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activation and auxin-induced growth of Jerusalem artichoke
tuber slicesUNIVERSITY/UNIVERSITÉ CarletonDEGREE FOR WHICH THESIS WAS PRESENTED/
GRADE POUR LEQUEL CETTE THÈSE FUT PRÉSENTÉE Ph.D.YEAR THIS DEGREE CONFERRED/ANNÉE D'OBTENTION DE CE DEGRÉ 1977NAME OF SUPERVISOR/NOM DU DIRECTEUR DE THÈSE Dr. G. Setterfield

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**LA THÈSE A ÉTÉ
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METABOLISM OF FREE AND MEMBRANE-BOUND RIBOSOMES
DURING ACTIVATION AND AUXIN-INDUCED GROWTH OF
JERUSALEM ARTICHOKE TUBER SLICES

by

JOACHIM SPARKUHL, B.Sc.

A thesis submitted to the Faculty of Graduate
Studies and Research in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy.

Department of Biology

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Ottawa, Ontario

April, 1977

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The undersigned hereby recommend to the
Faculty of Graduate Studies and Research acceptance of
this thesis, submitted by Joachim Sparkuhl, B.Sc., in
partial fulfilment of the requirements for the degree
of Doctor of Philosophy.

Chairman, Department of Biology

Supervisor

External Examiner

for my parents

And even I can remember
A day when the historians left blanks in their writings,
I mean for things they didn't know...

-Ezra Pound: Canto XIII

Ein wenig Weisheit ist schon möglich; aber diese selige
Sicherheit fand ich an allen Dingen: dass sie lieber
noch auf den Füßen des Zufalls - tanzen.

-Friedrich Nietzsche:
Also sprach Zarathustra

ABSTRACT

Cell fractionation techniques were used to follow changes in ribosome content, synthesis, and activity during activation and auxin-induced growth of Jerusalem artichoke tuber tissue. Excision of slices from dormant tubers resulted in activation of ribosome metabolism. During the first 24 h of incubation of slices in aerated water (aging), rRNA synthesis and turnover were initiated and a membrane-bound ribosome population appeared. Protein synthesis activity, as measured by polysome formation and KCl-stability, increased rapidly in both the free and membrane-bound ribosome populations reaching a maximum at 24 h aging. Aging in water beyond 24 h resulted in a decline in ribosome activity and in the proportion of membrane-bound ribosomes. Protein and rRNA content, and fresh weight of cells stabilized rapidly after excision and remained constant during aging in water up to 72 h.

The incubation of activated (24 h aged) tissue slices in 2,4-dichlorophenoxyacetic acid (2,4-D) plus kinetin resulted in an increase in fresh weight, accompanied by protein accumulation and a three-fold increase in rRNA content over 24 h of treatment. The proportions of active and membrane-bound ribosomes were maintained at the maximum levels reached during aging. 5-Fluorouracil, an inhibitor of ribosome synthesis, decreased incorpora-

tion of (^3H) uridine into ribosomes by 70% when supplied concurrently with 2,4-D plus kinetin but because of an apparent reduction in rRNA turnover had less effect on ribosome levels or protein accumulation.

The membrane-bound ribosome population which developed during aging appeared to be derived from free ribosomes on the basis of labelling kinetics. A membrane-fraction enriched in rough endoplasmic reticulum was isolated from both non-growing (water-aged) and growing (hormone-incubated) tuber slices by floatation fractionation. The membrane-bound ribosomes of growing cells transported 42% of their in vitro protein products into microsomes, while little vectorial transport was evident in membrane-bound ribosomes from non-growing tissue. Preliminary electrophoretic analysis suggested that some differences exist between the proteins made in vitro by free and membrane-bound ribosomes of growing cells.

ACKNOWLEDGEMENTS

It is a pleasure to thank Dr. George Setterfield for allowing me the freedom to pursue this project in my own way and at the same time teaching me a good deal about science. I also appreciate the numerous helpful suggestions made by Dr. Henry Byrne during our many enjoyable conversations.

Thanks to Sue Omar for generous assistance with the electron microscopy, and to Christine Wirta for excellent typing of the manuscript.

The financial assistance provided by the National Research Council of Canada in the form of a Post-graduate Scholarship is gratefully acknowledged.

I would like to express my appreciation to my friends at ELBA who suffered stoically through the genesis of this thesis during the past six months. Finally, a special thanks to Judith for moral support.

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ABBREVIATIONS

ATA	Aurin tricarboxylic acid
BTOA	Benzthiazolyloxyacetic acid
2,4-D	2,4-Dichlorophenoxyacetic acid
DEP	Diethylpyrocarbonate
EDTA	Ethylenediaminetetraacetic acid
ER ; RER	Endoplasmic reticulum ; rough endoplasmic reticulum
5-FU	5-Fluorouracil
GA ₂	Gibberellic acid.
HEPES	N-2-hydroxyethylpiperazine-N'-ethane-sulfonic acid
IAA	Indole acetic acid
IgG	Immunoglobulin G
LSU ; SSU	Large ribosomal subunit ; small ribosomal subunit
ma	Milliampere
mb	Membrane-bound
NADH	Nicotinamide adenine dinucleotide (reduced)
PCA	Perchloric acid
poly A	Poly-adenylic acid
POPOP	1,4-di(2-(5-Phenyloxazolyl))-benzene
PPO	2,5-Diphenyloxazole
SDS	Sodium docecyl sulfate
TCA	Trichloroacetic acid
Tris	Tris-hydroxymethylaminomethane.

INTRODUCTION

The induction of developmental changes in multicellular organisms often involves the activation of quiescent tissues by hormones or environmental stimulæ. The subcellular and molecular events involved in cell activation are consequently of fundamental importance to the understanding of development and have been investigated in a variety of biological systems.

The storage organs of many higher plants (Table 1) are well suited for such studies. Intact roots and tubers consist of essentially homogeneous populations of quiescent cells which can be activated by simply excising thin slices of tissue into a moist environment. Transition to a new biochemical balance occurs rapidly during subsequent incubation of slices in aerated aqueous medium. Such incubation, which is commonly referred to as "aging", allows for considerable experimental manipulation under defined conditions and has generated an extensive body of information in the 73 years since initial work in this field was reported (Nathansohn, 1904).

Table 1 lists plants which have been important in the study of tissue slice metabolism. Of these, white potato has been the most extensively used, primarily because of its wide availability. Jerusalem artichoke, the subject of this thesis, has also received consider-

able attention and possesses a number of characteristics which make it particularly useful for both cytological and biochemical investigations. The artichoke tuber consists primarily of parenchyma tissue with little vascular material, and contains no intrinsic pigmentation such as is found in carrot, beet, yam, etc. Its main storage polysaccharide is inulin, a fructose polymer which does not form massive accumulations akin to the starch grains which severely complicate analyses in potato. Cell expansion growth in Jerusalem artichoke slices has an absolute requirement for exogenous auxin, and the magnitude of the response exceeds that of most other plant storage tissues, allowing a clear separation between the physiological responses of aging and growth.

The synthesis of new structural and enzymic proteins is essential to any significant form of cellular differentiation, and has received widespread attention from biologists during the last few decades. Unfortunately, reliable information on the protein synthesis machinery of plant storage organs is fragmentary at best, and little work is available utilizing recent advances in cell fractionation methodology. Thorough cytological investigations of organelle changes, including nucleoli, ribosomes, and endoplasmic reticulum, have been done for Jerusalem artichoke and some preliminary biochemical work on RNA and protein synthesis has also been published (see Literature Review for details), but more information

is clearly needed if we are to understand the role of ribosome and protein production in the activation of artichoke tuber cells. With this in mind, the specific aims of the present study were:

- a) To measure changes in ribosome content, synthesis and activity during activation and auxin-induced expansion of Jerusalem artichoke tuber cells.
- b) To use cell fractionation techniques to isolate and characterize the free and membrane-bound ribosome populations of both activated and auxin-treated tissue.

Information on ribosome changes during development of Jerusalem artichoke tissue is pertinent not only to the study of excised tissue slices but also to RNA metabolism during activation and growth in intact organs of plants and animals. In fact, since both the nature and sequence of many metabolic responses are remarkably similar in such diverse systems as tuber slices, germinating seeds, hormone activated uterine cells, regenerating rat liver, and antigenically stimulated plasma cells (Hamilton, 1968; Kahl, 1973; Tata, 1971), results obtained from Jerusalem artichoke may well prove relevant to fundamental biological concerns including the formulation of general models of cell activation and growth.

A paper based on material from this thesis has been published (Sparkuhl et al., 1976), a second has been

accepted for publication (Sparkuhl and Setterfield,
1977), and a third is currently in preparation.

TABLE 1.

Plant species important in the study of tissue slice metabolism.

Modified from Kahl (1973).

Plant Species	Storage Organ
Carrot (<u>Daucus carota</u> L.)	Root
Chicory (<u>Cichorium intybus</u> L.)	
Dahlia (<u>Dahlia variabilis</u> Willd.)	
Sugar beet (<u>Beta vulgaris</u> L. var. <u>saccharifera</u> L.)	
Sweet potato (<u>Ipomoea batatas</u> Poir.)	
Yam (<u>Dioscorea batatas</u> Dcsne.)	
Radish (<u>Raphanus sativus</u> L. var. <u>radicula</u> Pers.)	Hypocotyl
Red beet (<u>Beta vulgaris</u> L. var. <u>esculenta</u> Gurke)	
Swede (<u>Brassica napus</u> L. var. <u>napobrassica</u> L.)	Hypocotyl/root
Turnip (<u>Brassica rapa</u> L.)	
Kohlrabi (<u>Brassica oleracea</u> L. var. <u>gongylodes</u> L.)	Aerial stem, tuber
Jerusalem artichoke (<u>Helianthus tuberosus</u> L.)	
White potato (<u>Solanum tuberosum</u> L.)	Underground stem tuber

LITERATURE REVIEW

Sections A, B, and C of this review deal primarily with the literature on metabolism in activated plant storage tissues. Recent surveys by Kahl (1973; 1974) and Van Steveninck (1975) cover many aspects of this field. Van Steveninck's review concerns itself mainly with transport related phenomena and is excellent. The more comprehensive overviews of metabolism by Kahl are thorough in their coverage but tend to be uncritical.

The present survey was concluded in January 1977 and is divided into four parts. Section A summarizes the general metabolic changes which occur during storage tissue activation, in an attempt to put into context the detailed examinations of growth regulators in Section B and of RNA and protein synthesis in Section C. Section D addresses the question of ribosome segregation through a comparative analysis of the structure and function of free and membrane-bound ribosomes in relation to development. Of necessity this last section draws upon work from a variety of plant and animal tissues since little relevant information is currently available from plant storage organs.

A. Metabolic Changes During Activation of Plant Storage Tissue

1) Energy Production

An increase in metabolic activity requires a concomitant increase in available energy. The rate of respiration as measured by O_2 uptake is low in quiescent roots and tubers (typically 30-40 $\mu l O_2/g$ tissue/h) but rises immediately after tissue excision. During subsequent incubation of slices at room temperature, respiration continues to increase to a maximum at 24-48 h and then slowly declines (Kahl, 1974). Adamson (1962) found a four to five-fold enhancement of respiration in Jerusalem artichoke after 20 h of aging. Similar increases have been reported for excised slices of potato (Sperling and Laties, 1963; Kahl *et al.*, 1966), carrot (Ap Rees and Beevers, 1960), yam (Coursey *et al.*, 1966), and sugar beet (Duda and Cherry, 1971). In general the maximum rate is three to five times that of quiescent tissue. This respiratory rise due to excision has variously been called "enhanced respiration", or "wound respiration", although the term "induced respiration" is gaining currency.

Induced respiration can be blocked by inhibitors of RNA and protein synthesis during the first 6 to 10 h of aging of potato (Click and Hackett, 1963; Laties, 1965) or carrot slices (Bryant and Ap Rees, 1971). If such

inhibitors are administered at later times they still interfere with synthesis but do not affect respiration, suggesting that the transcriptional and translational events leading to induced respiration are completed relatively early during aging. Cyanide is an inhibitor which blocks cytochrome a and a₃, reduction in the conventional electron transport chain of animal and plant mitochondria (Ikuma, 1972). The respiration of freshly sliced storage tissue is highly sensitive to cyanide, but during aging the sensitivity of potato slices to this inhibitor decreases progressively until by 24 h, respiration is reduced only 16 to 20% by cyanide (Hackett et al., 1960; Henry and Nyns, 1975). The development of cyanide-insensitive respiration has been reported for carrot, red beet, sweet potato, swede, and Jerusalem artichoke (Henry and Nyns, 1975; Ikuma, 1972). Respiration in isolated mitochondria from dormant potatoes is inhibited by cyanide, while uptake of O₂ by mitochondria from aged slices is not greatly affected (Hackett et al., 1960). The resistant organelles contain both the normal phosphorylative cyanide-sensitive electron transport chain as well as a non-phosphorylative cyanide-insensitive pathway (Ikuma, 1972). The physiological significance of this alternate pathway is not understood, but Ikuma (1972) suggests that it may help to regulate ATP concentration. Early in aging when energy requirements are high, phosphorylative transport is favored, and the

cyanide-insensitive pathway is blocked by high AMP and ADP levels. As ATP increases the non-phosphorylative route becomes operative, allowing a maintenance of high metabolic flux while the phosphorylative pathway continues to operate at a reduced level. Although this explanation is favored by both Ikuma (1972) and Van Steveninck (1975) it remains essentially speculative.

The respiratory quotient of induced respiration in fully activated tissue slices is approximately one (Kahl, 1974), indicating that the principal substrates being oxidized are carbohydrates. Starch represents the major storage polysaccharide in most roots and tubers, and its metabolism has been studied extensively in potato. A decrease in starch grains can be detected 9 to 12 h after excision of slices. Starch breakdown is O_2 dependent and is probably catalyzed by starch phosphorylase, with only a minor contribution by amylotic hydrolysis (Barker, 1965). The immediate products are glucose-1-phosphate and glucose-6-phosphate (Lange et al., 1970), the former probably being converted to sucrose prior to glycolytic breakdown (Barker, 1965).

Inulin (β 2,1 linked fructose) is the main storage polysaccharide in Jerusalem artichoke and chicory. Fructosan hydrolase activity has been detected in both intact and aged artichoke tubers and chicory roots (Flood et al., 1967; 1970). Data suggest that considerable inulin breakdown may occur in intact organs during

storage, with sucrose remaining as the principal storage sugar (Rutherford et al., 1969). Sucrose is an important storage product in other tissues as well and its hydrolysis by invertase is an initial step in respiratory development in carrot (Saunders and Paulson, 1968), sugar beet (Cherry, 1968), Jerusalem artichoke (Rutherford et al., 1969; Edelman and Hall, 1965), and other root and tubers.

The initial breakdown products of carbohydrate metabolism are rapidly degraded via glycolysis, the pentose phosphate pathway, and the Krebs cycle (see Kahl, 1974 for details). The enzymes involved in glycolysis undergo a bewildering variety of activity changes during aging (Kahl et al., 1969b) and it has been suggested that the ATP-transferases such as phosphofructokinase are rate limiting (Kahl, 1973; Black and Wedding, 1968). The Krebs cycle is blocked in intact tubers and becomes activated by slicing without any requirement for de novo synthesis. γ hydroxy- α -ketoglutarate, which is found in intact potatoes and which is an effective inhibitor of a number of Krebs cycle enzymes has been suggested to be the in vivo blocking agent (Laties, 1967).

Although carbohydrates are the main source of metabolites for induced respiration, lipids play an important role very early in the aging of potato slices. Isotope discrimination during biosynthesis results in different $^{13}\text{C}/^{12}\text{C}$ ratios for different classes of carbon

compounds in vivo and Jacobson et al. (1970a) have used this observation to estimate the relative contribution of lipids and carbohydrates to the CO_2 given off by potato cells. Seventy percent of the CO_2 released between 1 and 4 h after slicing was derived from lipids, probably by α -oxidation. Thereafter the contribution of lipids declined until by 20 h, 90% of the CO_2 released was derived from starch. In another paper the effect of cyanide on substrate utilization was studied in tissue which had developed cyanide resistance (20 h aged). Cyanide had little effect on total CO_2 given off but did induce a net shift from starch utilization to lipid degradation (Jacobson et al., 1970b).

2) Uptake and Synthesis of Small Molecules

Much work has been done on the uptake of small molecules into storage cells, but a review here would not be germane. The intention is simply to give some indication of the range of substances which can be picked up from the environment during tissue activation. Such information is important not only because it provides a probe for the investigation of metabolic changes but also because it relates to experimental design and interpretation, especially of labelling studies. Table 2 lists examples of uptake involving metabolic participation by the cell, i.e., examples in which inhibitors of respir-

TABLE 2.

Uptake of solutes into plant tissue slices during aging.

Solute Taken Up	Origin of Tissue Slices	References
Na ⁺ , K ⁺	Carrot Radish Red beet	Ellis, 1964; Ellis <u>et al.</u> , 1964 Younis <u>et al.</u> , 1970 Ellis, 1964; Ellis <u>et al.</u> , 1964
Cl ⁻	Carrot Potato Radish Red beet	Sutcliffe, 1960 Laties <u>et al.</u> , 1964 Younis <u>et al.</u> , 1970 Sutcliffe, 1960
Ca ⁺⁺ , Rb ⁺⁺	Carrot Potato	Chasson & Levitt, 1957 " "
H ₂ O	Chicory Jerusalem artichoke Potato	Flood <u>et al.</u> , 1970 Rutherford <u>et al.</u> , 1969 Sperling and Laties, 1963
Uridine	Jerusalem artichoke	Fraser, 1975
Uracil	Potato	Laties, 1965
Thymine	Potato	Watanabe and Imaseki, 1973
Alanine	Red beet	Ellis, 1964
Aspartate	Radish	Younis <u>et al.</u> , 1970
Glutamate	Carrot	Jacoby and Sutcliffe, 1962
Glycine	Carrot Red beet	Jacoby and Sutcliffe, 1962 Ellis, 1964
Isoleucine	Potato	Zucker, 1965
Leucine	Potato Red beet Sweet potato	Laties, 1965 Ellis, 1964 Sakano and Asahi, 1971
Proline	Carrot	Jacoby and Sutcliffe, 1962
Serine	Red beet	Ellis, 1964

ation, nucleic acid synthesis, or protein synthesis interfere with the development or course of solute uptake.

Although tissue slices are clearly capable of accumulating substances from the environment, the essential constitutional changes during activation require synthesis of a broad spectrum of molecules. Catabolism of storage products provides both the energy and the precursors necessary for such synthesis.

Slicing induces vigorous biosynthesis of phenolics in potato, sweet potato, swede, and Jerusalem artichoke (Bastin, 1968; Kahl, 1974). In fact storage tissues have been used to elucidate some of the steps in phenol production (Kojima et al., 1969; Hanson, 1966). Phenylalanine ammonia-lyase, polyphenol oxidases, and peroxidases are all activated by tissue excision (Bastin, 1969; Rhodes and Woollorton, 1973; Tomiyama et al., 1967; Zucker, 1965). Although they may be of considerable interest to phytochemists, the reactive phenols and quinones which accumulate during aging severely complicate numerous biochemical analyses by binding to and precipitating proteins, and by forming colored products which interfere with some colorimetric assays (Loomis, 1974). These considerations are still receiving insufficient attention from many investigators using plant storage organs.

Amino acids are synthesized by activated storage cells. Exogenously supplied radioactive Krebs cycle intermediates (citrate, fumarate, malate, pyruvate) are incorporated into aspartate and glutamate in potato slices (Laties, 1964). Transaminase enzymes necessary for amino acid interconversions have been reported in the same tissue (Sacher et al., 1972). Folate derivatives are required for the C-1 metabolism integral to a number of synthetic pathways including those for amino acid production. Fedec and Cossins (1976) have demonstrated that folates are synthesized during aging of beet, carrot, potato, and turnip slices.

We have already seen that lipids are used as substrates during early induced respiration. Evidence for the importance of lipid synthesis during activation will be considered in conjunction with membrane changes in Section D. The synthesis of RNA and protein bears directly on the experimental portion of this thesis and will be examined in detail in Section C.

B. Growth Regulators and Storage Tissue

Although many effects of exogenously added growth regulators on storage tissue have been documented, few direct measurements of the endogenous levels of such regulators are available. No gibberellic acid (GA) activity was detected in dormant tubers of potato or

Jerusalem artichoke, but slicing induced accumulation of GA in both (Bradshaw and Edelman, 1969; Rappaport and Sachs, 1967). GA is secreted into the incubation medium by artichoke slices during aging (Bradshaw and Edelman, 1969; Bradshaw et al., 1970). AMO 1618, an inhibitor of GA synthesis, blocks invertase production in sugar beet and Jerusalem artichoke (Cherry, 1968; Bradshaw and Edelman, 1971) and since invertase synthesis is stimulated by GA, AMO 1618 is assumed to be restricting the endogenous supply of hormone.

Abscisic acid has been found in potato tubers (Waring, 1969) and may be implicated in tuber dormancy. Potatoes also contain low levels of cytokinin activity (Van Staden and Dimalla, 1976). Measurements of indoles in kohlrabi were inconclusive as to the level of auxin activity (Kutacek and Kefeli, 1968). It is generally assumed that potato slices contain some endogenous auxin because both cell division and growth occur in the absence of added hormone (Hackett and Thimann, 1950; Lange, 1975). On the other hand growth or cell division in Jerusalem artichoke slices show an almost complete requirement for exogenous auxin (Adamson, 1962; Setterfield, 1963).

Numerous observations have clearly demonstrated that plant hormones have profound effects on various aspects of storage tissue physiology, but such observations have not in themselves proven particularly useful

in illuminating either the nature of hormone action or the controls in cell activation. An alternative approach, which has provided some insight into the activation process, has been the use of growth regulators as a probe in a defined system to affect the course of cell development. Jerusalem artichoke slices incubated in water undergo induced respiration and metabolic activation but show neither expansion growth nor cell division. Adamson (1962) and Setterfield (1963) have shown that auxin can promote cell expansion or cell division in this tissue, and that these two responses can be separated by environmental manipulation. The salient features of this system are as follows:

- a) Auxins (IAA, 2,4-D, BTOA) added to aqueous incubation medium promote cell expansion with little or no cell division (Adamson, 1962; Nooden, 1968; Setterfield, 1963). Expansion amounts to a 50-65% increase in fresh weight over 3-4 days and constitutes true cytoplasmic growth rather than merely uptake of water (Fowke and Setterfield, 1968).
- b) Auxins added to incubation medium containing 30-50 mM Ca^{++} or 300 mM mannitol promote cell division with little or no cell expansion (Adamson, 1962; Setterfield, 1963).

- c) Cell expansion and division appear to be competitive processes, with the latter occurring only under conditions which limit the former. The optimum concentration of auxin to promote division in a condition limiting expansion is the same as the optimum concentration for expansion in non-limiting conditions (Adamson, 1962; Adamson and Adamson, 1968).
- d) Kinetin by itself promotes neither expansion nor division but acts synergistically with auxins to enhance the level of the auxin effect (Setterfield, 1963).
- e) Gibberellic acid fails to promote expansion or division either by itself or in combination with auxin. It has a small synergistic effect when given with auxin plus kinetin, and also appears to depress auxin stimulated division (Setterfield, 1963).
- f) Tissue does not become competent to respond fully to auxin until it has been incubated in aerated water for about 24 h. The addition of auxin or auxin plus kinetin during the first 24 h of aging does not reduce this lag period (Adamson, 1962) although the application of GA may reduce the lag somewhat (Masuda, 1965a).

