the group of polycyclic aromatic hydrocarbons, nitrosamines and aflatoxins. This situation can be improved by in vitro activation of procarcinogens with such systems as a post mitochondrial supernatant fraction of mammalian livers. An incubation of dimethylnitrosamine and aflatoxin with a fortified activation mixture produced potent inducers of DNA alterations which subsequently led to a reduction of molecular weight and the initiation of an unscheduled repair synthesis (231). The inclusion of an activation procedure for procarcinogenesis seems to be a must in a prescreening program employing DNA repair synthesis of cultured mammalian cells.

Inspite of the early successes with the in vitro activation
tissues and cell populations from which tumors arise.

2.6.4 Teratogenesis:

Early reports on the ability of compounds to induce congenital malformations indicated that teratogenesis might be a fairly reliable and rapid indicator of carcinogenic potential (239). A variety of carcinogens including derivatives of the alkylating agents (239, 241) of urethane (240) and of 7,12 benz (a) anthracene (242) have all produced teratogenic changes in the offspring of treated female rodents. Of the direct acting carcinogens studied all but N-methyl-N-nitrosourethane produced congenital abnormalities, however, some of the carcinogens requiring metabolic activation also produced such abnormalities (240,241). However, no teratogenic effects could be noted in the progeny of a female rat given a dose of the hepatocarcinogen diethylnitrosamine (241). Few studies have been reported on the possible teratogenic effects of the carcinogenic aromatic amines, aminoazo dyes or polycyclic aromatic hydrocarbons. The major disadvantage of using teratogenicity in a screen for carcinogenicity is the large number of false positives detected by the method (240). Compounds which have been found teratogenic, but not carcinogenic include: 5-bromodeoxyuridine, 5-iododeoxyuridine, colchicine, aspirin, and hydroxyurea (243-245). Further, carcinogens apparently lacking teratogenic activity include N-methyl N-nitrosourethane (246). On the other hand derivatives of 5-fluorouracil, antimetabolites of folic acid and griseofulvin which have not been shown to attack DNA
procedures a certain reservation must be expressed. It appears unlikely that the activation preparations from livers will contain all enzyme systems required to activate all types of procarcinogens. Compounds with a highly specific organotropic action may only be changed into active metabolites in their target tissues by an organ specific enzyme system. Such potential carcinogens could produce "false negatives" when added to cultured fibroblasts with a wrong or incomplete activation mixture. Organotropic procarcinogens can be identified by a combined in vivo and in vitro system for estimating DNA alterations which result in a detectable level of DNA repair synthesis. The test compound is injected into mice; at various periods thereafter the mice are sacrificed; various organs and tissues are removed, teased into small pieces and incubated in a tissue culture medium containing tritiated thymidine. In this way, the compound is activated in vivo, exerts its organotropic action and the induced DNA damage is then estimated in vitro as DNA repair synthesis. Two carcinogens which differ in their organotropic action were examined with this novel procedure: 4-nitroquinoline-N-oxide which induces lung tumors (236), and dimethylnitrosamine, which causes hepatomas, lung carcinomas and to a lesser degree kidney tumors (237, 238) when injected into mice. A detectable level of unscheduled incorporation of tritiated thymidine occurred only in those
directly, have been reported to have some carcinogenic activity (247-250) and to be teratogenic (240).

2.6.5 **Cell transformation:**

Chemically induced *in vitro* cell transformation as a criterion of carcinogenesis holds great promise. The transformation of various cell lines has been achieved by several groups of investigators (226,251,252) using a variety of viral and chemical agents. The effects of some agents were found to be synergistic (253,254). The chemical carcinogens thus far successfully used in cell transformations include several polycyclic aromatic hydrocarbons (251,255,256), dimethylnitrosamine (257), 2-acetylaminofluorene and its N-oxidized derivatives (258), 4-nitroquinoline-N-oxide (259), urethane and aflatoxin. The *in vitro* high transforming activity of N-acetoxy-2-acetylaminofluorene (258) of the K-region epoxide and cis-dihydrodiol derivatives of benz(a)anthracene and dibenz(a)anthracene (226), and of 4-hydroxylaminoquinoline-N-oxide (259) reflects the requirement of their corresponding procarcinogens for metabolic activation. When urethane or diethylnitrosamine was incubated *in vitro* with mouse embryo fibroblasts, no significant cell transformation was seen; however, a high rate of transformation was found in the fibroblasts derived from embryonal cells when obtained from animals injected with diethylnitrosamine or urethane (258). The above studies have all been performed with mesenchymal or mixed cells, producing sarcomas in those instances where transformed cells were reintroduced into syngeneic hosts. The recent establishment of epidermoid cells in tissue culture
(252) is currently permitting detailed investigation of in vitro transformation in cell lines corresponding to the other of the two major classes of human and animal tumors, the carcinomas.