(g) Development of competence to respond to hormone is dependent upon metabolism during the lag period. If slices are aged at 4°C or in the presence of RNA or protein synthesis inhibitors, the tissue does not develop the capacity to expand in the presence of auxin (Masuda, 1966; Rose and Setterfield, 1971; Setterfield, 1963).

It will become evident in Sections C and D that hormone-induced expansion of Jerusalem artichoke has been very useful in the study of RNA and protein metabolism involved in the transition to growth. It has proven harder to examine division in this system since even under ideal conditions the majority of the cells fail to respond (Adamson, 1962). Yeoman et al. (1965) and Fraser et al. (1967) have claimed that cells of Jerusalem artichoke explants incubated in nutrient medium consisting of mineral salts, sucrose and 2,4-D undergo vigorous and synchronous division, and have used these conditions to investigate the metabolic events related to cell proliferation.

Masuda has attempted to establish a role for GA as a promoter of auxin-mediated cell expansion (Kamisaka and Masuda, 1968; 1970a; Masuda, 1964; 1965b; 1966; 1967). He presents evidence that GA supplied during the initial 24 h aging period reduces the lag time required for full tissue response to auxin.

Although these results have been repeated a number of times the promotive effect claimed for GA is quite variable, and the optimal concentration of the hormone varies some 60 fold between samples (Masuda, 1965a). Masuda has erroneously quoted Setterfield as reporting that GA given simultaneously with auxin synergistically enhances expansion (Masuda, 1967) and has attempted to demonstrate such synergism himself (Masuda 1965b; 1966). The differences between auxin and auxin plus GA treatments were marginally significant if at all, and have in any case not proven reproducible even in subsequent papers by the same laboratory (Kamisaka et al., 1973). It must be concluded that the nature of possible GA growth effects in artichoke tuber remains to be established.

The discovery of the central role played by cyclic AMP in animal hormone systems has stimulated the search for a role for this compound in phytohormone action. There have been six reports implicating cyclic AMP in Jerusalem artichoke tuber development (Kamisaka, 1970; Kamisaka and Masuda, 1970b; Kamisaka et al., 1973; Giannattasio et al., 1974a; 1974b; Giannattasio and Macchia, 1973). Cyclic AMP alone has no effect on cell growth, but is claimed to mimic GA enhancement of auxin action in some respects (Kamisaka, 1970). In view of the uncertainty of the GA effect (above) this suggestion is of questionable value. Despite claims to the contrary,

the effects obtained even with elevated levels of cyclic AMP (10^{-5} to 10^{-4} M) were probably not significant.

Where information was given, experiments consisted of three replicates with no indication of reproducibility and no details as to the time course of the response (Kamisaka et al., 1973). The effects claimed for GA and cyclic AMP appear to be additive even at high concentrations indicating that cyclic AMP could not be acting as a second messenger for the hormone. The authors find an enhancement of cyclic AMP action by theophylline and caffeine, two inhibitors of cyclic AMP phosphodiesterases in animal tissue. It is suggested that the inhibitors are blocking the breakdown of cyclic AMP by endogenous artichoke phosphodiesterases. This is puzzling since all known plant phosphodiesterases (Lin, 1974) including one reported in Jerusalem artichoke (Giannattasio and Macchia, 1973) are insensitive to theophylline and caffeine.

Giannattasio et al. (1973; 1974a; 1974b) have detected cyclic AMP, adenylate cyclase, and cyclic AMP phosphodiesterase activities in homogenates of Jerusalem artichoke tubers. Using a radioimmunoassay, cyclic AMP was detected at unusually high levels¹ in both dormant and activated tissue. This sort of work must be

¹10-60 pmoles/g fresh weight which equals 5-30 pmoles/mg protein. Mammalian tissues contain 0.5-2.5 pmoles/mg protein.

approached cautiously since the existence of cyclic AMP in higher plants is still a matter of some controversy. As Lin (1974) has pointed out in his critical review, the methodological difficulties are such that no single assay method is in itself sufficient proof of the presence of cyclic AMP in plants. Conversion of (^{32}P)-ATP to a presumed cyclic (^{32}P)-AMP form by homogenates of dormant tubers has been cited as evidence for adenylate cyclase in Jerusalem artichoke (Giannattasio and Macchia, 1973). Unfortunately the product of the reaction was insufficiently characterized to permit identification of the 3',5'-cyclic AMP form.

A cyclic nucleotide phosphodiesterase has been partially purified from Jerusalem artichoke tubers (Giannattasio et al., 1974b). The properties of this enzyme are quite different from mammalian cyclic AMP phosphodiesterases but similar to other plant cyclic nucleotide phosphodiesterases (Lin, 1974). The latter hydrolyze 3',5'-cyclic AMP to a mixture of 3'-AMP and 5'-AMP (the product of the mammalian enzymes is exclusively 5'-AMP), are insensitive to methylxanthines, have acidic pH optima, and most importantly, lack substrate specificity. Besides 3',5'-cyclic AMP, 2',3'-cyclic AMP and possibly cyclic forms of GMP, UMP, and CMP can be hydrolyzed by the plant enzymes (Giannattasio et al., 1974b; Lin, 1974). It has been suggested that the function of these enzymes in vivo is the hydrolysis

of 2',3'-nucleoside monophosphate products of RNase digestion rather than any role in cyclic AMP metabolism. From these considerations it is clear that no role has yet been established for cyclic AMP involvement in any aspect of Jerusalem artichoke metabolism.

C. RNA and Protein Synthesis

1) Template Availability and RNA Polymerase

The ability of dormant storage cells of potato and sugar beet to support DNA-dependent RNA synthesis is very low (Tuan and Bonner, 1964; Duda and Cherry, 1971) but increases after tissue excision. Reports of decreases in chromatin-associated basic proteins during aging of Jerusalem artichoke have been interpreted as indicating an increase in template activity (Kamisaka and Masuda, 1968; 1971). Such evidence is very indirect however, and no direct measurements of template availability have as yet been made in this tissue.

The RNA polymerase activity of isolated chromatin from sugar beet increases seven-fold over the first 24 h of aging in water, and is further stimulated by incubation in solutions containing gibberellic acid. In both cases the increase is due partly to enhanced template availability as measured by in vitro RNA synthesis at saturating concentrations of RNA polymerase (from E. coli), but activation of endogenous chromatin-

bound polymerases is also involved (Duda and Cherry, 1971). Based on amanitin sensitivity and DEAE-sephadex fractionation, the majority of the bound enzyme is RNA polymerase I (Cherry, 1975). Polymerase I from soybean is sensitive to auxin regulation (Rizzo and Cherry, 1976) and it is possible that sugar beet polymerase I may also be affected by this hormone. RNA polymerase activity from potato tuber chromatin is inhibited by aldehydes such as chloral, acetaldehyde, and propionaldehyde, and such inhibition has been proposed as a mechanism for repression of RNA synthesis in mature tubers (Graf, 1975). Such suggestions remain speculative and although it is clear that the RNA transcriptional capacity of storage cells increases during aging, we have no real understanding of what controls these changes.

2) Total RNA Synthesis

Slicing of storage tissue results in a rapid initiation of RNA synthesis. Using autoradiography, Setterfield (1963) detected a burst of RNA synthesis in Jerusalem artichoke tissue during the first 24 h of water aging. (^3H) cytidine incorporation was already evident at 2.5 h after excision (the earliest time tested). Pulse/chase experiments showed that label was initially localized over the nucleus and nucleolus, and

subsequently moved into the cytoplasm after a lag of 1.5 to 2 h. Identical experiments with essentially the same results were conducted later by Masuda (1966). More recent studies have detected (^3H) adenosine incorporation into RNA as early as 20 minutes (autoradiography; Byrne and Setterfield, 1977) and 10 minutes (cell fractionation; Byrne, 1977) after tissue excision. Fraser and Loening (1974) found that (^{32}P) orthophosphate was incorporated into RNA by explants of Jerusalem artichoke at an increasing rate over the first 24 h of incubation. Such incorporation was almost entirely abolished by low levels of actinomycin D (Fraser, 1975a). Aged, auxin-treated artichoke slices have also been shown to actively incorporate RNA precursors (Masuda, 1967; Rose and Setterfield, 1971).

Click and Hackett (1963) found that (^{14}C) uracil was incorporated in the RNA of potato cells at a slow but increasing rate during the first 24 h of aging. The low levels of incorporation obtained may in part be attributed to the extremely inefficient counting procedure employed (efficiency about 5%). Subsequent studies confirmed these results and showed that, as in Jerusalem artichoke, actinomycin D blocks incorporation when given early after excision (Laties, 1965; Sampson and Laties, 1968). Incorporation of ^{32}P into RNA in aging sugar beet (Cherry, 1968) and carrot slices (Leaver and Edelman, 1965) has also been reported.

Together the preceding investigations clearly show that cells of excised storage tissue actively synthesize RNA, and in some cases incorporate labelled precursors at an increasing rate during aging. It should be pointed out that rate of label incorporation and rate of RNA synthesis are not synonymous although they are often equated in the earlier literature. As presented, most of the studies cited do not permit the derivation of rate data due to non-quantitative recovery of label and/or failure to report pertinent values for uptake of labelled precursors (Bagni *et al.*, 1971; Leaver and Edelman, 1965; Masuda, 1967; Sampson and Laties, 1968). The latter consideration is particularly critical since uptake of many precursor molecules varies greatly with tissue state and the presence of inhibitors or hormones (Byrne and Setterfield, 1977; Click and Hackett, 1963; Fraser and Loening, 1974; Fraser, 1975a; Nooden, 1968; also see Table 2). Taking into account uptake of (^{32}P) orthophosphate Fraser and Loening (1974) found little net change in the rate of RNA synthesis in Jerusalem artichoke over the first 12 h of aging. Byrne and Setterfield (1977) came to the same conclusion about total RNA based on incorporation, (^3H) adenosine uptake, and ATP precursor pool specific activity.

There is still some controversy as to what happens to net RNA content of tissues during aging. Leaver and Edelman (1965) reported a 50% increase in

RNA per unit carrot tissue during 24 h of aging. However it was also found that the RNA content of dormant cells decreased by 75% during prolonged storage and that subsequent increases in response to excision were inversely correlated with initial content (Bryant and Ap Rees, 1971). In Jerusalem artichoke little change in RNA content occurs during storage of dormant tubers (Bagni et al., 1971; Gare, 1972). Using phenol extraction, a 30% increase in RNA was found after 20 h activation (Bagni et al., 1971). Masuda (1966) also reported an RNA increase during water aging but was not able to reproduce it in a subsequent paper (Kamisaka and Masuda, 1970a). In both cases methodology was inadequately documented.

Fraser and Loening (1974) and Gare (1972) reported a net drop in the RNA content of Jerusalem artichoke cells immediately after excision. Following this drop, RNA content remained stable for 48 h of water aging (Fraser and Loening, 1974; Gare, 1972; Yeoman and Mitchell, 1970). If auxin was included in the aging medium, RNA content doubled over a 40 h period. In Jerusalem artichoke at any rate, net RNA accumulation but not RNA synthesis seems to require auxin stimulation.

With the exception of ribosomal RNA, relatively little information is available on occurrence and changes of specific RNA species during storage tissue activation.

3) Ribosome Synthesis

Ribosomes contain most of the cell's cytoplasmic RNA and in general changes in synthesis and accumulation of ribosomal RNA (rRNA) during aging parallel those of total RNA already discussed. Two species of rRNA (approximately 18S and 26S) have been isolated from Jerusalem artichoke (Bagni et al., 1971; Masuda, 1967), sugar beet (Cherry, 1968; Duda and Cherry, 1971), and carrot (Leaver and Edelman, 1965), by MAK column chromatography; from potato (Click and Hackett, 1966; Kahl, 1971a; Sampson and Laties, 1968), and Jerusalem artichoke (Fraser, 1975a) by sucrose gradient centrifugation; and from carrot (Leaver and Key, 1970) and Jerusalem artichoke (Fraser and Loening, 1974; Rogers et al., 1970) by polyacrylamide gel electrophoresis. Although a number of papers assign S values to the extracted RNAs, these must be accepted as approximations since in no case was a proper determination of sedimentation coefficients carried out.

Incorporation of labelled precursors into rRNA occurs in aged potato (Click and Hackett, 1966; Kahl, 1971a; Sampson and Laties, 1968), carrot (Leaver and Key, 1970), sugar beet (Cherry, 1968) and Jerusalem artichoke (Bagni et al., 1972; Fraser, 1975a; 1975b; Fraser and Loening, 1974; Rogers et al., 1970). Although excision induces rRNA synthesis, dormant tubers already contain large quantities of ribosomes (Bagni et al., 1971; 1972;

Kahl, 1971a; Leaver and Key, 1967; Rose and Setterfield, 1971). Little is known about changes in rRNA levels (as distinct from total RNA) during aging. Bagni et al. (1971) found a slight increase in rRNA (20%) during 20 h water incubation of Jerusalem artichoke. Fraser and Loening (1974) and Gare (1972) using the same tissue, could detect no increase in rRNA content over this period. Auxin induces ribosome accumulation in many plant tissues. This effect has also been noted in storage tissue slices but has not been adequately quantitated (Bagni et al., 1971; Fowke and Setterfield, 1968; Fraser and Loening, 1974; Rose and Setterfield, 1971).

5-Fluorouracil (5-FU) is a pyrimidine analog which interferes with RNA synthesis and processing in plants. rRNA production is preferentially affected (Key, 1966; Fraser, 1975b), and consequently this inhibitor has been widely used to study ribosome metabolism in storage tissues. One must be careful not to infer that 5-FU effects are due solely to inhibition of ribosome synthesis however since other RNA species are also affected to some degree. In Jerusalem artichoke for example, incorporation of ^{32}P into rRNA is 90% inhibited in slices labelled for 1.5 h following 5 h of 5-FU treatment (Fraser, 1975b). Under the same conditions synthesis of polydisperse RNA (mRNA) is inhibited about 50% and nuclear RNA only 20%. This last result suggests

that 5-FU affects not only synthesis but also processing of RNA.

5-FU inhibits net RNA accumulation in carrot (Bryant and Ap Rees, 1971) and Jerusalem artichoke (Fraser, 1975b) presumably by interfering with ribosome metabolism. If the inhibitor is supplied to Jerusalem artichoke during the first 24 h of aging, development of the subsequent expansion response to auxin is abolished. If 5-FU is given after the first day, it has no effect on expansion although it still inhibits ribosome synthesis (Nooden, 1968; Rose and Setterfield, 1971; Setterfield, 1963). These observations have been interpreted to mean that RNA synthesis early in aging is necessary to prepare the tissue to respond to hormone but the increase in ribosome levels due to auxin is not a necessary part of cell expansion.

The processing of large precursor molecules into mature rRNA within the nucleus is well understood in many animals (Perry, 1976) but has been analyzed comprehensively in only a few plants (Cecchini and Miassod, 1976; Miassod and Cecchini, 1976). What little is known of the processing sequence in storage tissues seems consistent with the generally accepted schemes reported for other eukaryotes. Genes coding for rRNA are present in clusters of hundreds or thousands of copies. Jerusalem artichoke has about 250 copies per haploid complement, and interestingly there is no increase

or decrease in this number during cell activation (Ingle and Sinclair, 1972). In lower eukaryotes the initial rRNA precursor transcript has a molecular weight of $2.6-2.8 \times 10^6$, corresponding to a sedimentation coefficient of 36-38S (Perry, 1976). RNAs of 2.8 and 2.2×10^6 molecular weight have been detected in carrot nuclei (Leaver and Key, 1970), and a 2.3×10^6 species has been isolated from the nuclei of Jerusalem artichoke (Rogers et al., 1970). On the basis of labelling kinetics and base composition these molecules were proposed as precursors to the 1.4×10^6 and 0.7×10^6 molecular weight rRNAs. The 0.7×10^6 product accumulated rapidly in the cytoplasm while the 1.4×10^6 molecule was further processed to a 1.3×10^6 from before appearing in cytoplasm with a lag of about 20 min (Leaver and Key, 1970). Ribosomal proteins are known to be added to the large precursor RNAs in the eukaryotic nucleolus but virtually no information is available on this process in storage tissues.

The onset of ribosome production during activation can be correlated with a defined sequence of structural changes in the nucleolus. The nucleoli of dormant tuber cells are compact, consisting of a dense fibrillar zone (ribonucleoprotein) with prominent associated heterochromatic bodies (Fowke and Setterfield, 1968; Jordan and Chapman, 1971; 1973; Rose et al., 1972). Aging results in a pronounced increase in nucleolar

volume (100-200% over 24 h) in Jerusalem artichoke, carrot, and potato (Chapman and Jordan, 1971; Jordan and Chapman, 1973; Rose et al., 1971; Sponholz, 1975). This increase is enhanced by GA (Chapman and Jordan, 1971) and inhibited by actinomycin D (King and Chapman, 1972; Setterfield, 1977). Although the fibrillar zone remains, a distinct granular zone appears during periods of activation and ribosome synthesis (Barckhausen, 1975; Fowke and Setterfield, 1968; Jordan and Chapman, 1973; Rose et al., 1972). The particles in the granular zone are presumed to be pre-ribosomal particles at an advanced stage of processing. 5-FU which interferes with rRNA processing also prevents appearance of the granular zone (Rose et al., 1972). The nucleolar-associated heterochromatic bodies, which may represent the nucleolar organizing region of the DNA, become dispersed throughout the nucleolus during aging (Jordan and Chapman, 1973; Rose et al., 1972). Nucleolar vacuoles which are rare in dormant cells develop several hours after excision. Although these structures were thought to be involved in transport of RNA from the nucleolus, Rose et al. (1972) found no correlation between nucleolar synthetic activity and vacuole formation.

The morphological alterations seen in storage cell nucleoli are probably common to all plant cells during the transition to active ribosome production. Similar changes are observed in wheat embryos during

germination (Rose, 1974) and in comparing the nucleoli of synthetically inactive mature cells and active meristematic cells in pea root (Chaly and Setterfield, 1975).

4). Transfer RNA (tRNA)

An RNA fraction of low molecular weight (often designated sRNA in the earlier literature) has been identified in cells of aging sugar beet (Cherry, 1968), carrot (Leaver and Edelman, 1965; Leaver & Key, 1967), and Jerusalem artichoke (Masuda, 1967) by methylated albumin kieselguhr (MAK) chromatography; in potato (Click and Hackett, 1966; Kahl, 1971a), and Jerusalem artichoke (Fraser, 1975a) by sucrose gradient fractionation; in sugar beet (Stone et al., 1970) by Sephadex chromatography; and in Jerusalem artichoke by electrophoresis (Fraser and Loening, 1974). These low molecular weight fractions presumably contained tRNA, but in none of the above analyses was this directly demonstrated. MAK columns as used here did not adequately resolve tRNA from 5S ribosomal RNA components and in only one case (Rogers et al., 1970) was data available which permitted an estimation of sedimentation values. Leucine-tRNA acceptor activity was found in one sRNA fraction (Stone et al., 1970).

The low molecular weight RNAs of Jerusalem artichoke (Fraser, 1975a), potato Kahl, 1971a), and sugar beet (Cherry, 1968) become labelled with RNA precursors during aging. Fraser (1975a) isolated a putative tRNA fraction from Jerusalem artichoke which became labelled as early as 0.5-2.5 h after excision and which was less sensitive to actinomycin D than ribosomal RNA. The level of this "tRNA" remained essentially constant over the first 35 h of aging. Using MAK columns and gel electrophoresis King and Chapman (1973) were able to show preferential methylation of tRNA synthesized during the first 18 h of aging of Jerusalem artichoke. The methylation, which was not coordinated with synthesis during early activation, was suggested to be an important event in tRNA activation and the re-establishment of protein synthesis. Similar suggestions have been made for other systems (Tidwell, 1970) and Stone et al. (1970) found a positive correlation between methylation and an increase in leucine-tRNA acceptor activity in sugar beet slices.

5) Messenger RNA and Polysome Formation

Because it is found at low levels and as a heterogenous size class, messenger RNA (mRNA) can not be readily isolated as a discrete fraction by sedimentation or electrophoresis. Direct evidence for synthesis and metabolism of mRNA has consequently remained limited.

Kahl (1971a; 1971b) reported a non-ribosomal 15-18S RNA component from aged potato slices which stimulated incorporation of (^{14}C) leucine by potato ribosomes in vitro. The stimulatory effect of total RNA in the in vitro system was not documented so it is not clear to what degree the 15-18S fraction was enriched in messenger activity. Click and Hackett (1967) attempted to determine the functional stability of mRNA in potato by measuring the time required for actinomycin C to stop amino acid incorporation in vivo. An average half life of 1.6 h was found for message of both fresh and aged slices, but such values are tenuous since this method requires numerous assumptions and is open to alternate interpretations.

The discovery that many eukaryotic mRNAs contain 3' polyadenylic acid (poly-A) sequences allowed the development of a specific method for isolating mRNA by hybridizing the associated poly-A tracts to immobilized homopolymers such as oligo (dT)-cellulose (Aviv, 1972; Brawerman, 1974). This method has recently been employed to isolate mRNA from Jerusalem artichoke tissue (Byrne and Setterfield, 1977; Fraser, 1975a; 1975b). Poly-A containing RNA was found to be preferentially synthesized during the first few hours after excision and continued to be produced over the 12 h period examined. The poly-A containing fraction was more resistant to low levels of actinomycin D or 5-fluorouracil than was rRNA. A rapidly

labelled polydisperse non-poly-A containing RNA was also found and may represent another class of mRNA (Byrne and Setterfield, 1977; Fraser, 1975a). Neither putative mRNA has yet been examined for its ability to support amino acid incorporation in vitro.

The formation of polysomes provides good if indirect evidence for an increase in available mRNA. Dormant storage tissue contains significant numbers of ribosomes, but the vast majority of these are monosomes (Fowke, 1968; Fowke and Setterfield, 1968; Rose and Setterfield, 1971). Aging results in a rapid increase in the percentage of ribosomes in polysomal aggregates as determined from sucrose gradient profiles. Carrot increases from 10% polysomes in dormant tissue to 65% polysomes by 6 h aging (Leaver and Key, 1967), potato increases from 15% to 45% of ribosomes in polysomes over 12 h aging (Kahl, 1971a), and sugar beet increases from 35% to 65% in 4 h (Duda and Cherry, 1971; Cherry, 1968). Formation of polysomes in Jerusalem artichoke has been detected cytologically (Fowke and Setterfield, 1968; Rose and Setterfield, 1971) and by fractionation (Gare, 1972) although Bagni et al. (1972) failed to obtain meaningful polysomal profiles from this tissue.

The ability of cell-free ribosome preparations to support incorporation of amino acids into proteins in vitro changes dramatically during aging. Ribosomes extracted from dormant tissue of carrot (Leaver and Key,

1967), potato (Kahl, 1971b), red beet (Ellis and MacDonald, 1967), or Jerusalem artichoke (Chapman and Edelman, 1967) support protein synthesis only poorly whereas ribosomes from optimally aged tissues are much more active (about a ten-fold increase/unit RNA). The low activity of ribosomes from dormant tissue does not appear to be due to the ribosome particles themselves being defective since both "aged" and "unaged" ribosomes are equally efficient at incorporating (^{14}C) phenylalanine when supplied with poly-uridylic acid as message (Ellis and MacDonald, 1968; Kahl, 1971b; Leaver and Key, 1967). From these results Kahl (1973) has concluded that protein synthesis in dormant storage organs is restricted by lack of available mRNA. This is a possible interpretation, and one which is supported by previous observations that mRNA is synthesized preferentially early in activation. However it must be remembered that poly-uridylic acid translation is not subject to the same constraints as is the translation of native message (see Haselkorn, 1973) and consequently it remains possible that mRNA is present in dormant cells but that some limiting initiation or translocation factors are required for polysome formation. No systematic attempt has been made to look for stored or sequestered mRNAs in dormant storage tubers but Fraser (1975a) presents some preliminary evidence that a poly-A containing RNA fraction exists in unaged Jerusalem artichoke slices.