Major objections to the use of cell transformation as bioassay method include the high rate of spontaneous cell transformation, and the fact that in early studies rigorous criteria equating in vitro transformation with malignancy were absent. A high rate of spontaneous cell transformation may actually be considered as an advantage in carcinogenicity studies, for it would permit an investigation of the possible role in carcinogenesis of natural substances normally found in the nutrient medium (260, 261).

Recent findings of a high degree of correlation between the altered morphology of transformed fibroblasts and their capacity to produce sarcomas when transplanted into animals (258) further increase the prospective utility of cell transformation as a prescreen for chemical carcinogens.

2.6.6. Test- Reaction with nucleic acids:

Although the critical target(s) of chemical carcinogens have not been defined, there is a great deal of evidence supporting some degree of correlation between carcinogenicity and binding of chemical carcinogens to nucleic acids (193, 262, 263). Consideration of current facts suggests that a chemical which reacts with DNA or is bound to DNA is potentially carcinogenic. Therefore, examination whether there is a reaction of chemicals
with DNA might afford a useful means of screening for potential carcinogens. There are certain drawbacks with this method:

1. As a rule metabolic activation is required for those chemicals which are known to bind covalently to nucleic acids (193,262). Consequently, methods would have to be developed for selecting systems which would provide for the necessary metabolic activation. The exceptions to this rule are of alkylating agents and compounds, which yield alkylating agents by nonenzymatic reactions (262,263).

2. There are data to indicate that some specificity in the binding of a compound in the form of its activated metabolite to DNA may be required for a change in biological properties of the DNA to be expressed or detected. The nature of the base which is substituted (e.g. adenine vs guanine) and perhaps more important, the site of substitution on a specific base (e.g. O-6 alkylation of guanine might be more critical than N-7 or C-8 alkylation) are examples of such factors.

3. Some chemicals cause changes in the biological properties of DNA without covalent binding, e.g. by intercalation (264), which might lead to frame-shift mutations (265). These potential carcinogens might be missed in a screening procedure based on the detection of covalent binding to DNA.

There is probably sufficient knowledge in hand to be able to predict whether many compounds per se would react with and bind covalently to DNA. Such compounds with predictive reactivity should be tested for carcinogenicity regardless of their behaviour in the short-term screening study. The compounds of
most interest for short-term tests, are those for which reactivity could not be predicted and/or those which require metabolic activation (262,263).

Metabolic activation of carcinogens has been studied extensively in vivo, but such procedures by themselves would not be very useful in a screening program. Problems of species and tissue specificity exist, unless one were able to examine binding in most tissues of several species of animals, a formidable task. Another difficulty is the requirement that the compound after examination must be available in radioactive form with a high specific radioactivity, since the extent of binding of carcinogens to nucleic acids in vivo is extremely low, of the order of 1-2 molecules of carcinogen per ten or twenty thousand nucleotides.

Use of in vitro systems, such as selected cells in tissue culture (266), fortified liver microsomes (226) or chemical methods of activation such as making appropriate derivatives (262,263,267) or generation of free radicals (268) for the activation of compounds deserve further study as part of a screening procedure. Since in most instances, the compound of interest would not be available in radioactive form, practical methods for screening chemicals by examining reactivity with DNA in the in vitro systems described would require measurement with high sensitivity and specificity of some change in the properties of the DNA. Several possibilities could be explored: (a) change in base composition or the appearance of modified
bases (269); (b) changes in physico-chemical properties of the DNA, such as viscosity, sedimentation velocity or $T_m$ (270); or (c) changes in some biochemical properties of the DNA, such as priming ability, template activity or transforming ability.

2.6.7 Cytological alterations:

Ultrastructural changes have been detected in several cell organelles in response to chemical carcinogens. Perhaps of interest and potential relevance have been alterations observed in nucleolar structure. Aflatoxin (271), 4-nitroquinoline-$N$-oxide (272) actinomycin (273), ethionine (274), dimethylnitrosamine and $N,N'$-dimethyl-4-aminoazobenzene (271) cause segregation of the nucleolar fibrilar and granular components and nuclear cap formation. While thioacetamide feeding led to the enlargement of liver nuclei, a decrease in the size of the nucleolus is seen with acridine orange and actinomycin D (275-277). Although these ultrastructural alterations exhibit a degree of association with carcinogens and the changes can sometimes be correlated with such functional changes as the inhibition of RNA-polymerase (278) or maturation of ribosomes (275,276), the transient nature and reversibility of the induced changes has limited so far their applicability as carcinogen screen. Obviously more information on the effect of a variety of known carcinogens of different chemical types and varying potency is required.

An increased mitotic index in the target tissues has often been associated with chemical carcinogenesis (277,279) and has been proposed as playing an important role in the carcinogenic process,
especially in the steps subsequent to initiation. The absence of extensive structure-function studies relating to hyperplasia with increased tumor incidence discourages the use of hyperplasia as a screening procedure for carcinogens at this time.

2.6.8 Degranulation of rough endoplasmic reticulum:

In the phenomenon called "degranulation of rough endoplasmic reticulum" the membranes lose ribosomes which lie free in the cytoplasm. Degranulation is caused by many carcinogens with structures as diverse as the azodyes (280-282), dimethyl and diethyl nitrosamine, ethonine and aflatoxin B<sub>1</sub> (281) and 2-acetylaminofluorene (283). A single dose of dimethylnitrosamine causes a breakdown of the characteristic lamellar organisation of ER and a marked increase in the number of free ribosomes (284). Williams and Rabin (8,4) found that the degranulation effect could be reproduced in vitro using an isolated liver RER preparation and incubating with carcinogens.

The liver provides a good model for the study of carcinogen-induced degranulation for two reasons: firstly it is a rich source of RER and secondly it has the metabolic capacity required to generate active forms of carcinogens from precursors. The main difficulties experienced are in the methods employed to monitor ribosomal loss. Williams and Rabin (4) assayed protein disulfide isomerase (rearrangease) activity of membranes before and after treatment with carcinogens. As the ribosomes were removed, the enzyme was exposed and its activity estimated. However, the enzyme
assay is a multistage operation, depending upon several parameters. Protein disulfide isomerase catalyses the direct distribution of the disulphide bonds which maintain the tertiary structure of proteins. Therefore, a substrate with its disulphide bridges in the incorrect positions must be used for the assay. In practice (4), ribonuclease with randomly reoxidised disulphide bridges was used. The enzymic assay has been completely modified and shown to be useful for ribosome-membrane interaction studies by Dani et al. (9) employing double wavelength U.V. spectrophotometry.

A direct method of monitoring ribosome loss is by estimating RNA/protein ratios of membranes. This ratio is decreased in degranulated membranes due to the loss of RNA with the ribosomes. However, accurate RNA determinations are essential, as the changes in RNA content are small. As the RNA estimation takes a considerable length of time and due to the inherent difficulties with rearrangease assay, the use of membrane containing radiolabelled RNA was developed (285).

The exact mechanism of degranulation caused by carcinogens is not known. It has been suggested by Rabin and co-workers that certain steroids are implicated in the binding of ribosomes to the endoplasmic reticulum and that various carcinogens are able to degranulate RER by destroying the sites at which the steroids interact with the membrane. This suggestion, and the evidence supporting it, has not gained wide acceptance. Aflatoxin B1 appeared to cause the degranulation of rough endoplasmic reticulum in vivo (281). Rough microsomes were incubated with a high
concentration of the compound (12x10^{-5} M) and a marked increase (about 400 per cent) in the activity of rearrangase was observed. After centrifugation over 2 M sucrose to remove detached ribosomes, the RNA/protein ratio of the treated microsomes fell correspondingly. It therefore appeared that the degranulating potential of the toxin could be expressed in vitro.