6) Protein Synthesis

As would be expected from polysome analysis, the synthesis of proteins increases greatly during aging of storage tissue. Increasing (^3H) leucine incorporation has been detected autoradiographically during water incubation and during auxin treatment of Jerusalem artichoke tissue (Adams, 1967; Rose and Setterfield, 1971). Labelled amino acids are incorporated into protein by carrot (Jacoby and Sutcliffe, 1962), and potato slices (Click and Hackett, 1963; Laties, 1965; Sampson and Laties, 1968; Willemot and Stumpf, 1967a). (^{14}C) leucine rapidly becomes associated with polysomes from 15 h aged potato slices but not with ribosomes from fresh slices (Kahl, 1971a). Amino acid incorporation is blocked by protein synthesis inhibitors such as puromycin (Click and Hackett, 1963; Laties, 1965), cycloheximide (Willemot and Stumpf, 1967a), and in some reports even by chloramphenicol (Jacoby and Sutcliffe, 1962); by non-specific inhibitors such as chloral and ethionine (Laties, 1965; Zucker, 1963); and by the RNA synthesis inhibitor actinomycin D (Click and Hackett, 1963; Sampson and Laties, 1968; Willemot and Stumpf, 1967a). In general inhibitors of protein synthesis are effective throughout the water aging period but have their maximum effect only when applied prior to the development of active amino acid incorporation.

Actinomycin D blocks protein synthesis only when given during the initial 12 h after excision of potato slices, after which the RNA necessary for continued protein synthesis presumably remains stable.

As shown in Table 3, net protein content of tissues, as measured by Kjeldahl or Lowry assays, increases during aging. Although all tissues synthesize protein, accumulation is quite variable and is probably related to differing physiological requirements and rates of turnover. Protein accumulation is generally greater in the presence of auxin.

The development of protein synthesis during aging seems to be required for many metabolic processes. The appearance of selective ion transport in excised tissue requires protein production and Van Steveninck (1975) has suggested that the synthesis of specific carrier molecules or ion pumps is involved. Induced respiration can be blocked by protein synthesis inhibitors (Click and Hackett, 1963; Laties, 1965; Bryant and Ap Rees, 1971). RNA synthesis is sensitive to puromycin directly after excision indicating some requirement for new proteins such as perhaps polymerases or ribosomal proteins. The auxin-induced growth response of Jerusalem artichoke tuber cells can be blocked by puromycin, cycloheximide, or chloramphenicol. These inhibitors will stop expansion when applied simultaneously with, or 24 h after the auxin, indicating that protein syn-

TABLE 3.

Changes in protein content of plant tissue slices during aging.

Origin of Tissue Slices	Aging Medium	Increase in Protein per Unit Tissue	References
Beet	Water	130% in 3 days	MacDonald <u>et al.</u> , 1966
Carrot	Water	41% in 5 days	Jacoby & Sutcliffe, 1962
Chicory	Water	45% in 3 days	Flood <u>et al.</u> , 1970
	Water + auxin	103% in 3 days	" " "
Jerusalem artichoke	Water	33% in 3 days	Masuda, 1966
	Water + auxin	266% in 3 days	" "
	Salt medium	45% in 3 days	Yeoman & Mitchell, 1970
	Salt medium + auxin	255% in 3 days	" "
Potato	Water	0% in 2 days	Zucker, 1963
	Water	24% in 1 day	Tomiyama <u>et al.</u> , 1967
	Water	65% in 4 days	Thimann & Loos, 1957

thesis is required throughout the early expansion period (Nooden and Thimann, 1965; 1966; Nooden, 1968).

During a process as complex as metabolic activation many specific proteins must certainly be synthesized. Most attempts to identify new proteins during activation have focused on enzymes since they are more readily identifiable than are structural proteins. Several examples of increases in enzyme activity have already been cited in Section A, but in most studies it was not determined whether enhanced activity was due to activation of existing enzymes or to de novo synthesis.

- a) RNase - RNase activity in potato slices increases sharply during aging. Cycloheximide entirely abolishes this increase while actinomycin D has only a minor effect (Pitt and Galpin, 1971). This suggests that protein but not RNA synthesis is required for development of RNase activity. However attempts to demonstrate de novo RNase synthesis directly, by D_2O labelling or by quantitation with specific antibody radial diffusion, showed little or no synthesis of new enzyme (Pitt and Galpin, 1971; Pitt, 1971). This situation clearly shows the dangers of relying solely on data from inhibitors to infer de novo synthesis of a protein.

- b) Invertase - Increases in storage tissue invertase activity have already been mentioned in relation to carbohydrate metabolism. Such increases are stimulated by GA and are entirely abolished if actinomycin D is present immediately after excision (Cherry, 1968; King and Chapman, 1972). Delay in actinomycin D application by 4 h in Jerusalem artichoke or by 8 h in sugar beet results in much less efficient inhibition and suggests that "early" RNA synthesis (mRNA?) is required for the development of enzyme activity. Cycloheximide completely prevents invertase appearance in sugar beet over a 48 h period (Cherry, 1968). Chloramphenicol or thiouracil are 60-70% effective in blocking invertase in Jerusalem artichoke (Edelman and Hall, 1965).
- c) PAL - Phenylalanine ammonia-lyase activity increases during aging of potato slices (Zucker, 1965). Cycloheximide strongly inhibits this increase and also appears to interfere with the normal turnover of the enzyme. Sacher et al. (1972) have partially purified PAL and shown that no size change occurs during aging. Using D₂O labelling a net shift of PAL activity to a higher density was observed indicating that de novo synthesis

was probably occurring correlated with the increase in activity.

- d) Peroxidase - Slicing enhances peroxidase activity in Jerusalem artichoke and potato. Bastin (1968) has partially purified the enzyme and shown that cycloheximide but not chloramphenicol inhibits an increase in activity during the first 24 h of aging (Bastin and Unluer, 1972a). Actinomycin D inhibits peroxidase activity only when given during the first 6 h after excision; a situation similar to that found for invertase (Bastin and Unluer, 1972b). Decline in peroxidase activity in the absence of protein synthesis gives a half life of 8.3 h for the enzyme (Bastin and Unluer, 1972a).

It is clear even from this limited survey that at least some enzymes are synthesized in response to the excision stimulus.

D. Membrane-Bound and Free Ribosomes

1) Cell Membranes and Organelles

The role of membranes as active participants in cellular development is gradually being recognized. The extensive changes in some membranes during differentiation, the functional flexibility inherent in their

structure (Singer, 1974), the frequent membrane continuities between cells and organelles (Crotty and Ledbetter, 1973; Robards, 1975), and the established roles of golgi and endoplasmic reticulum in protein processing and export (Palade, 1975), all serve to illustrate the fundamental role which membranes play in development.

Disruption of plant storage tissue leads to rapid and extensive enzymatic degradation of membrane lipids. Homogenates of freshly sliced potato contain lipolytic hydrolases which are active even at 0°C (Galliard, 1970). An enzyme preparation which catalyzes deacylation of mono and di-acyl phospholipids, galactosyl diglycerides, as well as mono and diglycerides has been partially purified from the particle-free supernate of potatoes (Galliard, 1971a). These activities result in the release of free fatty acids and are particularly disruptive to membrane structure. The free fatty acids stimulate acyl hydrolase and are themselves metabolized by α -oxidation (Galliard, 1971b).

As a consequence of this rapid attack, most cytological preparations of "unaged" tissue probably show evidence of membrane degradation induced by cutting of tissue prior to fixation. Fowke and Setterfield (1968) found little endoplasmic reticulum (ER) in unaged Jerusalem artichoke slices, but more rapid fixation revealed a smooth membrane fraction which disappeared

rapidly following excision (Setterfield, 1977). Jackman and Van Steveninck (1967) reported that the lamellar ER of beetroot disappeared within 2 h of aging, with only small cytoplasmic vesicles remaining. Presumably membranes other than ER would also be affected by lipolytic activity but no cytological evidence for damage to other organelles has been reported (Fowke and Setterfield, 1968; Jackman and Van Steveninck, 1967; Rose and Setterfield, 1971). Another indication of membrane disruption following excision is the loss of microsome-associated enzyme activity. Slicing of beetroot, swede, or turnip resulted in a 20-100% loss of microsomal NADH dehydrogenase activities and a 20% decrease in microsomal protein within 10 minutes (Rungle and Wiskich, 1972). The significance of these extensive membrane disruptions early in aging is not yet clear.

A stimulation and change in pattern of lipid synthesis occurs during activation of plant storage cells. Linoleic, oleic, and palmitic acids are rapidly labelled from (^{14}C) acetate, while the distribution of label between different fatty acids changes as aging progresses (Willemot and Stumpf, 1967b). These changes are partially prevented by RNA and protein synthesis inhibitors (Willemot and Stumpf, 1967a). Labelled fatty acids are incorporated mainly into phospholipids (Tang and Castelfranco, 1968) while the level of total and mitochondrial phospholipid increases during aging of

potato (Ben Abdelkader et al., 1968; Ben Abdelkader, 1969). The appearance of lipid label in various membrane types has been followed by cell fractionation. Castelfranco et al. (1971) found that (^{14}C) choline was incorporated primarily into microsomes early in aging of potato, but by 24 h significant amounts of label also appeared in mitochondria. Ben Abdelkader and Mazliak (1970) also noted such a shift and reported that potato microsomes containing (^{14}C) phospholipids could transfer label to mitochondria directly when the two were incubated in vitro with potato supernatant fraction. These results show that new microsome and mitochondrial membrane components are synthesized during aging and that a transfer of material may occur between the two. It should be noted that the fractionation procedures used in the foregoing experiments were not sufficiently sensitive to resolve more than the two major membrane fractions. A shift in label from microsomes to mitochondria has also been found in studies with other plant tissues (Kagawa et al., 1973). The ER was shown to be the site of lecithin synthesis in castor bean endosperm (Lord et al., 1973) while the primary site of (^{14}C) choline incorporation in onion stem was the dictyosomes (which would not be resolved from microsomes in most fractionation protocols) (Morré, 1970).

The biogenesis and turnover of specific organelles has not been examined closely in plant storage

tissues (Van Steveninck, 1975). The number of mitochondria and dictyosomes increases during aging of potato (Barckhausen, 1975). In a cytological study of the organelles of Jerusalem artichoke, Fowke (1968) found no increase in mitochondria, dictyosomes or plastids during water aging although the numbers of all of these structures increased in the presence of auxin. ER, which was rare directly after excision, increased rapidly during 12-24 h aging of Jerusalem artichoke and beetroot (Fowke, 1968; Jackman and Van Steveninck, 1967; Rose and Setterfield, 1971).

2) Occurrence of Membrane-Bound Ribosomes

Ribosomes in many cells are closely associated with the ER and with other membranes. In the 20 years since this association was first reported in pancreas (Palade and Siekevitz, 1956) a vast literature has evolved to describe many aspects of the binding of ribosomes to membranes. The diverse nature and large volume of these investigations makes a comprehensive review unfeasible. Of principal interest here is the function of membrane-bound (mb) ribosomes and their relation to cell activation. There is no biochemical information on mb ribosomes in plant storage tissues, so to establish a basis for discussion, data from a variety of organisms are presented to illustrate the

generalizations which can be made and the questions which yet remain about ribosome/membrane association.

The majority of mb ribosomes are associated with the endoplasmic reticulum. Occasionally ribosomes are also found on the surface of the outer nuclear envelope especially when this envelope is continuous with the ER. Recently there have been a number of reports of 80S ribosomes binding to the outer membrane of yeast mitochondria (Kellems et al., 1974; Kellems and Butow, 1974) and 70S ribosomes binding to the thylakoids of chloroplasts (Margulies and Michaels, 1974, 1975; Tao and Jagendorf, 1973) and to mitochondrial membranes (Kuriyama and Luck, 1973). Cases of ribosomes associating with membranes other than ER are still rare and unless otherwise specified "mb ribosomes" refers to particles in the rough endoplasmic reticulum (RER).

Table 4 lists some of the organisms and tissues in which mb ribosomes have been studied. RER is found in mammals, birds, higher and lower plants, and it is probable that all eukaryotes contain RER during some phase of their development. Table 4a indicates a number of tissues in which mb ribosomes form the bulk of the total ribosome population. Such tissues are primarily specialized for the production and export of large quantities of protein. In the case of broad bean, storage products are not secreted but rather accumulated within the cotyledon. The tissues in 4b are very low in

Occurrence of membrane-bound ribosomes in plant and animal tissues.

Percentage values indicate the proportion of total ribosomes recovered with membranes after cell fractionation. In cases where no numerical values were available, approximate proportions were estimated from cytological or fractionation data.

+++ : Majority of ribosomes mb

++ : Half of ribosomes mb

++ : One quarter of ribosomes mb

+ : Few ribosomes mb

a) Tissues high in mb ribosomes.

b) Tissues low in mb ribosomes.

c) Tissues intermediate in mb ribosomes.

Tissue	Fraction of Ribosomes Membrane-Bound	References
a) Antithamnion secretory cells	+++	Feldmann & Guglielmi, 1976
Bat pancreatic acinar cells	++++	Fawcett, 1966
Broad bean cotyledon (during food reserve accumulation)	50%	Payne & Boulter, 1969
Chick liver	66%	O'Toole & Pollak, 1974
Guinea pig plasma cells	++++	Fawcett, 1966
Guinea pig pancreatic acinar cells	++++	Fawcett, 1966; Paladé, 1975
Hamster liver	++++	Fawcett, 1966
Mouse mammary epithelium	+++	Slaby & Brown, 1974
Rat liver	80%	Tata, 1971; Andrews & Tata, 1971

Tissue	Fraction of Ribosomes Membrane-Bound	References
b) Broad bean cotyledon (during seed dehydration)	7%	Payne & Boulter, 1969
Human peripheral lymphocytes	8%	Cooper et al., 1976
Jerusalem artichoke parenchyma	+	Rose & Setterfield, 1971 Fowke & Setterfield, 1968
Mouse fibroblasts (stationary phase)	+	Noll & Burger, 1974
Pea root meristem	+	Chaly & Setterfield, 1975
Rat skeletal muscle	7-10%	Tata, 1971; Andrews & Tata, 1971
Embryonic trophoblasts of rat, rabbit and guinea pig	+	Enders & Schlafke, 1967; 1969

Tissue	Fraction of Ribosomes Membrane-Bound	References
c) Beet root parenchyma	++	Jackman & Van Steveninck, 1967
Broad bean cotyledon (during cell division)	15%	Payne & Boulter, 1969
HeLa cells	15%	Rosbash & Penman, 1971
Jerusalem artichoke parenchyma	++	Fowke & Setterfield, 1968 Rose & Setterfield, 1971
Mouse myeloma cells (exponential growth)	18-20%	Mechler & Vassalli, 1975a
Mouse spleen	25%	Storb & Martin, 1972
Pea root elongation zone	++	Chaly & Setterfield, 1975
Rat cerebral cortex	15-18%	Tata, 1971; Andrews & Tata, 1971
Rat thymocytes	20%	Wettenhall & Slobbe, 1976
Rat uterus	15%	Khawaja, 1976
Sycamore callus culture	43%	Cella et al., 1976
Tetrahymena cells	++	Ronai & Wunderlich, 1975

RER. Some, such as embryos and meristems, are extremely active and probably require newly synthesized proteins for immediate internal use. Others, such as dormant tubers, exhibit low metabolism with little protein synthesis. Table 4c lists a variety of cases in which an intermediate sized mb ribosome population exists (15-25% mb). Examples here include growing, non-growing, and dividing tissues which have no single specialized function such as secretion in common. This intermediate category probably includes the majority of eukaryotic cell types.

Transition to a new physiological state often involves changes in the mb ribosome population in cells.

- a) During activation of Jerusalem artichoke tuber slices proliferation of ER is coordinated with ribosome synthesis to form RER (Rose and Setterfield, 1971). Similar coordination is seen in oestrogen stimulated rat uterus, liver following hepatectomy or testosterone stimulation, and rat mammary gland cultures following addition of corticosteroids (Tata, 1971).
- b) Maturation of broad bean cotyledons involves successive phases of cell division, storage product accumulation, and seed dehydration. The proportion of mb ribosomes rises from 15 to 50% during transition from division to storage accumulation and then falls to 7% during dehydration (Payne and Boulter, 1969). Similar

RER proliferation occurs during the early differentiation of aleurone tissue in wheat (Mares et al., 1976).

- c) Dividing cells at the meristem of pea root tip have only rudimentary RER. As cells elongate and differentiate, further down the root, RER becomes much more developed (Chaly and Setterfield, 1975).
- d) Exponentially growing 3T3 mouse fibroblasts transformed with polyoma virus maintain the same proportion of mb ribosomes as untransformed cells. During transition to the stationary state however, the normal cells lose most of the mb population while transformed cells maintain theirs (Noll and Burger, 1974).

Despite much work, the functions of the RER complex have only been partially resolved. Both free and mb ribosomes can be found in polysome form actively synthesizing proteins, but this does not explain why cells should need to maintain two distinct populations. That the ratio of free to mb ribosomes changes with the physiological state of the cell suggests that the functions of the two are not identical but gives no indication of what the differences might be. The attack on this problem has involved analyses of the proteins produced by different ribosome populations and an evaluation of

the structure of the membrane/ribosome complex. One view on these subjects is given in a recent review by Rolleston (1974).

3) The Protein Products of Free and Membrane-Bound Ribosomes

There is now considerable evidence that the types of proteins made on mb polysomes are different from those made on free polysomes. All of the major systems which have been examined for such differences are listed in Table 5. The methods used to localize the synthesis of specific proteins involve:

- a) Isolation of functional free and mb polysomes.
- b) Immunoprecipitation by specific antibodies of nascent polypeptides made in vivo.
- c) Alternatively, proteins are synthesized in vitro by completion of pre-existing nascent chains or translation of isolated polysomal mRNA. Products are then identified by electrophoresis, immunoprecipitation, or bioassay.

Specific experimental protocols vary and there has been criticism of the procedures used to obtain a few of the results listed in Table 5 (Eschenfeldt and Patterson, 1975; Rolleston, 1974; Shafritz, 1974a; 1974b). The bulk of the evidence nevertheless indicates that the distinctions observed are not artifactual. Most of the tissues

TABLE 5.

Synthesis of specific proteins on free and membrane-bound polysomes.

The relative contributions of free and mb polysomes to the synthesis of specific proteins were assayed and expressed in a variety of ways in the references cited. Activities in Table 5 are divided into four classes such that only relatively major differences are scored as significant.

+++ : Highest specific activity (cpm in specific protein/unit polysomal RNA) i.e. the polysome fraction which is most active in the synthesis of the protein under consideration.

++ : 25-50% of the specific activity of +++.

+ : 10-25% of the specific activity of +++.

- : Less than 10% of the specific activity of +++.

nt : Not tested.

Secretory Proteins	Type of Polysome ME	Tissue	Reference
Albumin ¹	+++	Rat liver	Tanaka & Ogata, 1971
"	+++	"	Takagi <u>et al.</u> , 1970
"	+++	"	Ikehara & Pitot, 1973
Amylase	+++	Pigeon pancreas	Redman <u>et al.</u> , 1966
Casein	+++	Ewe mammary epithelium	Houdebine & Gaye, 1975
Cellulase	+++	Pea epicotyl	Verma <u>et al.</u> , 1975
Collagen	+++	Chick embryo	Diegelmann <u>et al.</u> , 1973
"	+++	Fetal rabbit lung	Collins & Crystal, 1975
Fibroin	+++	<u>Bombix mori</u> silk glands	Daillet <u>et al.</u> , 1971
Immunoglobulin	+++	Mouse myeloma cells	Blobel & Dobberstein, 1975a
"	+++	Mouse plasmacytoma cells	Pryme, 1973; Pryme <u>et al.</u> , 1974
β lactoglobulin	+++	Ewe mammary epithelium	Gaye & Denamur, 1970
Proinsulin	+++	Rat pancreas	Permutt & Kipnis, 1972
Thyroglobulin	+++	Sheep thyroid	Bouchilloux <u>et al.</u> , 1974

¹For a more complete list of references to albumin and ferritin localization studies see Rolleston (1974).

Membrane Proteins	Type of Polysome MB	Free	Tissue	Reference
Glycoprotein	+++	-	Mouse Kidney	Priestly et al., 1969
NADPH cytochrome c reductase	+++	+++	Rat liver	Ragnotti et al., 1969
5' nucleotidase	+++	-	Mouse liver	Dergeron et al., 1975
Protein fraction (membrane derived)	+++	++	Rat liver	Elder & Morré, 1976
"	+	+++	Rat liver	Lowe & Hallinan, 1973
"	n.t.	+++	Rabbit reticulocytes	Lodish, 1973
Serine dehydratase	+++	++	Rat liver	Ikehara & Pitot, 1973

Internal Proteins	Type of Polysome MB	Free	Tissue	Reference
Arginase	+	+++	Rat liver	Tanaka & Ogata
Catalase	+++	+++	Rat liver	Takagi <u>et al.</u> , 1970
Ferritin	+	+++	Rat liver	Redman & Sabatini, 1966
Globin	++	+++	Rabbit reticulocytes	Brennessel & Goldstein, 1975
"	+++	+++	Mouse reticulocytes	Morrison & Lingrel, 1975
Histones ²	-	+++	Mouse reticulocytes	Zauderer <u>et al.</u> , 1973
Myelin encephal- itogenic protein	+++	++	Rat brain	Lim <u>et al.</u> , 1974
Myosin	+	+++	Rat skeletal muscle	Nihei, 1971
Ribosomal proteins ²	+	+++	Regenerating rat liver	Nabeshima, 1975
S-100 protein	++	+++	Rat brain	Amaldi <u>et al.</u> , 1973
Tubulin	+++	+++	Rat forebrain	Floor <u>et al.</u> , 1976

²In both histone and ribosome protein synthesis a "loosely bound" ribosome fraction is described which is KCl labile and synthesizes the specific protein at a higher specific activity than the normal mb ribosomes but at a lower specific activity than free ribosomes.

Miscellaneous Proteins	Type of Polysome MB	Free	Tissue	Reference
Cytochrome c (mitochondrial)	+++	+++	Rat liver	Gonzales-Cadavid & de Cordova, 1974
Protein fraction (mitochondrial)	+++	++	Rat liver	Shore & Tata, 1977
G protein (vesicular stomatitis virus VSV)	+++	-	VSV infected L cells	Toneguzzo & Ghosh, 1975
N, NS, and M proteins (vesicular stomatitis virus)	+++	+++	VSV Infected L cells	Toneguzzo & Ghosh, 1975
Rauscher leukemia virus (RSV) specific protein	+++	+	RSV infected mouse marrow cells	Gielkens et al., 1974

studied to date have been secretory because they tend to produce only a limited number of major proteins and are consequently easier to analyze.

The data show that proteins destined for export are made preferentially if not exclusively on mb polysomes. Membrane and mitochondrial proteins display no general segregation pattern with specific products being made on free, mb, or both polysome types. Most of the proteins intended for internal use are synthesized preferentially on free polysomes although the mb do contribute significantly to catalase, globin and MEP production. Certainly it is clear that varying degrees of product segregation exist between mb and free polysomes in secretory tissues. In the absence of sufficient examples we do not yet know whether an equivalent situation obtains in the large class of non-secretory cells containing an intermediate amount of RER (Table 4c).