The molecular shape of aflatoxin B₁ is similar to that of steroids. Therefore, corticosterone was included in the incubations to determine whether its presence could migrate the effects of aflatoxin by competition at the relevant site. Corticosterone (1.5x10^{-4} M) depressed the effect of aflatoxin B₁ by 40 per cent. It was discovered that corticosterone not only protected rough microsomes from the degranulatory properties of aflatoxin B₁ but it could actually generate in smooth microsomes the ability to bind added polyribosomes as judged by the enzyme assays. James et al. (286) concluded that oestradiol was also effective in promoting the binding of ribosomes to smooth membranes which resembled RER under the electron microscope.

It was further found (281) that the degranulation by aflatoxin B₁ abolished the ability of treated microsomes to bind untreated polyribosomes, unlike microsomes degranulated with EDTA. This was interpreted to mean that the aflatoxin-induced lesion was in the membrane and not in the ribosomes, and by analogy, that the generation of binding capacity by corticosterone and oestradiol was also brought about by an interaction of the steroids with the membrane.
Sunshine and co-workers (287) observed that whereas oestradiol was able to engender the creation of a binding capacity in male smooth microsomes for male polyribosomes, testosterone being ineffective, the converse held when microsomes and polyribosomes from female rats were used. On the basis of this sex-specificity in vitro, it was considered that the presence of opposite-sex steroid may control the extent of the ribosome membrane association in vivo. Roobol and Rabin (288) suggested that the pair of sex steroids was required for each interaction to take place, the opposite-sex steroid being required at a membrane site and the same sex steroid in the ribosome.

Purchase et al. (5) during the comparative studies of the six short term tests for the detection of carcinogens, checked the predictive values of degranulation test using the rough endoplasmic reticulum. Of the 58 carcinogens tested, 41 (71 per cent) gave a positive result. A correct negative result was obtained with 44 of 62 non-carcinogens tested (71 percent). The overall predictive value for all compounds was 71 per cent. Per cent degranulation observed on RNA/protein ratio basis was in the range of 3 to 25 per cent.

This in vitro technique though needs less time suffers from certain drawbacks. For example, the preparation of RER by ultracentrifuge at 105,000 'g' needs prolonged centrifugations resulting in lipid peroxidation, which would have a harmful effect on microsomal properties. Arstila et al. (10) demonstrated a correlation between the extent of lipid peroxidation of microsomal membranes and the breakdown and disappearance of membrane-bound
ribosomes. Wall pressure and high hydrostatic pressure generated during ultracentrifugation also leads to the removal of certain populations of the ribosomes from the membrane vesicles (64). Apart from these the high 'g' forces (105,000 'g') used for the preparations removed the loosely bound ribosomes from RER. The degree of removal of 'ultracentrifugal labile ribosomes depends on the length of time used (60). Palmer et al. (6) observed that the populations of the ribosomes which are removed by wall affect, high hydrostatic pressure, lipid peroxidation and ultracentrifugal forces are the same which are also removed by carcinogen treatments. The use of such membrane preparations measured only the detachment of the left over ribosomes by carcinogens and this can lead to ambiguous conclusions as some carcinogens with low potentialities could escape prediction. The results in this thesis show our endeavours to prepare microsomes with intact ribosomes. The use of these preparations for degranulation studies is expected to eliminate some of above mentioned drawbacks.

In addition to the above short-term techniques for the detection of environmental carcinogens, a number of other techniques like sebaceous gland test, sperm morphology assay, micronucleus test, tetrazolium reduction test and implant test have appeared in literature. Their predictive efficiencies on a large scale remain to be determined.

Evaluation of the short-term detection techniques for carcinogens is being made in a large number of laboratories. The
reports of these trials are published annually in the proceedings of various Symposia held by the Environmental Protection Agency of America.