4) Structure of the Membrane/Ribosome Complex

A number of attempts have been made to demonstrate structural differences between free and mb ribosomes. One-dimensional SDS electrophoresis of ribosomal proteins from rabbit reticulocytes indicated some differences between the two classes of ribosomes (Burka and Bulova, 1971), but samples were not adequately freed of cytoplasmic contaminants. A more thorough re-evaluation

of this system by two-dimensional electrophoresis showed that two proteins normally found in free ribosomes were absent from mb ribosomes and the latter contained three extra unidentified proteins (Fehlmann et al., 1975a; 1975b; 1975c). These are minor differences in an organelle containing of at least 75 distinct proteins. One-dimensional electrophoresis of chick embryo and rat liver cells revealed no qualitative differences between free and mb ribosomes (Borgese et al., 1973; Frilander and Wettstein, 1970), and two-dimensional electrophoresis of the latter gave the same results (Hanna et al., 1973; Hanna and Godin, 1974). "Fingerprint" patterns of 18S and 28S RNA digests (RNase treated) produced identical patterns for free and mb rRNA in HeLa cells (Khan and Maden, 1976). The kinetics of rRNA turnover are identical for free and mb ribosomes of rat liver and kidney cells (Izawa and Ichii, 1973; Mishra et al., 1972), a result consistent with free exchange between the two populations and mitigating against any structural ribosomal heterogeneity. These results taken as a whole do not support the contention that significant structural distinctions exist between free and mb ribosomes in the same cell.

The orientation of the ribosome relative to the ER membrane was established in a classic experiment by Sabatini et al. (1966). When low concentrations of EDTA were used to dissociate mb ribosomes only the 40S

small subunits (SSU) were released from the membrane. Large 60S subunits (LSU) and nascent polypeptides remained bound. The LSU could only be released by high EDTA or detergent treatment. Combined with electron microscopic evidence that the subunit groove ran parallel to the membrane, this finding indicated that the ribosomes were bound to the ER through the LSU (Fig. 1a), and suggested that nascent polypeptides might help to anchor the ribosome to the membrane. These conclusions have been confirmed by subsequent experiments. Baglioni et al. (1971) found that newly synthesized LSUs of mouse myeloma cells bound directly to ER in vivo. Such binding occurred even in the presence of protein synthesis inhibitors which prevented SSU association with the membrane. In vitro experiments showed that SSUs but not LSUs exchanged freely with corresponding free cytoplasmic particles (Borgese et al., 1973).

About 85% of ribosomes can be stripped from the RER of rat liver by a medium containing 0.75 M KCl and 1 mM puromycin. KCl alone removes up to 40% of mb ribosomes but puromycin alone has little effect (Adelman et al., 1973). This suggests that both ionic forces (KCl labile) and nascent polypeptide anchoring (puromycin labile) are involved in membrane/ribosome interaction. The ribosomes released by KCl alone are either inactive or bear relatively short nascent chains. Similar results have been reported for the RER of mouse myeloma (Mechler

and Vassalli, 1975c). Rough microsomes stripped of ribosomes by RNase, LiCl, or puromycin + KCl are capable of rebinding exogenous 60S subunits (Borgese et al., 1974; Rolleston, 1972; Shires et al., 1975), 80S ribosomes (Borgese et al., 1974) and polysomes (Rolleston and Mak, 1973; Shires et al., 1973; 1974a; 1975) in vitro. Such reconstituted microsomes have been used to investigate ribosome binding with the following results.

- a) Stripped microsomes bind exogenous polysomes or ribosomes much better than do native rough microsomes (Borgese et al., 1974; Shires et al., 1975). This is interpreted to mean that the membranes contain ribosome binding sites which are mostly occupied by endogenous ribosomes in native microsomes but become available when such ribosomes are removed. Shires et al. (1975) have investigated the stoichiometry of these binding sites and have demonstrated competition between labelled and unlabelled exogenous polysomes for the sites.
- b) Ribosome binding sites are selective to the extent that they bind LSUs more efficiently than SSUs (Rolleston, 1972; Borgese et al., 1974; Davis and Morris, 1976). This is consistent with the ribosome orientation shown in Fig. 1. The binding sites do not however distinguish between different classes of ribo-

somes and will bind polysomes derived from mb or free fractions or even ribosomes from other tissues with equal efficiency (Rolleston and Mak, 1973; Shires et al., 1973; 1974a).

- c) Phospholipase C treatment of microsomes destroys phospholipids but does not affect in vitro ribosome binding capacity (Borgese et al., 1974; Jothy et al., 1973). Mild proteolysis (Jothy et al., 1974) or neuraminidase treatment (Scott-Burden and Hawtrey, 1973) destroys most of the membrane's binding capacity. This suggests that proteins or glycoproteins but not phospholipids are principal constituents of the ribosome binding sites.

5) Vectorial Transport and the Function of Membrane-Bound Ribosomes

Peptides made on mb ribosomes of liver or pancreas cells are not released directly into the surrounding medium, but rather remain associated with the ER complex (Andrews and Tata, 1971; Blobel and Sabatini, 1970b; Redman and Sabatini, 1966; Redman et al., 1966), and can be released experimentally by disruption of the membranes with detergent or sonication. Nascent polypeptides of mb ribosomes are protected from proteolytic attack to a much greater degree than are the

polypeptides of free ribosomes (Blobel and Sabatini, 1970a; 1970b). It was concluded from these observations that mb ribosomes in secretory cells vectorially transport their protein products into the ER cisternae or into the microsomal space in vitro (see Fig. 1a), although it is not clear whether such proteins are free in the cisternal space or buried within the membrane (Rolleston, 1974; Sauer and Burrow, 1972). The percentage of protein made on mb ribosomes which is vectorially transported appears to vary, but recent findings suggest that all molecules of secretory proteins such as albumin enter the membrane fraction (Shore, 1977).

The concept of vectorial transport fits well with Palade's widely known model of protein secretion. On the basis of autoradiography and cell fractionation data it was concluded that secretory proteins accumulate in the ER cisternae directly after synthesis, are processed (proteolysis, glycosylation etc.) within the ER/golgi complex, and finally packaged into vesicles for export (Palade, 1975). This model is generally accepted, although recent criticisms indicate that revision of some of its assumptions may be required (Rothman, 1975).

We have already seen that secretory proteins are made preferentially on mb polysomes. The existence of vectorial transport indicates that one function of the membrane/ribosome association is to permit direct

entry of specific proteins into the endomembrane system as the first step in the secretory process. The role of mb ribosomes in non-secretory tissues remains controversial however. The only representatives of this class which have been closely examined for vectorial transport are rat brain and muscle. In both cases vectorial transport was evident only to a minor degree if at all (Andrews and Tata, 1971). Until more data are available no generalizations can be made about the mode of protein discharge in non-secretory cells. There has been much speculation that a general function of mb ribosomes may be the synthesis of integral membrane or organelle proteins. Some recent evidence favors this hypothesis (Elhammer et al., 1975; Shore and Tata, 1977; and see Table 5).

Tata (1971) has proposed that membrane association may be used to physically compartmentalize or segregate different classes of ribosomes synthesizing different types of proteins. Certainly the work on ribosome structure (see Section D, 4) does not support the existence of physically distinct classes of ribosomes and it is therefore unlikely that mb ribosomes are programmed to recognize and selectively translate specific messages. The fact remains that free and mb ribosomes do synthesize distinct classes of proteins and some mechanism must be invoked to explain this. One possible explanation is the recently formulated "signal

hypothesis" of Blobel (Blobel, and Sabatini, 1971; Blobel and Dobberstein, 1975a). The salient features of this hypothesis are:

- a) Translational specificity is determined not by ribosomes but by a signal codon sequence at the 5' end of mRNAs coding for proteins made on mb ribosomes.
- b) Initiation of such messages begins on free ribosomes and the amino acid sequence coded for by the signal mediates the binding of ribosomes (by the amino terminal of the nascent chain) to sites on the ER (Fig. 1b).
- c) Only mRNAs containing a signal will result in functional ribosome binding involving attachment and vectorial transport. Upon completion of the peptide, the ribosome is released from the membrane into the free ribosome pool while the peptide remains with the ER. The ribosome binding cycle proposed for the "signal hypothesis" as illustrated in Fig. 1b is similar to an independently derived polysome binding model discussed by Rolleston (1974).

The hypothesis predicts that the initial transcript of "signal-containing" mRNA should be larger than the functional secretory protein due to the presence of the signal amino acid sequence. Immunoglobulin (IgG) synthesized in vitro by isolated mouse myeloma mRNA in

a heterologous reticulocyte system has a molecular weight of 25,000 whereas the in vivo secretory product has a molecular weight of 21,000 (Blobel and Dobberstein, 1975a). Isolated microsomes from mouse myeloma synthesize only the 21,000 MW product but removal of the membrane leaves polysomes which produce primarily 25,000 MW IgG. This has been interpreted to mean that proteolytic processing to remove the signal amino acid sequence normally occurs during synthesis as a function of the membrane.

In a similar system a precursor/product relationship has been demonstrated between pre-trypsinogen 2 and trypsinogen 2 in dog pancreas. The former, which can be isolated only in vitro due to rapid conversion to trypsinogen in vivo, has an additional 16 amino acids at the amino terminal. Extensive homology between these residues and the amino terminal sequences of other putative secretory protein precursors suggests a common "signal sequence" on pancreatic mRNAs coding for secretory proteins (Devillers-Thiery et al., 1975).

A heterologous system consisting of stripped ER from dog pancreas and reticulocyte ribosomal subunits will translate IgG message from mouse myeloma and vectorially transport and process the transcript to produce IgG. mRNA for globin (a non-secreted protein) can also be translated but the product is neither vectorially transported or processed (Blobel and

Dobberstein, 1975b). This indicates that the specificity for mRNA segregation and vectorial transport of protein resides in the mRNA itself and not in the ribosomes. Other papers bearing on the "signal hypothesis" are currently in preparation but have not appeared in time for inclusion in this review. This hypothesis is concerned primarily with membrane binding in secretory tissues. It can be extended to cover vectorial transport in non-secretory cells but does not account for the possibility of translational specificity of mb ribosome complexes which do not vectorially transport their products. The alternatives in this last case will be considered in the Discussion section of this thesis.

The "Membron" model is another recent attempt to explain the translational specificity of mb ribosomes (Shires et al., 1974). The model predicts that the membrane plays a role in translational regulation by specifically binding particular classes of mRNA (presumably to protein binding sites on the ER). Here again specificity resides in the message but unlike the "signal hypothesis" mRNA binding to ER is assumed to be direct and not necessarily dependent on protein synthesis.

Some evidence consistent with this model has appeared within the last few years. Transport of mRNA to the RER of Ehrlich-Ascites tumor cells is not significantly affected by inhibitors of protein synthesis

such as puromycin or cycloheximide (van Venrooij et al., 1975). Direct mRNA/membrane association was suggested in mouse myeloma on the basis of kinetic evidence (Mechler and Vassalli, 1975a; 1975b; 1975c). When the RER of human fibroblasts is stripped of polysomes the bulk of the mRNA remains with the ER (Adesnik et al., 1976; Lande et al., 1975). Using puromycin plus KCl to remove ribosomes from rat liver ER, Cardelli et al. (1976) found that about half of the poly-A containing RNA remained membrane-associated. The critical demonstration of specific recognition of a message by membranes has yet to be accomplished and consequently the Membron model remains largely hypothetical.

FIGURE 1. Binding of ribosomes to endoplasmic reticulum.

- a) Orientation of mb ribosome on an ER membrane. Vectorial transport of the nascent polypeptide (n); large ribosomal subunit (LSU); small ribosomal subunit (SSU); cisternal space (c). Modified from Tata (1971).
- b) The ribosome binding cycle based on the "Signal hypothesis". Signal region on mRNA is represented by a zig-zag line. The signal sequence on the nascent polypeptide is represented by a dashed line. Ribosome binding sites are shown as solid bars. Modified from Blobel and Dobberstein (1975a).

(a)

(b)

MATERIALS AND METHODS

A. Preparation and Treatment of Tissue Slices

Jerusalem artichoke (Helianthus tuberosus L.) plants were grown outdoors at Carleton University. Tubers were harvested annually in October, stored at 0-4°C in vermiculite, and used for experiments after storage for a minimum of 6 weeks and a maximum of 11 months. All experiments were done with tissue from plants which were originally derived from a single tuber (laboratory designation: Clone #3). The procedures used for short-term culture were essentially those of Adamson (1962). Tubers were washed in water, peeled at room temperature, and using a hand microtome were cut perpendicular to the longitudinal axis into 0.75 mm thick slices. The central pith and outer cortical regions were removed leaving essentially homogenous parenchyma tissue. Each slice was radially divided into six to eight smaller segments which constituted the basic experimental material, subsequently called "slices".

Estimates of the quantity of slices used were made on a gram fresh weight basis. Slices were blotted lightly with absorbent paper and weighed immediately before use. Changes in fresh weight during growth experiments were periodically monitored in the same way. One g fresh weight of freshly excised tissue contains

approximately 2×10^6 cells and about 2 mg of protein.

Tissue slices were incubated, i.e. aged, in aerated "water medium" (distilled water containing 0.05 mM CaCl_2), in the dark at room temperature. Aeration with filtered air was carried out in round bottom flasks containing 1 liter of solution and a maximum of 30 g of tissue slices. The solution was changed three times during 24 h of aging. Hormone treatment consisted of replacing the "water medium" with "hormone medium" (0.05 mM CaCl_2 solution containing 1 $\mu\text{g/ml}$ each of 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin).

Alternately in small scale growth experiments, 10 slices were placed on two thicknesses of Whatman #1 filter paper sitting on a glass ring in a 9 cm petri plate containing 20 ml of hormone medium.

For labelling of tissue with radioactive precursors of RNA or protein, 10 g of slices were placed on two thickness of Whatman #1 filter paper in a 14 cm petri plate containing 10 ml of either water medium or hormone medium plus radioactive precursors (10 $\mu\text{Ci/ml}$ ($5\text{-}^3\text{H}$) uridine; 30 Ci/mM; or 10 $\mu\text{Ci/ml}$ L(4,5- ^3H (N)) leucine; 60 Ci/mM, New England Nuclear).

B. Fractionation Protocols

1) Isolation of Total Ribosomes

To determine the total ribosome content of Jerusalem artichoke slices, tissue was homogenized in two volumes (i.e. 2 ml/g tissue) of G1 solution¹ for 1 min in a Sorvall Omni-Mixer. All operations were carried out at 0-4°C. The homogenate was filtered through four layers of cheesecloth and Triton X-100 detergent was added to a final concentration of 1%. Cell debris, nuclei, and mitochondria were pelleted by centrifugation at 8,000 xg for 15 min in a Beckman-Spinco #30 rotor². The supernatant fraction was layered over a 3 ml cushion of 0.8 M sucrose in R1 solution and centrifuged for 3.5 h at 140,000 xg in a #40 rotor. The resulting "total ribosome" pellet was resuspended in R1 solution for analysis of RNA content. The same procedure was used to isolate total polysomes except that tissue was homogenized with a mortar and pestle since mechanical homogenization resulted in poor polysome preservation.

¹The contents of all solutions used in fractionation are given in Section E.

²Unless otherwise specified, all rotor numbers refer to Beckman-Spinco rotors used in Model L Beckman-Spinco ultracentrifuge at 2°C.

2) Isolation of Total Protein

Tissue slices were washed in distilled water and stored in five volumes of 80% ethanol at -20°C until use. Material was homogenized in a Sorvall Omni-Mixer for 1 min in five volumes of 80% ethanol at 0°C . Alternatively, homogenization of fresh slices in 10% TCA proved equally effective and was employed in some experiments. Ethanol or TCA precipitated material was pelleted at 8,000 xg in an International 870 rotor for 20 min. Pellets were resuspended in 1:1 acetone/ether and were recentrifuged. The resulting pellets were air dried and dissolved in 0.9 N NaOH for determination of protein content by the Lowry method.

3) Isolation of Free and Membrane-Bound Ribosomes

Tissue slices were homogenized in a mortar and pestle in two volumes of G1 (Procedure A) or one volume of G2 (Procedure B). All operations were carried out at $0-4^{\circ}\text{C}$. The homogenate was filtered through two layers of cheesecloth and centrifuged at 8,000 xg for 10 min in a #30 rotor. The resulting supernate was transferred to a second tube and centrifuged at 8,000 xg for 5 min.

Procedure A: 13 ml of post-mitochondrial supernate was layered over 6 ml of 0.8 M sucrose made up in R1 solution

and centrifuged for 20 min at 100,000 xg in a #30 rotor. This pelleted membranes, mb ribosomes, and large free polysomes while monosomes and smaller free polysomes remained in the supernatant fraction. The total supernate (including the sucrose cushion), designated S-1, was decanted and saved. The pellet was resuspended in 2 ml of R1 solution and layered on a 20 ml 15-30% (w/v) linear sucrose gradient¹ made up in R1. Centrifugation for 45 min at 78,000 xg in an SW 25.1 rotor produced a pellet containing mb ribosomes and cell membranes while the largest free polysomes failed to penetrate the bottom third of the sucrose gradient. The pellet was resuspended in 5 ml of R1 solution containing 0.7% each of Triton X-100 and sodium deoxycholate. The ribosomes liberated from the membranes in this way were centrifuged for 2.5 h at 100,000 xg in a #30 rotor and the resulting pellet (P) was designated the "membrane-bound ribosome fraction". The ribosomes remaining in the 15-30% sucrose gradient (S-2) were combined with S-1 and the whole fraction was diluted with an equal volume of R1 solution and centrifuged for 2.5 h at 100,000 xg in a #30 rotor. The pellet was designated the "free ribosome fraction".

Procedure B: 6 ml of post-mitochondrial supernate was layered directly on an 18 ml 15-30% linear sucrose

¹All sucrose concentrations given as percentages are weight/volume. Sucrose used for gradients and buffered solutions was Ultrapure RNase free grade (Schwarz/Mann).

gradient overlaying a 5 ml cushion of 65% sucrose, both made up in R1. The gradient was centrifuged for 50 min at 78,000 xg in an SW 25.1 rotor. All but the last ml of the 15-30% sucrose fraction was removed, diluted with two volumes of R1, and centrifuged for 3 h at 100,000 xg in a #30 rotor to obtain the free ribosomes. The membranes which had accumulated at the gradient/cushion interface were collected (final volume = 3 ml) and diluted with 2.5 volumes of R1 solution. Four ml of this preparation were layered over 1 ml of 65% sucrose in R1 in a 5.5 ml tube and centrifuged for 45 min at 175,000 xg in an SW 39L or 50.1 rotor. This concentrated all membranes at the supernate/cushion interface. All but the last half ml of supernate was carefully removed leaving the membrane band undisturbed. Then 0.75 ml each of 48, 40, and 30% sucrose in R1 was layered over the membranes causing discrete membrane density fractions to float to the 65/48%, 48/40%, and 40/30% sucrose interfaces. Tubes were centrifuged for 20 min at 175,000 xg to insure equilibration and the components at each interface were collected by pasteur pipet for analysis (see Fig. 2). Fraction III (65/48% interface) contained the membranes enriched in RER (i.e., mb ribosomes). Free or mb ribosomes isolated in this way could be stored in 30% glycerol at -20°C for up to 6 weeks without losing their ability to support in vitro protein synthesis.

FIGURE 2.

Protocol for the isolation of RER-enriched membranes
by Procedure B.

75,000 xg for 45 min.

Collect membranes at 30/65% sucrose interface.

Concentrate membranes over 65% sucrose cushion,
175,000 xg for 45 min.

Layer discontinuous gradient over membrane band.

175,000 xg for 20 min.

Collect membranes at interfaces I, II, and III.

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4) Polysome and "Activity" Profiles

Polysome profiles were obtained by layering 0.5 ml of resuspended ribosomes on a 5 ml linear 15-40% sucrose gradient made up in R1 solution. To obtain reproducible results it was necessary to process polysomes immediately after isolation without intervening storage. The ribosomes layered on each gradient constituted the extract from 3-6 g of slices. Gradients were centrifuged for 2 h at 80,000 xg in an SW 39L rotor and fractionated on an ISCO Model D density gradient fractionator at a pumping speed of 0.25 ml/min. Absorbance at 254 nm was continuously monitored by an ISCO UA-2 ultraviolet analyzer and recorded on an ISCO or Perkin-Elmer chart recorder.

To determine the proportion of ribosomes bearing nascent polypeptides (active ribosomes), ribosomes were extracted as described in B (3) except that resuspension solution R2 was substituted for R1. Ribosomes from 5-10 g of tissue were resuspended in 1 ml of R2 and processed essentially as described by Martin (1973). A 0.75 ml sample of this suspension was incubated at 0°C with 2 µg pancreatic ribonuclease (Worthington Biochemical Corp.). After 2 min, 0.25 ml of 2.5 M KCl was added and a 0.2-0.5 ml aliquot of this treated sample was immediately layered on a 10-30% linear sucrose gradient made up in R3 solution. The gradient was centrifuged for 2.25 h at 175,000 xg in

an SW 39L rotor and then fractionated and monitored as described above. The portion of the gradient corresponding to each peak in the absorbance profile (254 nm) was collected separately for determination of RNA content and/or radioactivity. The area under each peak in the profile was determined from cutouts of the recorder tracings. Once calibrated by direct RNA measurements, the weights of such cutouts proved to be good estimates of the RNA content of the collected profile peak fractions and were determined routinely.

C. Analysis of Ribosomes and their Protein Products

1). Electron Microscopy

Pelleted membrane fractions obtained from isolation Procedures A or B (Section B, 3) were fixed at room temperature in 1.6% glutaraldehyde for 2 h followed by 6% glutaraldehyde for 14 h. The fixative was buffered with 0.1 M Na cacodylate pH 7.2. Following fixation, the pellets were washed over a 6 h period at 0°C in four changes of cacodylate buffer and then post-fixed for 15 h in 1% OsO₄ buffered as for glutaraldehyde. Material was dehydrated in a graded ethanol series followed by propylene oxide, and then embedded in Epon-Araldite resin. Sections were cut with a diamond knife, stained with uranyl acetate and lead citrate, and examined with a Siemens 1A or 101 microscope.

2) Protein Synthesis In Vitro

Incorporation of labelled amino acids into protein by isolated polysomes or rough microsomes was carried out using a modification of the wheat embryo system developed by Marcus et al. (1974a; 1974b). Ribosomes isolated with G2 solution were used in all experiments. The constituents of the incubation medium are given in Table 6. Transfer RNA was prepared by phenol extraction from commercial wheat germ (Marcus et al., 1974a) and stored at -20°C . The S-140 fraction which contained the protein factors necessary for ribosome function was prepared as follows:

0.6 g of wheat embryos prepared according to Marcus et al. (1974a; 1974b) was homogenized at 0°C with 6.6 ml of G3 solution in a mortar containing a small quantity of sand. The homogenate was centrifuged for 10 min at 23,500 xg in a #40 rotor. The supernate was removed taking care to exclude the lipid layer, and centrifuged for 2 h at 140,000 xg in a #40 rotor. The resulting ribosome-free supernate, designated S-140, was passed through a $0.45\ \mu\text{m}$ millipore filter and frozen at -20°C . In this form it could be stored up to 2 months prior to use without appreciable loss of activity. Just before use, 1 ml of S-140 was thawed and dialyzed for

TABLE 6.

Standard incubation medium for in vitro protein synthesis by Jerusalem artichoke polysomes.

Stock solutions and S-140 were prepared according to Marcus et al., (1974a: 1974b), and stored at -20°C .

Final volume of the reaction mixture was 0.28 ml.

Disodium ATP (Sigma Chem.)	1.4 mM
Creatine phosphate (Calbiochem.)	11.2 mM
Creatine Kinase (Boehringer/Mannheim)	16.0 μg
Trilithium GTP (Boehringer/Mannheim)	35.0 μM
KCl	48.0 mM
Magnesium acetate	4.3 mM
Tris buffer, pH 7.2	50.0 mM
Dithiothreitol	3.2 mM
(^{14}C) leucine (New England Nuclear)	0.5 μCi
S-140 fraction	2.0 mg protein
tRNA	0.06 mg RNA
Microsomes or polysomes	0.05-0.1 mg RNA

100 min against 500 ml of R4 solution at 0-4°C.

Reactions were started by addition of ATP/creatine kinase/creatine phosphate, and allowed to proceed for 30 min at 30°C. In vectorial transport experiments 1 mM puromycin was added during the last 15 min of incubation. Incorporation was terminated by addition of 0.1 ml of 0.1 M unlabelled leucine and 1 ml of R1 solution at 0°C. For timecourse and inhibitor studies 2 ml of 10% TCA were then added directly. For all other experiments ribosomes were pelleted at 140,000 xg for 2.5 h in a #40 rotor and 2 ml of 10% TCA added to the supernatant and pellet fractions. In some cases aliquots of supernate were taken prior to precipitation and fractionated into high and low molecular weight polypeptides on a 7.5 ml G-25 Sephadex column. G-25 (fine, which has an exclusion limit of 5,000 MW, was made up in R1 solution. The void volume contained high MW polypeptides while peptides smaller than 5,000 MW were retained on the column and were removed by washing with buffer. Fractions collected were precipitated with 10% TCA. TCA precipitates from all of the above treatments were processed for electrophoresis or assayed for radioactivity as described below.

3) Electrophoresis

Precipitated proteins, labelled in vitro were pelleted at 8,000 xg in an International 870 rotor for 15 min, washed with 1:1 acetone/ether followed by ether alone to remove water and TCA, and then air dried. Dried pellets were resuspended in 0.1 ml of E1 solution and solubilized by heating to 60°C for 30 min. Five µl of 0.05% bromophenol blue were then added and each sample was layered over an 8% polyacrylamide gel (100 mm x 5 mm diameter) made up in E2 solution. Samples were carefully overlaid and the upper and lower chambers of the electrophoresis cell filled with 0.1 M phosphate buffer pH 7.2 containing 1% sodium dodecyl sulfate (SDS).

Electrophoretic separation was carried out at room temperature. Current from a Buchler 3-1155 power supply was adjusted to 1 ma/gel for 30 min and then raised to 5 ma/gel for 6.5 h. At the completion of the run, gels were removed from their glass tubes and fixed for 30 min in 10% acetic acid. For staining, gels were placed in large test tubes containing 0.25% Coomassie Brilliant Blue R250 (BioRad Laboratories) in 50% methanol and 7% acetic acid for 1-2 h. Destaining was carried out by diffusion in 5% methanol plus 7% acetic acid. Gels to be fractionated were left unstained. After fixation they were frozen in foil troughs over powdered dry ice, cut into uniform 1 mm slices with a Mickle Gel

Slicer, and placed in scintillation vials for processing.

4) Determination of Radioactivity

The radioactivity of all samples was determined using a Packard Tri-Carb Liquid Scintillation Spectrometer. Counting efficiency of the instrument was 86% for ^{14}C and 48% for ^3H . Half ml fractions of (^3H) uridine labelled ribosomes taken from KCl-containing sucrose gradients were diluted with an equal volume of water and added directly to 10 ml of either Aquasol LSC (New England Nuclear) or XLSC (750 ml xylene, 250 ml Triton X-100, 3 g PPO, 0.2 g POPOP). Vials containing LSC and samples were usually kept at 4°C in the dark for 1 h prior to determination of radioactivity.

TCA precipitated proteins from in vitro incorporation experiments were heated to 80°C for 20 min, cooled to 0°C and resuspended in 5% TCA with a teflon homogenizer. The suspension was filtered through a GF/C glass fiber filter (Whatman) by suction, resulting in trapping of the TCA precipitate on the surface of the filter disc. Filters were washed 3 times with cold 5% TCA, dried in an oven at 80°C , and placed in vials containing 6 ml of TLSC (5 g PPO, 0.3 g POPOP, in 1000 ml toluene) for determination of radioactivity. Efficiency of counting using this method approached the maximum efficiency of the spectrometer.

Slices from polyacrylamide gels containing ^{14}C -labelled proteins were solublized by incubation overnight at 60°C in sealed vials containing 0.2 ml of 30% H_2O_2 . Six ml of Aquasol LSC were then added to each vial and samples were counted after 1 h in the dark at 4°C .

D. Chemical and Enzyme Determinations

1) Ribonucleic Acid

The RNA content of ribosome fractions was measured using the orcinol color reaction for ribose sugars. Ribosomes were precipitated with 10% TCA at 0°C and pelleted at 8,000 xg in an 870 International rotor. The pellets were washed twice with 5% TCA and air dried. Two ml of orcinol reagent (25 mg orcinol, 0.7 mg $\text{CuCl}_2 \cdot \text{H}_2\text{O}$, in 10 ml of concentrated HCl made up just prior to use) diluted by an equal volume of water were added to each pellet and tubes were incubated for 20 min in a boiling water bath. Samples were cooled and absorbance at 660 nm was measured within 1 h, using a Unicam SP-800 or a Spectronic 70 spectrophotometer. Absolute RNA values were determined from yeast RNA (Sigma Chemical Co.) standards processed identically. With the volumes employed, the A_{660} standard curve was linear between 5 and 200 μg RNA/tube.

Alternatively, TCA precipitated ribosomes were pelleted as above and washed successively with 5% TCA,

80% ethanol, and 1:1 acetone/ether. After air drying, precipitates were hydrolyzed for 1 h at 70°C in 2 ml of 0.5 N PCA and absorbance at 260 and 290 nm was measured. $A_{(260 - 290)}$ was used to estimate RNA from a standard curve of hydrolyzed yeast RNA. For the linear portion of the curve:

$$\Delta 0.1 A_{(260 - 290)} = 5.6 \mu\text{g/ml sample}$$

2) Protein

The protein content of tissue slices or isolated fractions was determined by a modified Lowry procedure (Lowry et al., 1951). Plant phenols can produce spurious values in the Lowry reaction resulting in over-estimation of protein content (Loomis, 1974).

The standard protocol was consequently modified according to Potty (1969) to eliminate this problem. TCA precipitated proteins were pelleted at 8,000 xg in an 870⁶ International rotor and washed with ether. The precipitate was then solubilized in 0.9 N NaOH. A 0.4 ml aliquot of sample was added to 3.6 ml of Reagent C (2% Na_2CO_3 , 0.02% sodium potassium tartrate, 0.01% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) and incubated at 20°C for 10 min. Then 0.4 ml of 1 N Folin reagent was added to each tube and after 30 min, absorbance at 750 nm was measured on a Spectronic 70 spectrophotometer. A second set of identical samples was processed in the same way except that Reagent C

(Reagent C minus CuSO_4) was used. These latter samples gave an estimate of absorbance due to phenols alone and their values were subtracted from the initial readings to give absorbance due to protein. Absolute protein values were determined from bovine serum albumin standards processed identically.

3) Phospholipids

Phospholipid content of membranes was estimated as 25 times the lipid phosphorous content (Andrews and Tata, 1971). Membrane phosphorous content was measured as illustrated in Fig. 3 (Chen et al., 1956). Values were determined from a curve of phosphorous standards processed by this method.

4) NADH Cytochrome c Reductase Activity

One half ml of freshly isolated membrane suspension (corresponding to 20-70 μg phospholipid) was added to 3.5 ml of freshly made assay medium (0.39 mg KCN, 1.3 mg cytochrome c in 3.5 ml of R1 solution pH 7.6); 0.5 mg of β NADH (Sigma Chemical Co.) was added to start the reaction and absorbance at 550 nm was monitored at 30 second intervals for the duration of the reaction. Values were plotted and change in A_{550}/min in the linear part of the reaction curve was used as a measure of activity. Enzyme activity was defined in arbitrary

FIGURE 3.

Protocol for the extraction and measurement of phosphate from membrane lipids.

Precipitate membranes with 10% TCA at 0°C and pellet at 8,000 xg for 15 min in an International 870 rotor.

↓

Resuspend pellet in 3:1 ethanol/ether and heat to 80°C for 5 min.

↓

Cool to 0°C and centrifuge at 8,000 xg for 15 min.

Supernate contains lipids. → Pellet contains RNA and protein.

↓

Evaporate to dryness and add 100 µl each of H₂O and concentrated H₂SO₄.

↓

Heat gently until white gas (SO₃) is evolved

↓

Add 50 µl concentrated PCA and heat until solution clears, then add 200 µl of 10 N NaOH and make sample up to 5 ml with H₂O.

↓

Add 1 volume of diluted sample to 2 volumes of fresh ascorbic acid reagent (2% ascorbic acid, 0.5% (NH₄)₆Mo₇O₂₄·4H₂O, in 1.2 N H₂SO₄).

↓

Incubate for 2 h at 37°C.

↓

Measure absorbance at 820 nm.

units; 1 unit resulting in a change in A_{550} of 8/min.

This corresponds to a reduction of approximately 1 μ mole of oxidized cytochrome c/min per reaction tube¹.

Cytochrome c reductase from pig heart (Sigma Chemical Co.) with activity of 0.3 units/mg was used as a standard.

5) RNase Activity

Artichoke tuber slices were homogenized in a Sorvall Omni-Mixer in one volume of acetate buffer (70 mM sodium acetate pH 5, 20 mM mercaptoethanol) at 0°C and the post-ribosomal supernate was collected. RNase activity was measured in the crude extract by the spectrophotometric method of Zollner and Hobom (1965). One ml of 2% yeast RNA (Sigma Chemical Co.) was rapidly mixed with 3 ml of ~~extract~~ diluted 1:5 or 1:20 with acetate buffer. The decrease in absorbance at 300 nm with time was monitored until the reaction had gone to completion.

Enzyme activity was expressed in arbitrary units, 1 unit being the amount of enzyme needed to decrease A_{300} from the initial value (E_0) to the final value (E_f), i.e. when the reaction has gone to completion, in 1 h. The rate $\Delta A_{300}/\Delta t$ (min) was derived from the linear portion of the reaction curve.

¹Sigma Chem. Co., 1975 catalog, p. 131.

$$1 \text{ unit} = (\Delta A_{300} / \Delta t) \times (60 / E_o - E_f)$$

The activity measured was considered to be primarily due to RNase since the pH optimum of the reaction was around 5. Non-specific nucleases and phosphatases, generally have pH optima above 6.5.

E. Buffered Solutions

G1: used to homogenize Jerusalem artichoke tuber tissue for ribosome and polysome extraction.

HEPES buffer, pH 7.6	50	mM
Bentonite ¹	0.6	mg/ml
Diethylpyrocarbonate	0.1%	(v/v)
Dithiothreitol	10	mM
KCl	30	mM
KOH	28	mM
Magnesium acetate	10	mM
Mercaptoethanol	10	mM
Sucrose	250	mM

G2: used to homogenize Jerusalem artichoke tuber tissue for isolation of polysomes and microsomes for in vitro protein synthesis.

HEPES buffer, pH 7.6	50	mM
Bentonite	0.6	mg/ml
Dithiothreitol	15	mM
KCl	30	mM
KOH	28	mM
Magnesium acetate	10	mM
Sucrose	250	mM

¹Bentonite was prepared according to the method of Watts and Mathias (1967).

G3: used to homogenize wheat germ embryos for S-140 fraction of in vitro protein synthesis system.

CaCl ₂	2 mM
KCl	90 mM
KHCO ₃	6 mM
Magnesium acetate	1 mM

R1: ribosome resuspension solution.

R2: ribosome resuspension solution for "activity" assay.

R3: gradient solution for "activity" assay.

R4: dialysis solution for S-140 fraction.

	R1	R2	R3	R4
Tris/HCl, pH 7.6	50 mM	50 mM	50 mM	1 mM
KCl	15 mM	45 mM	800 mM	50 mM
Magnesium acetate	5 mM	15 mM	15 mM	2 mM
Mercaptoethanol	-	-	-	4 mM

E1: used to resuspend and dissociate proteins for SDS electrophoresis.

Phosphate buffer, pH 7.2	100 mM
Glycerol	20% (w/v)
Mercaptoethanol	1% (v/v)
Sodium dodecyl sulfate	1% (w/v)

E2: used to make polyacrylamide gels for SDS electrophoresis.

Phosphate buffer, pH 7.2	100 mM
Acrylamide	8.0% (w/v)
Ammonium persulfate	5.0% (w/v)
Bis acrylamide	0.25% (w/v)
Sodium dodecyl sulfate	1.0% (w/v)
TEMED	0.15% (v/v)

RESULTS

A. Analysis of Procedures1) RNA Measurement

RNA content of ribosomes was determined from the orcinol color assay. Since orcinol can produce a positive reaction with some sugars besides ribose, it was necessary to test the reliability of the values obtained. Artichoke slices were labelled for 4 h with (^3H) uridine and then aged for 20 h in water. Total ribosomes were extracted and divided into three equal portions. The first was processed for RNA by the orcinol method, and the second by the PCA procedure (Materials and Methods). The third was precipitated with 10% TCA, washed successively in acetone, acetone/ether, and ether. RNA was extracted in 10% NaCl for 40 min at 100°C and then precipitated in 80% ethanol. Absorbance of the resuspended RNA was measured at 260 nm. Prior to RNA determination the radioactivity of aliquots from each of the three extracts was measured. The orcinol and PCA methods gave the same values for RNA content (140 μg RNA/g fresh weight). The salt extraction (which is non-quantitative) gave a lower estimate, but the specific activities (cpm/ μg RNA) of all three samples were essentially identical. This indicates that orcinol gives a reliable measure of RNA content of Jerusalem

artichoke ribosomes precipitated with TCA.

2) Polysome Extraction

The effectiveness of various buffered solutions in maintaining polysome structure during extraction was evaluated. Both HEPES and Tris-HCl buffers at 50 mM maintained the desired pH in extracts during homogenization. The high pH, high ionic strength solution (200 mM Tris-HCl, pH 8.5, 30 mM MgCl₂, 60 mM KCl, 250 mM sucrose) recommended by Davies et al. (1972) for isolation of pea polysomes, gave poor recovery in artichoke (Fig. 4a). Results were improved somewhat by the addition of 0.4 mg/ml of bentonite (Fig. 4b). HEPES buffered solution (50 mM HEPES pH 7.6, 10 mM magnesium acetate, 30 mM KCl, 250 mM sucrose, 10 mM mercaptoethanol) led to poor polysome preservation until supplemented with bentonite and diethylpyrocarbonate (DEP) as RNase inhibitors (Anderson and Key, 1971). This combination produced the best results (Fig. 4c) and was designated G1 solution. G2 solution which contained the above ingredients with 0.6 mg/ml bentonite but no DEP also resulted in good polysome preservation.

FIGURE 4. Polysomes from 24 h aged tissue slices homogenized in various solutions.

Profiles of ribosome distribution in 5 ml, 15-40% linear sucrose gradients centrifuged for 2 h at 80,000 xg. Relative distance down the gradient is given on the abscissa.

- a) Total ribosomes isolated in Davies' buffer (see text).
- b) Total ribosomes isolated in Davies' buffer supplemented with 0.4 mg/ml bentonite.
- c) Total ribosomes isolated in G1 solution.

Top

3) Fractionation Procedure A

The fractionation of ribosomes into free and mb populations was initially attempted by a widely used isopycnic centrifugation method. Total polysomes were extracted in G1 solution and pelleted. After resuspension in R1 solution the material was layered over a cushion of 2 M sucrose and centrifuged for 16 h at 60,000 xg in an SW 25.1 rotor. The membranes and associated ribosomes collected over the sucrose cushion, while free ribosomes pelleted. Extensive degradation was observed when mb polysomes were released from isolated membranes by detergent treatment and centrifuged on 10-30% linear sucrose gradients. When isolated membranes were pelleted without detergent and the supernate analyzed on gradients, degraded polysomes were also seen, suggesting that the membrane fraction was extensively contaminated with free or loosely bound ribosomes (Fig. 5).

The rapid fractionation procedure which was developed to overcome these problems is described in Materials and Methods (Procedure A). For good separation of the two populations it was necessary that the largest free ribosomes did not pellet through the 15-30% sucrose gradient at the third centrifugation step. Fig. 6 shows that free ribosomes did not enter the bottom third of the gradient under the conditions used and thus

FIGURE 5. Membrane-bound ribosomes isolated from 24 h aged tissue slices by isopycnic centrifugation.

Membrane-bound ribosomes were isolated by layering post-mitochondrial supernate over 2 M sucrose and centrifugation for 16 h at 60,000 $\times g$. Membranes at the supernate/2 M sucrose interface were collected and layered over a 10-30% linear sucrose gradient either directly or after treatment with Triton X-100 and Sodium deoxycholate (0.7% each). Polyosomes were resolved after centrifugation at 80,000 $\times g$ for 100 min.

..... = No detergent treatment
| = Detergent treatment

Top

FIGURE 6. Sedimentation of free ribosomes on a 15-30% linear sucrose gradient during Procedure A.

This gradient represents the third centrifugation step in Procedure A (Materials and Methods). After centrifugation at 78,000 xg for 45 min the largest free polysomes have not penetrated the bottom third of the gradient. Relative distance down the gradient given by abscissa.

Top

FIGURE 7. Polysomes from ribosome fractions isolated at each step in Procedure A.

Polysomes from 24 h aged tissue slices were resolved on 15-40% sucrose gradients as for Fig. 4.

- a) S-1 : free ribosomes from first supernate (see Procedure A; Materials and Methods).
- b) S-2 : free ribosomes from second supernate.
- c) S-3 : free ribosomes from third supernate.
- P : membrane-bound ribosomes from final pellet.

did not contaminate the membrane pellet. The polysome profiles of ribosomes isolated at each step in the fractionation procedure are shown in Figs. 7a-c. The monosomes and smaller free polysomes were found primarily in the S-1 fraction while S-2 was enriched in large free polysomes. The pellet, P fraction, contained polysomes associated with membranes. When this last fraction was recentrifuged virtually all ribosomes were found in the pellet and not in the supernate (S-3) indicating that P was not contaminated with free or loosely bound ribosomes.

4) Bacterial Contamination

Artichoke tissue was not treated aseptically during aging and fractionation, and although bacteria do not readily proliferate in the incubation medium, it was necessary to determine whether any residual bacteria on slices would be carried over into various steps of the fractionation procedure. Samples of the bacteria which are normally found on artichoke were isolated and cultured in liquid nutrient medium containing 5 μ Ci/ml (3 H) thymidine. Cells were harvested after 24 h, washed repeatedly by centrifugation and inoculated on Jerusalem artichoke tissue slices. Slices were immediately processed by fractionation Procedure A. Table 7 shows that bacteria were apparently not disrupted by homogenization with mortar and pestle, and collected in the initial

TABLE 7.

Localization of labelled bacteria in subcellular fractions obtained from inoculated Jerusalem artichoke slices by Procedure A.

Fraction	Radioactivity (cpm)	Total Counts
Total bacterial counts added	426,394	100.0
8,000 xg pellet	422,534	99.1
8,000 xg supernate	1,302	0.3
Fraction S-1	0	0.0
Fraction S-2	0	0.0
Fraction P	0	0.0
Total counts recovered	423,836	99.4

8,000 xg pellet with virtually no carry-over into the post-mitochondrial fractions.

B. Ribosome Content of Jerusalem Artichoke Slices
During Activation and Growth

Ribosome changes were followed during two distinct phases of tuber tissue development; cell activation, and the transition to cell growth.

- a) Activation - Excision and water aging resulted in rapid metabolic activation of Jerusalem artichoke tuber slices (see Literature Review and Results below) but no growth as measured by fresh weight increase occurred under these conditions (Fig. 8a)
- b) Growth - Activated (24 h aged) tissue slices could be stimulated to grow by the addition of 2,4-D plus kinetin to the incubation medium (Fig. 8a). It was not necessary for the hormones to be present continuously for optimum growth. 24 h aged slices given 2,4-D plus kinetin for as little as 18 h and then transferred to water, showed as much fresh weight gain over 96 h as did slices kept on hormones continuously (Fig. 8b). 2,4-D plus kinetin given to unaged tissue during the first 24 h after excision resulted in poor

FIGURE 8. Change in fresh weight of tuber slices incubated in water or 2,4-D plus kinetin.

a) Timecourse for fresh weight changes.

Addition of 2,4-D plus kinetin is indicated by arrow.

□ = water

■ = 2,4-D plus kinetin

b) Change in fresh weight, after 120 h, induced by varying periods of treatment with 2,4-D plus kinetin. Activated (24 h aged) slices were incubated with hormones for the indicated number of hours and then transferred to water. Fresh weight was measured 120 h after excision.

a = Hormone treatment during the activation period (0 to 24 h) only.

c = Activated slices incubated in hormones continuously up to 120 h (control).

expansion showing that competence to respond to the growth stimulus required prior metabolic activation.

1) Total Ribosome Content

The RNA content of total ribosome extracts from tissue slices aged for various periods of time is shown in Figs. 9a and 9b. Quiescent tissue (0 h) contained a relatively high level of rRNA per gram fresh weight. This level stayed essentially constant for the 11 month period during which intact tubers were kept in storage. Excision and aging of slices resulted in a drop in rRNA of about 30% during the first 2 h of incubation. No further change in rRNA content occurred during subsequent aging in water up to 72 h. This indicates that after an initial drop, the level of ribosomes remained constant during activation and aging of artichoke tissue. On the other hand, treatment of 24 h aged slices with 2,4-D plus kinetin resulted in a rapid accumulation of ribosomes, amounting to a three-fold increase over controls by 24 h (Fig. 9b). The rRNA content of hormone stimulated tissue dropped somewhat during the second 24 h of treatment, but remained at least twice that of slices aged in water.

FIGURE 9. Change in rRNA content of tissue slices during aging and growth.

- a) Activation period (0 to 24 h in water).
- b) Post-activation period in water or 2,4-D plus kinetin.

□ = water

■ = 2,4-D plus kinetin

2) Membrane-Bound Ribosomes

The ribosomes of the P fraction from Procedure A were identified as membrane-bound on the basis of their sedimentation with membranes in sucrose gradients, their release from membranes upon treatment with detergents and their appearance under the electron microscope. Fig. 10 shows typical views of membrane vesicles and fragments both in sectional and tangential view. Some membranes have numerous associated ribosomes and presumably represent material derived from RER. Since membranes were purposely not subfractionated, many smooth vesicles are also evident. Virtually all ribosomes seen in such preparations appear to be membrane associated.

Although the total ribosome content of Jerusalem artichoke tuber tissue does not rise during water aging, the proportion of ribosomes bound to membranes increases sharply. Fig. 11 shows that only about 5% of ribosomes in dormant tissue were mb. This increased to a maximum of 25% after 24 h of aging. Subsequent incubation of slices in water resulted in a decline in mb ribosomes. When aged tissue was stimulated to grow by the addition of 2,4-D plus kinetin, this decline was prevented and the proportion of ribosomes in the membrane fraction was stabilized at about the maximum level reached during aging. Since the total ribosome content of hormone

FIGURE 10. Electron micrographs of sections through the membrane fraction (P) isolated by Procedure A prior to removal of membranes by detergent.

Cross-sections of numerous vesicles, some without and some with ribosomes (r). Insets show two rough microsomes (rm) at a higher magnification. Scale lines represent 0.4 μm

FIGURE 11. Change in the percentage of ribosomes bound to membranes in tissue slices aged in water or incubated in 2,4-D plus kinetin.

Hormones were supplied to slices after 24 h aging (arrow). Ribosomes were separated into free and mb by Procedure A and orcinol reactions were carried out on the resulting fractions.

□ = Slices incubated in water.

■ = Slices incubated in 2,4-D plus kinetin.

treated tissue increased however (Fig. 9), the absolute size of the mb population grew three-fold during transition to cell expansion.

C. Ribosome Activity During Activation and Growth

1) Polysome Profiles

Although dormant Jerusalem artichoke tuber tissue contains abundant ribosomes very few of these are synthesizing protein. Fig. 12a shows the sucrose density gradient profile of a total ribosome extract from freshly excised slices. A large single peak corresponding to monosomes is evident, with virtually none of the ribosomes sedimenting in the polysome region of the gradient. By contrast, Figs. 12b and 12c show that polysomes are found in aged tissue. After 4 h activation a significant proportion of both free and mb ribosome fractions sedimented as polysomes. The relative proportion of ribosomes in polysomes increased from 10% to about 70% during 24 h of aging, at which time the size class of maximum peak height for free polysomes was the heptamer. In all cases the profiles obtained from the mb fraction showed excessive dimer and trimer peaks suggestive of partial degradation, but otherwise polysome development and hence the development of protein synthesis were quite comparable to the situation seen for free ribosomes. By 48 h aging considerable

FIGURE 12. Polysomes from tissue slices aged in water for various periods.

Free and mb ribosome fractions were isolated by Procedure A and processed as for Fig. 4.

- a) Total ribosomes from unaged tissue.
- b) Free ribosomes after 4, 12, 24, and 48 h aging.
- c) Membrane-bound ribosomes after same aging times as b.

apparent polysome depletion was seen in both ribosome fractions.

Such depletion was probably due to the enhanced RNase activity of aged tissue. Although RNase inhibitors were required for polysome isolation even at early times, degradative activity in the homogenate increased sharply by 48 h. The RNase activity of Jerusalem artichoke extracts was consequently measured directly in dormant and aged tissue. Between 0 and 48 h such activity increased almost ten-fold, from 10.2 to 96 units per gram fresh weight.

2) "Activity" Profiles

Attempts to eliminate polysome degradation through increased concentrations of RNase inhibitors proved unsatisfactory. Consequently an alternate method of estimating ribosome activity, described by Martin (1973), was investigated. This method is based on the observation that ribosomes not engaged in protein synthesis dissociate into 40S and 60S subunits in the presence of 0.5 - 1.0 M KCl, while ribosomes active in protein synthesis, i.e. those derived from polysomes, are resistant to dissociation due to the stabilizing effect of their nascent polypeptides (Martin et al., 1969). Two classes of ribosomes can thus be separated on sucrose density gradients containing an appropriate

concentration of KCl. "Inactive" ribosomes sediment as 40S and 60S subunits while synthetically "active" ribosomes sediment as 80S particles with a small proportion of dimers (Martin, 1973; Storb and Martin, 1972). Unlike polysome analysis, the results from this method are not sensitive to moderate levels of RNase activity. Another advantage is that most mRNA is removed from ribosomes during processing, making analysis of rRNA labelling easier..

When Jerusalem artichoke tuber ribosomes were treated according to this procedure and sedimented through 0.8 M KCl containing, 10-30% linear sucrose gradients, the results shown in Figs. 13, 14, and 15 were obtained. Peaks A and B represent the small (40S) and large (60S) ribosomal subunits respectively. Peak C is intact monoribosomes while peak D represents ribosome dimers.

The profiles of total ribosomes from freshly sliced and 1 h aged tissues are shown in Figs. 13a and 13b. Virtually all of the material from fresh tissue sedimented as subunits, indicating that very few active ribosomes were present in the intact tuber. Protein synthesis appeared to start up quite rapidly however; since after only 1 h of aging significant quantities of ribosomes bearing nascent polypeptides were present. Further aging resulted in a considerable shift of RNA into synthetically active particles as shown in Figs.

FIGURE 13. Activity profiles from total ribosomes from freshly excised and 1 h aged tissue slices.

Profiles show distribution of ribosomes in 10-30% linear sucrose gradients containing 0.8 M KCl. Ribosome samples were treated with pancreatic ribonuclease for 2 min at 0°C prior to centrifugation for 2.25 h at 175,000 xg. Peaks A and B represent the small and large ribosomal subunits respectively; peak C represents intact monoribosomes and peak D ribosome dimers. Relative distance down the gradient is given on the abscissa.

- a) Ribosomes from unaged tissue.
- b) Ribosomes from tissue slices aged for 1 h.

Abs. 254 nm

Top

FIGURE 14. Activity profiles of free and membrane-bound ribosomes from tissue slices aged in water for various periods.

Free and mb ribosomes were isolated by

Procedure A and processed as for Fig. 13.

a) Free ribosomes after 4, 12, 24, and 48 h aging.

b) Membrane-bound ribosomes after same aging times as in a.

FIGURE 15. Activity profiles of total ribosomes from tissue slices incubated in water or 2,4-D plus kinetin in the absence or presence of 5-FU.

Ribosome samples processed as for Fig. 13.

- a) Ribosomes from tissue slices aged for 48 h in water.
- b) Ribosomes from tissue slices aged for 24 h in water followed by 24 h in 2,4-D plus kinetin.
- c) Ribosomes from tissue slices aged as in b with 100 $\mu\text{g/ml}$ 5-FU added for the last 24 h.

Abs. 254 nm

Top

14a and 14b, with the relative proportion of active ribosomes reaching a maximum at 24 h, similar to the results obtained from the polysome profiles. Figs. 15a-c show a comparison between activity in non-growing and growing tissue at 48 h after excision. 2,4-D plus kinetin stimulate the active ribosome fraction relative to water aged cells. The rRNA synthesis inhibitor 5-FU has no apparent effect on the hormone induced stimulation (see Section C, 3).

Quantitative values for the percentage of ribosomes active in protein synthesis can be derived from RNA measurements of the fractions from gradient profiles, just described (Tables 8 and 9). Active ribosomes increased 3.5 fold over the first 4 h after excision, and reached a maximum of about 68% of the total population by 24 h aging. Again the results were very similar for free and mb ribosomes. Further water aging resulted in a decrease in activity. The application of 2,4-D plus kinetin temporarily prevented this decline and even resulted in a slight enhancement during the first 24 h of treatment. By 48 h in hormone however, values had declined to the level of water controls for both free and mb ribosomes.

The significance of ribosome activity expressed as percentages can be misleading due to the change in total rRNA content of expanding cells. Fig. 16 shows the above data recalculated as the relative number of

TABLE 8.

Percentage of ribosomes bearing nascent polypeptides during cell activation.

Slices were incubated in water for various times and free and mb ribosomes extracted by Procedure A. Values represent the percentage of rRNA remaining in monosomes or dimers after exposure of ribosomes to 0.8 M KCl, and were measured as areas under the profile peaks of KCl-containing sucrose gradients (Fig. 14). Individual results are the average of two to six separate determinations.

Hours Aging In Water	Type of Ribosome	
	Free	MB
0	12.7	-
1	29.6	-
4	45.9	46.5
12	64.7	66.4
24	69.2	68.0

TABLE 9.

Percentage of ribosomes bearing nascent polypeptides during aging and auxin induced cell growth.

Activated (24 h aged) tissue slices were incubated for various times in water or 1 ppm 2,4-D plus kinetin. Values for the percentage of ribosomes bearing nascent polypeptides were obtained as for Table 8. Individual results are the average of three to five separate determinations.

Incubation Medium	Type of Ribosome	Hours of Treatment		
		0	24	48
Water	Free	69.2	54.8	55.0
	MB	68.0	53.0	54.0
2,4-D + kinetin	Free	69.2	74.4	50.0
	MB	68.0	70.5	58.2

FIGURE 16. Size of the synthetically active ribosome fraction in tissue slices incubated in water or 2,4-D plus kinetin.

Hormones were supplied to slices after 24 h aging (arrow). Points on this graph were calculated from data in Fig. 5 and Tables 8 and 9.

□ = Slices incubated in water.

■ = Slices incubated in 2,4-D plus kinetin.

FIGURE 17. Protein content of tissue slices incubated in water or 2,4-D plus kinetin in the absence or presence of 5-FU.

Hormones and 5-FU were supplied to slices after 24 h aging (arrow). Protein measured by Lowry reaction on TCA insoluble extracts.

▲ = Protein per g fresh weight.

◻ = Protein per 2×10^6 cells.

◻ = Incubation in water.

▨ = Incubation in 2,4-D plus kinetin.

▨ = Incubation in 2,4-D plus kinetin + 5-FU.

ribosomes synthesizing protein per unit tissue. Presented in this way it becomes clear that hormone treatment significantly increases the number of synthetically active ribosomes and hence the protein synthesis capacity of aged tissue. This conclusion is supported by measurements of total cellular protein content presented in Fig. 17. Water treated slices displayed an essentially constant protein level throughout aging, while in growing cells protein per gram fresh weight doubled during 48 h of hormone treatment.

D. Ribosome Synthesis

1) Total Ribosomes

Initiation of RNA synthesis is one of the early events in the activation of Jerusalem artichoke cells. It has been demonstrated that the RNA made during the first 2 - 4 h after excision is primarily non-ribosomal (Byrne and Setterfield, 1977). To determine when rRNA was being made and incorporated into cytoplasmic ribosomes, tissue slices were aged for 2, 12, or 22 h, supplied with (^3H) uridine for an additional 2 h, and then homogenized. The RNA content and radioactivity of total ribosome extracts was then determined. Table 10 shows that even during the earliest period tested (2 - 4 h), cells incorporated significant amounts of labelled precursor into ribosomes. The rate of (^3H)

TABLE 10.

Incorporation of (^3H) uridine into ribosomes during aging.

Tissue slices were aged for 2, 12, or 22 h, supplied with 10 $\mu\text{Ci/ml}$ (^3H) uridine for an additional 2 h, and then processed to extract total ribosomes. RNA content was determined by the orcinol procedure and radioactivity was measured by direct counting in Aquasol. Uptake was determined as TCA soluble counts remaining in the post-ribosomal supernate derived from 1 g fresh weight of slices. Results are the average of three separate determinations.

(^3H) uridine	Hours of Labelling		
	2-4	12-14	22-24
Incorporation (cpm/ μg rRNA)	54	227	210
Uptake (cpm/mg fresh wt.)	118	480	504
Ratio Incorporation/Uptake	0.46	0.47	0.42

FIGURE 18. Distribution of ribosomal subunits on a 10-20% linear sucrose gradient containing 0.8 M KCl.

(³H) Uridine was supplied to slices between hours 12 and 14 of aging and ribosomes were extracted and processed as for Fig. 13.

Gradient fractions were collected directly into Aquasol for determination of radioactivity.

uridine incorporation into rRNA increased during 24 h of water aging. At the same time the uptake of radioactive precursor, as measured by the amount of TCA soluble label in washed slices, also increased in an equivalent fashion. The ratio of uptake to incorporation thus remained essentially constant, indicating that the net rate of rRNA synthesis was stable between 2 - 4 and 22 - 24 h of aging.

When ribosomes labelled at 12 - 14 h with (^3H) uridine were dissociated on 10-20% sucrose gradients containing 0.8 M KCl, the small ribosomal subunits had a considerably higher specific activity than did the large subunits (Fig. 18). This result is consistent with the generally accepted scheme of rRNA processing in eukaryotes in which newly made 18S RNA enters the cytoplasm more rapidly than 28S RNA.

2) Free and Membrane-Bound Ribosomes

To determine whether there was any difference between label incorporation into free and mb ribosomes, slices were aged and labelled for 2 h as in Table 10. Free and mb ribosomes were isolated by Procedure A and processed on gradients as for Fig. 13. Table 11 shows that both free and mb fractions contained labelled rRNA at each of the times tested. At 12 - 14 and 22 - 24 h the specific activity of total free ribosomes was con-

siderably higher than that of mb ribosomes. To determine if this difference was due preferentially to synthetically active or inactive ribosomes, each population was subfractionated into KCl-dissociable and KCl-stable particles and the radioactivity and RNA content of each was measured. The results presented in Table 11 show that in both cases (active and inactive) the specific activity of the free population was significantly greater than that of the mb population at 12 - 14 and 22 - 24 h, although the difference was somewhat more pronounced in the inactive fractions.

In a separate experiment, 12 h aged slices were labelled with (^3H) uridine for 2 h, then incubated in unlabelled medium for 12 more hours and processed as above. After the 12 h "chase" period there was little difference between the specific activities of free and mb populations or subfractions. From Table 11 it is clear that directly after a 2 h pulse of label, newly synthesized ribosomes were found preferentially in the free fraction. If such a pulse was followed by a 12 h chase period there appeared to be an equilibration of labelled ribosomes resulting in similar specific activities for both populations.

TABLE 11.

Specific activity of free and membrane-bound ribosome fractions during aging.

Tissue slices were aged and labelled as for Table 10.

Free and mb ribosomes were extracted by Procedure A and processed as for Fig. 13. Fractions corresponding to the profile peaks were collected and their radioactivity and RNA content determined as for Table 10.

Results are the average of two separate determinations.

Specific Activity of Ribosomes (cpm/ μ g rRNA)	Hours of Labelling			
	2-4	12-14	22-24	12-14 + 12 h chase
Free-Total	56	246	238	1087
MB-Total	48	148	118	1004
Ratio Free/MB	1.17	1.66	2.01	1.08
Free-Active	46	172	171	945
MB-Active	45	121	96	853
Free-Inactive	64	380	434	1455
MB-Inactive	53	210	166	1295

3) The Effects of 5-FU on Ribosome Synthesis and Activity

5-FU is a pyrimidine analog which interferes preferentially with rRNA synthesis. Since the auxin-mediated growth response in Jerusalem artichoke is accompanied by a large increase in ribosome and protein synthesis, and yet expansion itself is not inhibited by 5-FU given concurrently with 2,4-D plus kinetin (Rose and Setterfield, 1971), it was of interest to determine the effect of this inhibitor on the RNA metabolism of excised tissue.

To examine the effect of 5-FU on the incorporation of labelled RNA precursor into ribosomes, slices aged for 24 h in water were transferred to a medium containing 2,4-D plus kinetin with or without 5-FU for 2 h. (^3H) uridine was then supplied to each sample for 4 h, after which the slices were transferred back to their corresponding unlabelled medium for 18 h further incubation. Preliminary experiments had shown that after 4 h exposure to (^3H) uridine, the specific activity of ribosomes continued to increase throughout the subsequent 18 h in unlabelled medium, presumably because of label accumulated in RNA precursor pools. This phenomenon, which was also noted by Rose and Setterfield (1971), makes it impossible to perform meaningful pulse chase experiments with this tissue.

Following incubation ribosomes were extracted and fractionated as for Fig. 13. Table 12 shows that incorporation of (^3H) uridine into total ribosomes was much higher in tissue treated only with 2,4-D plus kinetin than in material where 5-FU was also added. Uptake of labelled precursor into tissue, measured as counts in TCA soluble material, was identical for the two treatments. When total ribosomes were subfractionated on sucrose gradients containing 0.8 M KCl little difference was found between the specific activities of inactive and active ribosome populations derived from hormone treated tissue. This result indicates that newly synthesized ribosomes are distributed randomly between the two populations. The situation is different for tissue treated with 5-FU. In this case, the inactive ribosomes had almost 3 times the specific activity of the active, indicating that newly synthesized ribosomes accumulate preferentially in the inactive population.

Despite its marked effect on incorporation of (^3H) uridine into ribosomes, 5-FU administered concurrently with 2,4-D plus kinetin, did not result in ribosome depletion; in fact it only reduced hormone stimulated rRNA accumulation by less than 50% after 24 h treatment (Table 13). The proportion of ribosomes active in protein synthesis was also not significantly altered by the inhibitor (Fig. 13, Table 13). 5-FU treated tissue therefore had twice as much rRNA and three times as many

TABLE 12.

Incorporation of (^3H) uridine into ribosomes during cell growth in the absence and presence of 5-FU.

Activated (24 h aged) tissue slices were incubated for 24 h in 2,4-D plus kinetin in the absence or presence of 5-FU, with 10 $\mu\text{Ci/ml}$ (^3H) uridine supplied between hours 2 and 6 of incubation. Total ribosomes were extracted and processed as for Fig. 13. Radioactivity and RNA content were determined as for Table 10.

Treatment	Specific Activity (cpm/ μg RNA)		
	Total Ribosomes	Inactive Ribosomes	Active Ribosomes
2,4-D + kinetin	970	1104	902
2,4-D + kinetin plus 5-FU	292	630	229

TABLE 13.

Ribosome activity during cell growth in the absence and presence of 5-FU.

Activated (24 h aged) tissue slices were incubated for 24 h in water or in 2,4-D plus kinetin in the absence or presence of 5-FU. Total ribosomes were extracted and processed as for Fig. 13. RNA was measured by the orcinol procedure.

Treatment	Total rRNA ($\mu\text{g/g}$ fr. wt.)	rRNA in Active Ribosomes	% Ribosomes Bearing Nascent Polypeptides
Water	140	76	54
2,4-D + kinetin	430	335	78
2,4-D + kinetin plus 5-FU	289	223	77

synthetically active ribosomes as an equivalent amount of water aged slices. This shows that 5-FU was only partially effective in blocking the increase in the capacity for protein synthesis which develops in the presence of 2,4-D plus kinetin. Further evidence for this is provided in Fig. 17 which shows that the inhibitor did not alter cellular protein accumulation over the first 24 h of treatment (24 - 48 h incubation) and even after 48 h in 5-FU inhibition was only partial.

E. Isolation of an RER-Enriched Membrane Fraction

1) Fractionation Protocol

The mb ribosome fraction isolated by Procedure A was purposely not subfractionated from non-ER membranes so as to maximize the yield of mb ribosomes. For further characterization and product analysis where fractionation losses were not critical, it was desirable to obtain a more purified RER preparation.

In preliminary experiments 24 h aged tuber slices were homogenized in G2 solution by grinding in a mortar and pestle or by chopping with a razor blade. The post-mitochondrial supernate was layered on a 20-55% linear sucrose gradient and centrifuged for 2.5 h at 90,000 xg in an SW 39L rotor. Based on turbidity and absorbance at 280 nm no discrete membrane bands were visible on the gradient; material was present throughout,

although it was more concentrated in the region of 40-55% sucrose. A pellet of dense material without an absorbance peak in the 250-290 nm range was also present.

As an alternative, a rapid floatation method was developed in which all membranes within a particular range of densities were collected at an interface of a discontinuous sucrose gradient. It was hoped that a density range could be found which was enriched in microsomes derived from rough endoplasmic reticulum. Initial experiments showed that the most reproducible separations were obtained with Procedure B and the discontinuous gradient shown in Figs. 2 and 19b-c.

It should be noted that in this procedure the actual fractionation was achieved by displacement or floatation of membranes during the layering of different concentrations of sucrose over the total membrane band, and not by centrifugation. Subsequent centrifugation up to 90 min did not substantially alter the banding observed (Fig. 19c). A final 20 min spin at 175,000 xg was included merely to prevent artifacts of incomplete displacement due to membrane fragments sticking to the sides of the centrifuge tube etc. Fractionation similar to that obtained by floatation was also achieved by layering total membrane samples over a discontinuous gradient (1 ml each of 30, 40, 50, and 65% sucrose layers) and centrifugation at 175,000 xg in an SW 39L rotor for 90 min. Since far less material per tube

FIGURE 19. Photographs of gradient tubes showing separation of Fractions I, II, and III during isolation Procedure B.

- a) Total membranes from 24 h aged tissue over 65% sucrose cushion after centrifugation at 170,000 for 45 min (see Fig. 2).
- b) Separation of membranes into three major fractions (I, II, III) following layering of 48, 40, 30, and 20% sucrose solutions over membranes in a.
- c) Discontinuous gradient in b after 90 min centrifugation at 170,000 xg.

could be processed this way however, the floatation procedure was chosen for the main analyses.

To demonstrate that Fractions I, II, and III obtained by Procedure B (Fig. 19) constituted defined density classes, isolated membranes from each fraction were layered over separate discontinuous gradients of 30, 40, 48, and 65% sucrose and centrifuged for 90 min at 175,000 xg in an SW 39L or SW 50.1 rotor. Visual inspection showed that the membrane components of each tube sedimented to the interface corresponding to the one from which they had originally been isolated (i.e. Fraction III banded at the 48/65% interface etc.), with little material evident in other parts of the gradient. No pellets were found in any of the three tubes.

2) Characterization of Fractions I, II, and III

Fig. 20a-c shows electron micrographs of sections through membrane pellets derived from Fractions I, II, and III of 24 h aged tissue. Fraction I consists of smooth vesicles and membrane fragments with a few dense particles which may be detached ribosomes. Both Fractions II and III contain large numbers of rough microsomes as well as some smooth membranes. From examination of a number of preparations it appeared that Fraction III was more enriched in mb ribosomes although this was purely a qualitative judgement.

FIGURE 20. Electron micrographs of sections through membrane pellets derived from Fractions I, II, and III isolated from 24 h aged tissue slices by Procedure B.

Scale lines represent 0.4 μm .

- a) Fraction I showing primarily smooth vesicles and membrane fragments, with some detached ribosomes (r) and one rough microsome (rm).
- b) Fraction II showing numerous vesicles both with and without attached ribosomes.
- c) Fraction III showing primarily rough microsomes (rm) both in cross section and tangential section. Some smooth vesicles are still present.

a

(b)

(c)

Table 14 presents the results of a chemical analysis of the membrane fractions isolated by Procedure B from 24 h aged tissue. Since phospholipid content is a good measure of the total amount of membranes present, protein, RNA, and NADH cytochrome c reductase activity are all expressed per unit phospholipid. It is clear that Fraction III has the highest protein/phospholipid and RNA/phospholipid ratios. Since the RNA and some of the protein sedimented with ribosomal particles if membranes were disrupted by detergent, it appears that Fraction III is enriched in mb ribosomes. NADH cytochrome c reductase activity, which is found on ER membranes, was also enriched at the 48/65% sucrose interface (Fig. 21).

From these data it was concluded that Fraction III was preferentially enriched in RER-derived microsomes. Equivalent fractions with similar RNA/phospholipid ratios were also isolated from tuber slices aged for 24 h in water followed by 24 h in 2,4-D plus kinetin. Comparison of the values of mb RNA per unit tissue in Table 14 with those of Figs. 5 and 11 indicates that the yield of mb ribosomes is less for Procedure B than for Procedure A. Losses were undoubtedly incurred in the former due to added subfractionation, and incomplete recovery of fractions from gradients where some material had to be excluded to avoid cross contamination.

TABLE 14.

Properties of membrane Fractions I, II, and III isolated by Procedure B.

24 h aged tissue slices were processed by Procedure B and the resulting membrane fractions were isolated and characterized. Protein was measured on TCA precipitated samples by the Lowry method and RNA was determined by the PCA procedure. Phospholipid was calculated as 25 times the lipid phosphate concentration measured by the ascorbic acid assay. Enzyme activity was measured by the ability of freshly isolated membrane suspensions to reduce cytochrome c (Fig. 21). Results are the average of three to four separate determinations.

	Fraction		
	I	II	III
Density (g/cc)	1.1150 to 1.1519	1.1520 to 1.1829	1.1830 to 1.2400
Protein ($\mu\text{g}/10 \text{ g fr. wt.}$)	43.2	131.0	227.0
RNA ($\mu\text{g}/10 \text{ g fr. wt.}$)	4.3	26.6	61.9
Phospholipid ($\mu\text{g}/10 \text{ g fr. wt.}$)	73.0	190.0	222.0
NADH cyto. c reductase (units $\times 10^{-3}/10 \text{ g}$ fr. wt.)	1.41	5.56	7.57
Protein/Phosphol.	0.59	0.69	1.02
RNA/Phosphol.	0.06	0.14	0.28
NADH cyto. c reductase/Phosphol. ($\times 10^{-2}$)	1.93	2.94	3.41

150

FIGURE 21. NADH cytochrome c reductase activity of Fractions I, II, and III from 24 h aged tissue slices.

Graph shows increase in optical density at 550 nm due to reduction of cytochrome c by Fraction I, II, and III membranes isolated by Procedure B from 3 g fresh weight of 24 h aged tissue slices.

F. Protein Synthesis In Vitro by Free and Membrane-Bound Ribosomes

1) A Homologous System from Jerusalem Artichoke

In order to examine protein synthesis in isolated free and mb polysomes from Jerusalem artichoke it was necessary to develop a suitable in vitro translation system. Initial attempts utilized the components shown in Table 15, with tuber tissue as a source of protein factors as well as polysomes. Aged slices (24 h) were homogenized with one volume of R1 solution containing 20 mM mercaptoethanol and 0.6 mg/ml bentonite. The post-ribosomal supernate (S-100) was dialyzed overnight at 0°C against 100 volumes of R1 solution containing 20 mM mercaptoethanol, and used immediately. Incubation was for 30 min at 25°C. Due to the extremely low level of incorporation obtained, attempts to use this system were abandoned and a more efficient heterologous translation system was employed.

2) A Heterologous System from Wheat Embryo

Protein factors from the ribosome-free supernate of wheat embryos, prepared according to Marcus et al. (1974a; 1974b), were used to support protein synthesis by polysomes isolated from aged Jerusalem artichoke tuber slices. Total polysomes (90 µg RNA/reaction) or

TABLE 15.

Incubation medium for a homologous Jerusalem artichoke
in vitro protein synthesis system.

Reaction Mixture	Concentration
ATP	0.9 mM
GTP	0.3 mM
Phosphoenolpyruvate	3.1 mM
Pyruvate kinase	20.0 μ g
17 Amino acids	0.1 mM
(³ H) Amino acids	10.0 μ Ci
Tris buffer, pH 7.6	50.0 mM
Fraction S-100	1.0 mg protein
Polysomes	200.0 μ g RNA

Reaction volume = 1 ml

Incorporation/30 min incubation = 275 cpm

rough microsomes (12 μ g RNA/reaction) isolated by Procedure B were incubated for varying periods of time with the components shown in Table 6. Incorporation of (14 C) leucine into polypeptides was linear for only a short period and essentially complete by 20-30 min (Fig. 22). Net incorporation was nevertheless sufficient to justify further experiments.

A series of control reactions were run to characterize the system. Samples were incubated for 30 min at 30°C in the standard reaction mixture (Table 6) with the additions or omissions listed in Table 16. Incorporation was absolutely dependent on added polysomes showing that the wheat embryo extract was free of active endogenous polysomes. Little polypeptide synthesis occurred at 0°C or in the absence of an energy generating system. Added amino acids only enhanced incorporation by 7% which indicates that endogenous amino acids from wheat were not limiting. Since rapid termination of incorporation might be attributed to residual RNase activity (Fig. 22), 0.05% diethylpyrrocarbonate was added to one reaction. This actually reduced incorporation substantially possibly because of DEP attack on RNA or an adverse effect on pH. Cycloheximide, an inhibitor of eukaryote protein synthesis, virtually abolished incorporation whereas chloramphenicol, a prokaryote inhibitor, had no effect. Puromycin which causes premature chain termination in both eukaryotes

FIGURE 22. Timecourse for in vitro incorporation of (^{14}C) leucine into protein by total and membrane-bound ribosomes.

Rough microsomes (Fraction III; 12 μg RNA/reaction) and total polysomes (90 μg RNA/reaction) were incubated in the wheat embryo translation system (Table 6) for various periods and TCA insoluble label was collected and counted on glass fiber filters.

Δ = total polysomes

$\blacktriangle$ = mb polysomes

O = no polysomes

TABLE 16.

Effect of inhibitors and changes in the wheat embryo incubation medium on in vitro protein synthesis by Jerusalem artichoke polysomes.

Reactions were carried out for 30 min in the standard incubation medium (Table 6) with the additions or deletions noted. TCA insoluble material was collected and washed on glass fiber filters and radioactivity determined.

Incubation Conditions	Incorporation (100% = 25,000 cpm)
Complete system (Table 6) 30°C	100.0%
Complete system 0°C	9.3%
Minus polysomes	0.8%
Minus energy (ATP, creatine phosphate, creatine kinase)	5.4%
Plus 16 amino acids (40 µM each)	107.0%
Plus 0.05% diethylpyrocarbonate	57.9%
Plus cycloheximide (330 µg/ml)	3.1%
Plus chloramphenicol (10 µg/ml)	102.1%
Plus puromycin (25 µg/ml)	8.4%
Plus ATA (533 µg/ml)	101.2%

and prokaryotes blocked most polypeptide synthesis. ATA, an inhibitor of initiation, had little effect on incorporation indicating that although this heterologous system permits translation of polysomal mRNA (i.e. pre-initiated), it does not support re-initiation. Taken together these results clearly demonstrate that incorporation was sensitive to the expected inhibitors, dependent on added Jerusalem artichoke polysomes, and not due to bacteria.

3) Release of Nascent Polypeptides from Membrane-Bound Ribosomes

The route of discharge of nascent polypeptides from mb ribosomes derived from growing and non-growing tuber tissue was investigated using the in vitro system just described. As has been discussed earlier, mb ribosomes can release their products either directly into the surrounding medium or discharge them into the adjacent membrane or microsomal space (vectorial transport; see Fig. 1). To differentiate between these two alternatives, the following experiment was designed:

Rough microsomes isolated by Procedure B were incubated for 30 min as in Table 6 with 1 mM puromycin added for the last 15 min. Incorporation was stopped by the addition of 0.1 ml of 0.1 M leucine and 1 ml of R1 at 0°C. The contents of 4-6 tubes were pooled and divided

into two equal portions. One portion was made 1% with Triton X-100 while the other received an equal amount of water. Samples were then centrifuged for 2.5 h at 175,000 xg in an SW 39L rotor to pellet ribosomes and membranes. The supernate and resuspended pellet from each sample were precipitated with 10% TCA and processed for radioactivity on glass fiber filters as outlined in Materials and Methods.

The rationale for this procedure was that any nascent polypeptides released directly into the surrounding medium during in vitro incubation would be found in the supernate of the sample which had not received any detergent (S_0), while proteins vectorially transported into the microsomes would be in the pellet (P_0). Detergent treatment, which disrupts membranes would be expected to release microsome associated polypeptides into the supernate (S_d) with only non-releasable label remaining in the pellet (P_d). The difference between S_d and S_0 therefore gives the amount of incorporated label which was vectorially transported.

Table 17 shows the results of experiments using microsomes from non-growing (24 h aged) and growing (24 h aged + 24 h 2,4-D plus kinetin) tissue. It is clear that mb ribosomes from non-growing cells released most of their polypeptide products directly into the incubation medium with only 12% of the releasable label

TABLE 17.

Vectorial transport of proteins by membrane-bound ribosomes from non-growing and growing Jerusalem artichoke tuber tissue.

Fraction III membranes were isolated by Procedure B from non-growing (24 h aged) and growing (24 h aged, then 24 h in 2,4-D plus kinetin) tissue slices. Microsomes were incubated in the standard incubation medium (Table 6) for 30 min with 1 mM puromycin present during the last 15 min. Microsomes were then pelleted in the absence or presence of 1% Triton X-100 and TCA insoluble label collected and measured on glass fiber filters.

$$\% \text{ Vectorial Transport} = 100 (S_d - S_o) / S_d$$

Results for non-growing and growing tissues represent the average of four and three experiments respectively.

Fraction	Non-Growing Tissue		Growing Tissue	
	CPM	%	CPM	%
Pellet - detergent (P_o)	1468	33.2	3614	59.1
Pellet + detergent (P_d)	1075	24.2	1831	29.7
Supernate - detergent (S_o)	2953	66.8	2501	40.9
Supernate + detergent (S_d)	3375	75.8	4334	70.3
Total - detergent	4421	100.0	6115	100.0
Total + detergent	4450	100.0	6165	100.0
% Vectorial transport	11.9		41.8	

remaining with the microsomes. The situation was very different in rough microsomes from growing hormone-treated tissue. Here 42% of newly synthesized proteins were associated with the membrane fraction and required detergent treatment to be released. In both types of microsomes a significant amount of label was still associated with the detergent-treated ribosome pellet even in the presence of puromycin. This probably represents incomplete polypeptides on ribosomes where translation was prematurely terminated but where normal release did not occur.

A separate experiment performed as in Table 17 without puromycin, gave the same distribution for vectorial transport but resulted in a higher percentage of total incorporated label sedimenting with the detergent treated ribosomes. As a further control free ribosomes were incubated as above, and detergent treatment was found to have no effect on the release of labelled polypeptides from the ribosome fraction.

The existence of vectorial transport was also indicated by the reduced susceptibility of newly made microsome-associated polypeptides to protease digestion. Microsomes from growing tissue were incubated for 30 min as in Table 6 without puromycin and pelleted for 60 min at 140,000 xg in a 440 rotor. Pellets were resuspended in R1 and aliquots incubated for 30 min at 30°C with one half volume of trypsin plus chymotrypsin.

TABLE 18

Protease digestion of proteins made in vitro by Jerusalem artichoke polysomes.

Fraction III microsomes isolated by Procedure B were incubated for 30 min as for Table 6 and then pelleted for 1 h at 140,000 xg. Labelled protein associated with resuspended microsome pellets (membrane-associated) and supernate (membrane-free) were incubated for 30 min at 30° with 0.5 volumes of water, Proteinase K, or trypsin/chymotrypsin. The TCA precipitable label in the void volume after Sephadex G-25 filtration was collected and counted on glass fiber filters.

	Membrane-associated protein		Membrane-free protein	
	cpm	%	cpm	%
Water	18,763	100	13,606	100
Proteinase K (10 µg/ml)	10,542	56	2,540	19
Trypsin/chymotrypsin (10 µg/ml each)	9,482	51	-	-

(10 µg/ml each) or Proteinase K (Beckman Instruments Inc.: 10 µg/ml) or water. Control samples containing membrane-free in vitro labelled proteins were incubated in the same way. After incubation samples were fractionated on a Sephadex G-25 column and TCA precipitable label in the void volume was determined. While 81% of membrane-free proteins were degraded to TCA soluble products, 51-56% of the microsome-associated labelled proteins were precipitated by TCA after treatment with trypsin/chymotrypsin or Proteinase K (Table 18).

From the above experiments it was concluded that significant vectorial transport of proteins made by mb ribosomes occurred in growing but not in non-growing Jerusalem artichoke tuber cells.

4) Electrophoresis of In Vitro Products of Jerusalem Artichoke Ribosomes

A preliminary attempt was made to compare the in vitro protein products of different populations of Jerusalem artichoke ribosomes by one dimensional SDS polyacrylamide gel electrophoresis. In order to minimize contamination of labelled protein samples by incomplete polypeptides, polysomes were allowed to incorporate (¹⁴C) leucine for 30 min in the absence of puromycin. Ribosomes and any attached nascent polypeptides were pelleted at 140,000 xg for 3.5 h in a #40 rotor and discarded while

the supernate was collected for analysis. Filtration of the supernates on a Sephadex G-25 column resulted in 94% of the TCA precipitable label eluting in the void volume. Since G-25 has an exclusion limit of 5,000, this showed that virtually all of the polypeptides in the sample had a molecular weight greater than 5,000. If the supernate had been significantly contaminated with incomplete low molecular weight peptides a larger proportion of the label should have been retained on the column.

Prior to analysis of in vitro products, the soluble in vivo protein constituents of non-growing and growing tissue were compared. Fig. 23 shows drawings of the protein bands visible on gels after staining with Coomassie Blue. Both tissues displayed a complex array of proteins with no obvious qualitative differences between the two treatments. In order to construct a scale for molecular weights from the SDS gels, known protein standards were run separately and in combination and a standard curve of their relative mobilities was drawn (Fig. 24).

Fig. 25 shows a comparison of labelled proteins made in vitro by total polysomes of non-growing and growing tissue. Polysome preparations were freed of membranes prior to incubation by detergent treatment followed by pelleting and resuspension in R1 solution. At least seven major protein species were resolved

FIGURE 23. Drawings of SDS polyacrylamide gels following electrophoresis of soluble proteins of non-growing and growing Jerusalem artichoke tissue slices.

Protein bands were visualized by staining gels with Coomassie Blue. Bromophenol blue dye front indicated by arrows.

- a) Protein constituents of the post-ribosomal supernate from slices aged for 24 h in water (non-growing).
- b) Protein constituents of the post-ribosomal supernate from slices aged for 24 h in water followed by 24 h in 2,4-D plus kinetin (growing).

FIGURE 24. Migration of proteins of known molecular weight on SDS polyacrylamide gels.

Protein standards (Sigma Chem. Co.) were electrophoresed at pH 7.2 on 8% polyacrylamide gels containing 1% SDS, and visualized by staining with Coomassie Blue.

- 1) Bovine serum albumin (68,000 MW)
- 2) Ovalbumin (42,000 MW)
- 3) Trypsin (23,000 MW)
- 4) Cytochrome c (13,000 MW)
- 5) Dye front (bromophenol blue).

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TABLE 19.

Molecular weights of polypeptides made in vitro by Jerusalem artichoke polysomes.

"a" through "g" represent the peaks of label seen in SDS polyacrylamide gel profiles of the products of total, free, and mb polysomes (Figs. 25 and 26). Mobility values (relative to bromophenol blue dye) are based on the ranges obtained from four separate experiments and MW values were calculated from the curve of protein standards (Fig. 24).

Peak	Relative Mobility	Molecular Weight
a	0.25-0.27	66,100-63,900
b	0.44-0.46	40,300-38,900
c	0.54-0.56	31,300-30,200
d	0.66-0.68	23,200-21,900
e	0.73-0.75	19,500-18,400
f	0.78-0.81	17,000-15,800
g	0.91-0.95	12,300-11,100

ranging in molecular weight from 12,000 to 66,000 (Table 19). As was the case for in vivo proteins; the patterns seen here for the two tissues were remarkably similar.

Fig. 26 compares the in vitro protein products of free and mb polysomes (freed of membranes prior to incubation as above) from hormone-stimulated growing tissue. Again the overall patterns were similar but several apparent differences were evident. The 19,000, 22,000 and 65,000 MW species seen in Fig. 25 were present in both fractions here. The 12,000 MW peak was found only in the mb products however while the 30,000 and 40,000 MW species were clearly defined only in the free ribosome products.

FIGURE 25. SDS polyacrylamide gel profiles of labelled proteins made in vitro by total polysomes from non-growing and growing tissue slices.

Labelled proteins isolated from the wheat embryo translation system programmed with Jerusalem artichoke polysomes were electrophoresed as in Fig. 24. Gels were cut into 2 mm segments. Each segment was solubilized in H_2O_2 and its radioactivity determined. Molecular weights of major peaks (a-g) are given in Table 19.

□ = Proteins made by polysomes of tissue slices aged for 24 h in water (non-growing).

■ = Proteins made by polysomes of tissue slices aged for 24 h in water followed by 24 h in 2,4-D plus kinetin (growing).

FIGURE 26. SDS polyacrylamide gel profiles of labelled proteins made in vitro by free and membrane-bound polysomes from growing tissue slices.

Free and mb polysomes were isolated by Procedure B from tissue slices aged for 24 h in water followed by 24 h in 2,4-D plus kinetin. Proteins made by these polysomes in wheat embryo system were analyzed as for Fig. 25.

Δ = Proteins made by free polysomes.

▲ = Proteins made by mb polysomes.

DISCUSSION

A. Ribosomal Changes During Activation and Growth of Jerusalem Artichoke Tuber Slices1) Activation During Aging in Water

A review of the literature has shown that the first 12 to 24 h of aging constitute a period of metabolic activation for excised plant storage tissues. Respiration and energy production, uptake and synthesis of small molecules, and synthesis and degradation of macromolecules all increase during this time. Incubation in water beyond 24 h seems to result in a gradual decline in these activities although this has not been examined in any detail.

In the present study excised Jerusalem artichoke tuber slices were monitored during aging for changes in cell growth, rRNA content and synthesis, ribosome activity in protein synthesis, protein content, and size and distribution of free and mb ribosome populations. During water aging these parameters fell into two groups: those which were stable or reached equilibrium shortly after tissue excision, and those which continued to change throughout the aging period.

The fresh weight of Jerusalem artichoke slices remained unchanged during aging in water (Fig. 8a), as did cellular protein content (Fig. 17). This is in

agreement with cytological studies by Fowke (1968) which showed that cytoplasmic volume and the number of organelles do not increase under these conditions, and indicates that no net growth occurs during aging. The rRNA content of tissue slices dropped 30% during the first two hours after excision, but then stabilized and remained essentially constant during the next 70 h in water (Fig. 9).

Labelled uridine was incorporated into ribosomes by 2 - 4 h after excision, and the rate of rRNA synthesis was stable between 2 and 24 h of aging. Since rRNA was being synthesized, while the total ribosome population did not increase, some turnover of ribosomes must have been occurring with a balance between synthesis and degradation established after the first 2 h. From cytological analysis of 5-FU treated tissue, Rose and Setterfield (1971) also concluded that fairly extensive ribosome turnover was occurring during prolonged aging of artichoke slices. The purpose of such turnover is still unclear but it has been proposed that pre-existing ribosomes (i.e. those present in tissue prior to activation) are incapable of protein synthesis or membrane association to form RLR. As will become evident below these suggestions are now untenable.

A drop in rRNA directly after excision similar to that in Fig. 9a was reported by Fraser and Loening (1974) and has been attributed to loss of RNA from

surface cells damaged by excision. On the basis of microscopic examination of the cut surfaces and estimation of the proportion of damaged cells, this explanation could not account for the rRNA loss seen in the slices used in the current studies. It is more likely that the drop was primarily due to rapid initiation of turnover resulting in ribosome depletion during the 2 h lag prior to the appearance of appreciable numbers of newly synthesized ribosomes in the cytoplasm.

In contrast to the stable ribosome content, the proportion of ribosomes engaged in protein synthesis changed rapidly following excision. Both polysome and high KCl activity profiles indicated that protein synthesis was very low in dormant cells but increased during aging, reaching a maximum at 24 h (Figs. 12 and 14). The mb ribosome population was small in freshly sliced tissue but represented 25% of total ribosomes by 24 h aging (Fig. 11). Further incubation in water resulted in a gradual decline in membrane association and in the activity of both free and mb ribosomes, but values never dropped to the levels found in intact tubers. During extended aging the RNA degradative activity, probably RNase, of Jerusalem artichoke extracts increased sharply, although its significance in vivo remains to be established.

In summary it has been shown that the rRNA and protein content of non-growing water-aged slices of

Jerusalem artichoke remained stable during prolonged aging. The rate of rRNA synthesis also remained constant between 2 and 24 h whereas ribosome activity and mb ribosome content of tissue increased during the initial 24 h period normally associated with metabolic activation, and then declined slowly.

2) Treatment of Activated Tissue with 2,4-D plus Kinetin

The addition of 2,4-D plus kinetin to 24 h aged (i.e. activated) tissue disrupted the equilibrium established during incubation in water. The most obvious effect was the pronounced gain in fresh weight by treated slices (Fig. 8). The rRNA content, which remained stable in water, increased three-fold over the first 24 h of treatment with hormones. Protein levels (Fig. 17) as well as cytoplasmic volume and organelles also increased (Fowke, 1968) showing that true cellular expansion was occurring in the presence of hormones.

The decline in the proportion of active ribosomes and mb ribosomes which normally occurred between 24 and 48 h aging was prevented by 2,4-D plus kinetin, with both parameters remaining at the maximum levels reached during activation. Davies and Larkins (1973) have shown a similar effect in peas, where auxin plus kinetin delay the decrease in polysomes seen in untreated stem segments. Because of the accumulation of rRNA, the

absolute number of active ribosomes and hence the protein synthesis capacity of expanding artichoke tuber tissue increased substantially during 24 h treatment with hormones.

In Jerusalem artichoke both growth and accumulation of RNA and protein have an absolute requirement for exogenous auxin. This is not the case in potato, carrot or beet slices where no discrete separation is found between activation and growth (see Literature Review and Table 3). Whether this difference is due to the presence of active endogenous auxin in these tissues or to genuine differences in regulation is not known.

Ribosome and protein values given in all figures were calculated on a gram fresh weight basis, to indicate their concentrations in the tissue, although such values could also have been expressed on a per cell basis. In water incubated slices the two measures are equivalent since no growth or cell division occurs, but in expanding tissue, estimates based on per cell calculations result in larger apparent changes because of growth without increase in cell number. This is illustrated in Fig. 17 where protein accumulation is presented both per cell and per gram fresh weight.

B. Ribosome Activity and Synthesis in Jerusalem
Artichoke Tuber Slices

1) Ribosome Activity

The ribosomes extracted from dormant cells were virtually all monomers (Figs. 12a and 13a), in agreement with earlier cytological observations by Rose and Setterfield (1971). Excision resulted in the rapid formation of polysomes and an increase in the proportion of ribosomes in polysomes up to 24 h. These profiles were consistent with reports of rapid increases in protein synthesis by excised plant tissue slices during aging (Kahl, 1973; Setterfield, 1963). Only extensively degraded polysome profiles have previously been published for Jerusalem artichoke (Bagni et al., 1972), although relatively intact polysomes have been extracted from a variety of other plant storage tissues (see Kahl, 1971a; 1973 and Literature Review). The polysomes obtained from free and mb fractions in the present study were both intact and functionally active as shown by their ability to support protein synthesis in vitro (Fig. 22).

The KCl dissociation method employed here to determine the fraction of ribosomes bearing nascent polypeptides has been used successfully with a number of plant and animal tissues. RNase activity which degrades the mRNA of polysomes does not affect the dissociation assay since nascent polypeptides are not released from

ribosomes even after considerable RNA degradation (Martin, 1973). The sucrose gradient profile in high KCl for freshly sliced tissue (Fig. 13a) provides a good internal check on the completeness of dissociation of inactive ribosomes under assay conditions. Measurements on polysome profiles from freshly sliced tissue (Fig. 12a) showed that 87% of the ribosomes sedimented as monosomes and would thus be expected to dissociate in KCl. In Table 6, 12.7% of ribosomes from unaged tissue were stable in KCl and 87.3% sedimented as subunits indicating that essentially complete dissociation of inactive ribosomes had occurred.

The increase in the percentage of ribosomes engaged in protein synthesis, as determined by resistance to KCl dissociation (Table 6), was consistent with qualitative results from direct polysome isolation. About 29% were already synthesizing protein by 1 h after excision. Since this was prior to the appearance of significant numbers of newly synthesized ribosomes in the cytoplasm, pre-existing ribosomes, i.e. those present in intact tubers, must have been participating at least in the initial development of protein synthesis. This conclusion agrees with observations by Kahl (1971b) and Leaver and Key (1967) that ribosomes from dormant tissue are functional and can support protein synthesis when supplied with synthetic message in vitro.

The maximum values of 68-69% active ribosomes after 24 h aging and 70-74% after 24 h hormone treatment compare with values of 60% for chick muscle, 53% for acites cells, and 35% for barley aleurone, based on the KCl dissociation method (Martin, 1973). One interesting feature of the present polysome and activity profiles is that the patterns seen during both aging and growth were virtually identical for the free and mb ribosome populations. Few comparisons between the activities of free and mb ribosomes have been carried out, but in mouse spleen the mb fraction was found to have a higher percentage of active ribosomes (56-71%) than did the free fraction (36-55%) (Storv and Martin, 1972).

The decrease in ribosomes sedimenting as polysomes between 24 and 48 h (Figs. 12b and 12c) was probably due in part to RNase activity during homogenization since the KCl dissociation profiles showed only a small drop in activity (from 68% to 54%) during this period. The latter probably give a better estimate of true changes in ribosome activity during periods when RNase levels rise.

2) Ribosome Synthesis

Incorporation studies with labelled uridine indicated that new ribosomes were made throughout the aging and hormone treatment periods tested. The rate

of rRNA synthesis appeared to be constant during activation since uptake of (^3H) uridine into tissue increased in the same fashion as incorporation into ribosomes (Table 10). Fraser and Loening (1974) obtained similar results with (^{32}P) orthophosphate label over a 12 h incubation period with Jerusalem artichoke explants. The present incorporation results agree with RNA labelling in autoradiographs (Rose and Setterfield, 1971; Setterfield, 1963) and with observations on nucleolar structural changes (Jordan and Chapman, 1971; Rose et al., 1972), which suggested considerable ribosome synthesis during aging. The lag period of about 1 - 2 h between excision and the appearance of new ribosomes was probably due to the time required for processing of the rRNA in the nucleus and does not necessarily reflect a delay in the initiation of rRNA synthesis. Nucleolar RNA labelling can be detected very early after excision but does not move to the cytoplasm for some time (Byrne and Setterfield, 1977; Setterfield, 1963). Preferential labelling of small ribosomal subunit RNA immediately after a 2 h pulse of label (Fig. 18) was consistent with the eukaryotic sequence of nucleolar RNA processing in which the 18S rRNA enters the cytoplasm more rapidly than the 28S species. In carrot root a lag of about 20 min has been found between the first appearance of 18S and 28S rRNAs in the cytoplasm (Leaver and Key, 1970).

Since pre-existing ribosomes are probably active in protein synthesis (Section B, 2) it is not immediately obvious why synthesis of new ribosomes should occur throughout aging. Perhaps "old" ribosomes have to be replaced because they are unstable or translationally inefficient. If the "lifetime" of ribosomes is restricted to a given number of rounds of translation and the ribosomes in quiescent tubers are near the end of their effective lifetimes then the substantial reactivation of protein synthesis during aging may in itself bring most pre-existing ribosomes to their turnover point. It has also been suggested previously that "old" ribosomes might be unable to associate with membranes to form RER (Gare, 1972).

Following a 2 h pulse of (^3H) uridine at both 12 - 24 and 22 - 24 h the free ribosomes showed a significantly higher specific activity than did the mb fraction. This difference disappeared if labelling was followed by a 12 h incubation period without label (Table 11). Sub-fractionation of total free and mb ribosomes showed that the specific activity differences between the two populations were present in both of their respective active and inactive fractions. The simplest model to explain the higher specific activity of free ribosomes is one in which newly synthesized ribosomes enter the free population directly, with some subsequently becoming membrane-bound through exchange between the two populations. The

lag required for equilibration would then result in a higher specific activity for free ribosomes immediately after a pulse of label while 12 h in unlabelled medium allows enough time for complete equilibration. The fact that little difference existed between labelling of free and mb ribosomes after a pulse at 2 - 4 h probably reflects rapid equilibration during a period when the mb population was still small but growing very rapidly. This model is consistent with observations in other tissues which indicate that exchange can occur between free and mb ribosomes and subunits both in vivo and in vitro (Izawa and Ichii, 1973; Mishra et al., 1972; Borgese et al., 1973). On the basis of kinetic data, Mechler and Yassalli (1975a; 1975b; 1975c) proposed that mb ribosomes from mouse myeloma cells were derived from a pool of free subunits.

If pre-existing ribosomes were incapable of binding to membranes the mb fraction should be composed almost entirely of newly synthesized ribosomes and should thus have a higher specific activity than the free population after a pulse of label given early in aging. This is clearly not the case (Table 11), and it is therefore likely that pre-existing ribosomes do contribute to the formation of RER during activation. These conclusions are consistent with the current view that mb ribosomes do not constitute a structurally distinct class, and do in fact exchange with free ribosomes.

3) The Effect of 5-FU on Ribosome Synthesis and Activity

The pyrimidine analog 5-FU has been found to reduce incorporation of label into Jerusalem artichoke ribosomes without at the same time affecting auxin-induced cell expansion (Fraser, 1975b; Rose and Setterfield, 1971). The present results show that incorporation of (^3H) uridine into ribosomes was inhibited 70% by 5-FU given concurrently with 2,4-D plus kinetin (Table 12). Since label was incorporated into ribosomes of hormone-treated tissue both during the 4 h labelling period and also during the subsequent 18 h in unlabelled medium, the inhibition reflects the net effect of 5-FU on incorporation over 24 h of treatment. Uptake of (^3H) uridine into tissue was not affected by the inhibitor, indicating that decreased incorporation was not the result of reduced availability of radioactive precursor, but was due to a reduction in ribosome production and/or a decrease in the stability of ribosomes made in the presence of 5-FU. Other studies on Jerusalem artichoke have shown that during treatment with this inhibitor, the granular zone disappears from the nucleolus (Rose et al., 1972) while labelling of nucleolar RNA is not as greatly affected as is rRNA (Fraser, 1975b). This suggests that 5-FU exerts at least part of its effect by interfering with processing of rRNA precursors at

the nucleolar level, leading to a net reduction in ribosome production. Reduced stability of ribosomes containing 5-FU may also contribute to the reduction of RNA label.

Interestingly, on the basis of specific activity measurements (Table 12), ribosomes which are produced in the presence of 5-FU accumulate preferentially in the inactive population. This is in contrast to the situation seen with hormones alone and is consistent with suggestions that incorporation of 5-FU into rRNA results in defective ribosomes (Rose and Setterfield, 1971).

Despite its profound effects on accumulation and activity of new ribosomes however, 5-FU only decreased the content of total and active ribosomes in the tissue by less than one third as compared to hormone-treated material (Table 13). Since the bulk of the ribosome population is normally synthesized during the period when 5-FU was applied (Fig. 9b), it becomes necessary to reconcile these two observations. One possible explanation is that 5-FU directly or indirectly interferes with turnover as well as production of functionally competent ribosomes. This would result in the situation seen in Tables 12 and 13 where, with hormones plus 5-FU, the ribosome population increases over the base level in water, but is composed to a larger extent of "old", i.e. unlabelled ribosomes, than is the population of cells incubated in hormones alone. Unfortunately

since pulse/chase experiments have not yet been successful in this tissue, it has not been possible to test this hypothesis by measuring rRNA turnover directly.

Regardless of the explanation, 5-FU incubated tissue does maintain much of its capacity for protein synthesis as measured by rRNA in active ribosomes (Table 13), and it does accumulate protein at a level comparable to tissue treated with 2,4-D plus kinetin only (Fig. 17). Since protein synthesis is required for cell expansion (Nooden, 1968), the insensitivity of expansion to 5-FU probably reflects the fact that the translational process, as distinct from ribosome production, is relatively unaffected by the inhibitor.

C. Membrane-Bound Ribosomes

1) Development of Membrane-Bound Ribosomes

Earlier cytological analyses have revealed that a population of ER-associated ribosomes appears during aging of Jerusalem artichoke tuber slices (Fowke and Setterfield, 1968; Rose and Setterfield, 1971). In the present study these observations have been confirmed and extended by the use of fractionation techniques to isolate and characterize the mb ribosomes. Membrane-bound ribosomes were found to be rare in quiescent cells but appeared rapidly during the activation period and by 24 h constituted one quarter of total tissue ribosomes

(Fig. 11). This increase appeared to be triggered by slicing and aeration of tissue and was not dependent on induction by exogenous hormones. A similar proliferation of mb ribosomes occurs in a variety of tissues including pea root, bean cotyledon, cultured fibroblasts, and liver slices, during periods of developmental change (see Tata, 1971; and Literature Review).

The value of 25% mb ribosomes in activated Jerusalem artichoke confirms the earlier tentative inclusion of this tissue in the large and diverse group of cell types with intermediate RER content (Table 4c). Jerusalem artichoke cells held in water past the 24 h activation period slowly lose mb ribosomes. The induction of growth by 2,4-D plus kinetin stabilizes the proportion of mb ribosomes at about the maximum level reached during activation and results in a three-fold increase in the absolute size of this population.

The values for percentage of ribosomes bound to membranes were derived from Procedure A fractionation and must be regarded as minimum estimates due to the possibility of some selective loss of RER during isolation, especially during the initial low speed spins. Experiments in which the percentage of mb ribosomes was estimated from differential rRNA yields before and after membrane solubilization (Gare, 1972) suggested that Procedure A leads to 70-80% recovery of RER. Few fractionation procedures have been clearly demonstrated to give

quantitative recovery of RER and those which appear to do so are not widely applicable (O'Toole and Pollak, 1974).

Procedure B resulted in less efficient recovery than Procedure A but was designed to subfractionate an RER-enriched fraction (Fraction III) from total membranes. Examination of Fraction III by electron microscopy revealed numerous rough microsomes, although some contamination by smooth vesicles was still evident (Fig. 20). The RNA/phospholipid and RNA/protein ratios of Fraction III were in the same range as those reported for rough microsome preparations isolated from rat liver (Adelman et al., 1974), and guinea pig pancreas (Tartakoff and Jamison, 1974).

NADH cytochrome c reductase, a membrane-bound enzyme which is associated with ER of higher plants (Lord et al., 1973) was also enriched in Fraction III. These data combined with density and sedimentation characteristics, and the lability of the membrane/ribosome association in detergents, confirm that the material isolated consisted of mb ribosomes derived from RER. The possibility that some free ribosomes were trapped by membranes during isolation can not be entirely excluded but is not likely to be significant. Non-specific association of ribosomes and membranes would be a function of the quantity of free ribosomes and membranes present and would thus be expected to be

highest in dormant artichoke tissue which in fact produces very few ribosomes sedimenting with membranes under the conditions used. Non-random NADH cytochrome c reductase distribution, and the kinetics of mb ribosome labelling are also not consistent with trapping (Table 11).

Some newly synthesized mb ribosomes dissociated in 0.8 M KCl or sedimented as monosomes in polysome gradients (Figs. 12a and 13a) and were thus considered to be inactive in protein synthesis. Non-synthesizing mb ribosomes have also been seen in other tissues (Storb and Martin, 1972) but have not been explicitly commented upon although they raise an interesting point. Despite such observations and despite the fact that in vitro ribosome binding to membranes can occur in the absence of protein synthesis (Baglioni et al., 1971) the prevailing models of the ribosome/membrane binding cycle (see for example Fig. 1b and Rolleston, 1974) propose that binding is necessarily associated with translation and do not account for bound ribosomes without nascent polypeptides. Clearly a general model of membrane/ribosome association has yet to be devised.

2) The Significance of Membrane-Bound Ribosomes in
Jerusalem Artichoke

What is the function of mb ribosomes in Jerusalem artichoke? Tables 8 and 9 show that the mb population synthesizes proteins as actively as free ribosomes, but this does not tell us whether the former has a specific role to play in tissue development. The prevailing view is that mb ribosomes synthesize specific classes of proteins and segregate them in the endomembrane system by vectorial transport. Secretory and membrane proteins are two classes which have been found to originate preferentially on mb ribosomes (see Rolleston, 1974 and Table 5). The evidence for this comes almost exclusively from work with animal secretory tissues such as liver and pancreas, and it is not yet known to what extent the model applies to tissues such as Jerusalem artichoke tuber and those listed in Table 4b and 4c. The present study presents evidence that mb ribosomes from the parenchyma cells of a higher plant behave similarly to those of animal secretory cells in some respects.

At least some of the proteins made in vitro by ribosomes of growing Jerusalem artichoke cells were vectorially transported into the microsomes on the basis of detergent release experiments (Table 17). The degree of protection of such proteins from protease attack (Table 18) was similar to that found in other vectorial

transport systems (Blobel and Dobberstein, 1975b; Shore 1977). To the author's knowledge the present report constitutes the first demonstration of vectorial transport by cytoplasmic ribosomes of a higher plant. Preliminary electrophoresis experiments (Fig. 26) also suggested that some differences exist between the in vitro products of free and mb ribosomes from such cells. Although the differences proved reproducible the complexity of the profiles obtained necessitates further investigation by higher resolution techniques such as two dimensional electrophoresis or immunologic analysis.

Since Jerusalem artichoke tuber cells do not produce significant quantities of what would normally be classed as secretory proteins, one of the established functions of mb ribosomes may be ruled out in the present case. Palade (1975) however has suggested that all plant cells can be considered to be secretory to the extent that they produce the components of their extracellular walls. For example cellulase, an exported enzyme important in cell wall extension, is made preferentially on RER in pea stems (Shore, 1974; Verma et al., 1975). It is possible therefore that the vectorially transported proteins in Jerusalem artichoke are in some way involved in wall modification during cell expansion.

Membrane-bound ribosomes may also be producing proteins for the membrane synthesis which occurs during activation and especially during growth of Jerusalem

artichoke and other tuber slices, although no direct evidence is yet available on this point. Considerable secondary processing of vectorially transported proteins destined for secretion occurs within the ER and golgi (Palade, 1975), but such processing is not necessarily restricted to secretory proteins. Glycoproteins and other modified polypeptides destined for internal use may well follow the same route. It is suggestive that dictyosomes, which are rare in non-growing artichoke cells where vectorial transport is low, increase rapidly in growing cells where vectorial transport is high.

Activated but non-growing cells showed little or no vectorial transport by mb ribosomes (Table 17), which would be expected if transported proteins were concerned primarily with cell growth. None of the currently available models assigns a function to non-transporting mb ribosomes, although they apparently exist in secretory systems and form the bulk of the mb population in rat muscle and brain (Andrews and Tata, 1971) and in 24 h aged Jerusalem artichoke.

Possibly protein segregation of the type suggested by Tata (1971) can occur in vivo without the need for vectorial transport. Merely the topological restriction involved in the translation of specific messages only on RER may be sufficient to compartmentalize the protein products. This would be consistent with suggestions by Rothman (1975) that some pancreatic

secretory proteins can be synthesized, processed, and exported without the necessity for vectorial transport or the continuous physical segregation of the proteins within the ER-golgi complex.

Another alternative is that non-transporting mb ribosomes synthesize the same proteins as free ribosomes. Although no product comparisons have yet been made between free and mb ribosomes from non-growing Jerusalem artichoke or similar tissues, such a situation could arise on the basis of the following model:

Translational segregation and vectorial transport are probably directed by mRNA rather than ribosomes. Under physiological conditions where little vectorial transport is required and hence little message directing such transport is present, polysomes containing "cytoplasmic" mRNA (i.e. derived from the free population) might attach to vacant ribosome binding sites on the ER. Such polysomes would consequently be producing the same proteins as free polysomes, and on the basis of the signal hypothesis, would be releasing their products directly into the cytoplasm. Non-selective binding of free and mb derived ribosomes by vacant ER binding sites has been demonstrated in vitro (see Shires et al., 1975 and Literature Review) and contrary to some suggestions (Blobel and Dobberstein, 1975a; 1975b) ribosome-ER associa-

tion does not appear to require vectorial transport since non-transporting mb ribosomes have been reported here and in other studies (Andrews and Tata, 1971).

Any definite assignment of function to mb ribosomes of activated or growing Jerusalem artichoke cells must await the identification and isolation of specific proteins, although in view of the variety of proteins made, this may prove difficult. Nevertheless, the deficiencies pointed out above for models based exclusively on data from secretory tissue, make it clear that any truly general understanding of mb ribosome function will require the added perspective of information derived from the class of intermediate RER containing cell types of which Jerusalem artichoke is a member.

D. Cell Growth

From data here and in the literature, the transition of Jerusalem artichoke tuber cells from quiescence to growth can be described as a three stage process, consisting of activation, growth induction by auxin, and cell expansion growth.

Excision of tissue slices leads to physiological activation, during which the capacity to respond to exogenous auxin develops. Tissue does not become competent to respond to auxin until approximately 24 h

after excision. This is illustrated in Fig. 8b which shows that 2,4-D plus kinetin supplied during the first 24 h did not effectively promote subsequent cell expansion. The development of competence is dependent on metabolism and the synthesis of macromolecules, since tissue incubated at 4°C (Adamson, 1962), or in the presence of puromycin (Masuda, 1966) or 5-FU (Rose and Setterfield, 1971) fails to develop the capacity to grow. The growth response is also greatly reduced when tissue is aged for more than 48 h prior to the application of auxin (Adamson, 1962) indicating that competence is restricted to a specific period of tissue development (24 to 48 h after excision).

The present results show that both ribosome activity and the occurrence of nb ribosomes reach a maximum at the time that tissue develops the ability to respond to hormones. Whether this is a fortuitous correlation or indicative of a causal relationship is unclear, although the subsequent development of vectorial transport in response to 2,4-D plus kinetin suggests that the appearance of nb ribosomes may be important to hormone-induced growth.

Stage two involves induction of growth in activated tissue by exogenous auxin. The optimum induction period occurs in the 24 h immediately following the 24 h activation period, and is characterized by a relatively low rate of cell expansion accompanied by

rapid accumulation of protein and rRNA (Fig. 9 and Table 17). When hormones were removed after the 24 h induction period, subsequent cell expansion proceeded normally (Fig. 8b) showing that auxin is required to induce but not to maintain growth.

RNA made during induction is probably critical to further expansion since actinomycin D is effective in preventing growth if given concurrently with auxin but not if its application is delayed for 24 h (Nooden, 1968). Inhibition of protein synthesis during this period also prevents growth (Nooden and Thimann, 1965; 1966). The fact that 5-FU does not affect expansion when given during the induction period has been used to argue that hormone-induced ribosome accumulation is not essential to growth in Jerusalem artichoke. However the present work has shown that this insensitivity is probably due to the fact that ribosome accumulation (as distinct from rRNA labelling) and protein synthesis are not greatly affected by 5-FU.

Some proteins made on mb ribosomes during induction of growth are vectorially transported into the ER (Tables 17 and 18). Since significant vectorial transport is seen only in expanding artichoke tuber cells, it is tempting to speculate that the transported proteins may be involved in growth-related processes such as membrane production or cell wall extension (Section C), but this remains to be established.

The third stage is characterized by rapid auxin-independent cell expansion. This occurs between hours 48 and 96 (Fig. 8) reaching a maximum rate of 0.9-1.2% increase in fresh weight per h. After hour 96 the rate declines and growth gradually ceases, probably due to depletion of endogenous storage reserves since increase in fresh weight occurs at the expense of stored materials and is paralleled by a decrease in dry weight (Masuda, 1966).

The stages of activation, induction, and cell expansion described above are not as clearly defined during the growth of roots and coleoptiles. Since the sequence leading to growth in such organs is usually preceded by active metabolism and cell division rather than quiescence, no discrete activation phase is evident. The maximum auxin-induced expansion rate in corn and oat coleoptiles or pea stem sections develops rapidly (within a few hours) without the 24 h induction period of slow growth seen in Jerusalem artichoke (Nooden, 1968; Nooden and Thimann, 1965; 1966). This may reflect a shorter induction period in the former, but may also be due to the fact that the stem sections expand to some extent even prior to the addition of exogenous auxin. Inhibitors of RNA and protein synthesis decrease auxin-induced growth in corn and oat coleoptiles but unlike Jerusalem artichoke, expansion in corn segments does not become less sensitive to actinomycin D as auxin treatment pro-

gresses (see Nooden, 1968).

Cell expansion in Jerusalem artichoke tuber slices is clearly preceded and accompanied by extensive metabolic changes. Interestingly however, on the basis of the present results, the effect of 2,4-D plus kinetin on RNA and protein was primarily quantitative. The distribution of ribosomes between active and inactive or free and mb fractions was not substantially altered by hormones, although the absolute size of each fraction increased due to ribosome accumulation. One dimensional SDS polyacrylamide electrophoresis of proteins made in vivo and in vitro indicated that auxin did not induce any major changes in the molecular weight distribution of polypeptides in Jerusalem artichoke cells (Figs. 23 and 25). Changes in enzyme activity and minor protein components undoubtedly did occur but would not have been detected by this method; for example, enzyme activation can occur without any shift in molecular weight. Nevertheless it appears that the effect of 2,4-D plus kinetin was to a large extent quantitative rather than qualitative, i.e. increase in existing components rather than drastic change in the types of components made.

CONCLUSIONS

- 1) rRNA and protein content of Jerusalem artichoke tissue stabilized rapidly after excision and remained constant during activation and aging in water up to 72 h. The addition of 2,4-D plus kinetin to activated tissue slices resulted in cell expansion accompanied by protein accumulation and a three-fold increase in rRNA after 24 h of treatment.
- 2) A mb ribosome population appeared during aging and by 24 h constituted 25% of total ribosomes. Subsequent incubation in water resulted in a decline in the proportion of mb ribosomes. In cells stimulated to grow by 2,4-D plus kinetin the proportion of mb ribosomes was maintained at the maximum level reached during aging, while the absolute size of the mb population increased three-fold.
- 3) Ribosome activity, as judged by polysome formation and KCl stability, increased rapidly during aging, reached a maximum at 24 h, and then slowly declined. The addition of 2,4-D plus kinetin to 24 h aged slices prevented the decline in protein synthesis activity. Free and mb ribosomes showed similar levels of activity during both water aging and treatment with hormones.

4) Ribosomes were synthesized at a constant rate throughout the first 24 h of aging. New ribosomes entered the free population directly, with some subsequently becoming mb through exchange between the two populations. Ribosomes present in tissue prior to excision probably participated in the formation of RER.

5) 5-FU supplied concurrently with 2,4-D plus kinetin, reduced incorporation of (^3H) uridine into ribosomes by 70% during 24 h of treatment. The inhibitor had a lesser effect on ribosome content and protein accumulation by the tissue, and probably affected turnover as well as production of ribosomes.

6) An RER fraction was isolated from both non-growing and growing Jerusalem artichoke slices. Membrane-bound ribosomes from growing cells transported 42% of their in vitro protein products into the microsomal vesicles, while mb ribosomes from non-growing tissue showed a low level of vectorial transport. Electrophoresis suggested the existence of some differences between the proteins produced by free and mb ribosomes from auxin-treated cells.

7) The transition of Jerusalem artichoke tuber cells from quiescence to growth is a three stage process consisting of activation, induction of growth by auxin,

and cell expansion. Physiological activation without growth occurs during the first 24 h after excision. Induction of growth by 2,4-D plus kinetin occurs during the subsequent 24 h period which is characterized by rapid accumulation of rRNA and protein, but a low rate of expansion. The final stage consists of rapid auxin-independent cell expansion over a 48 h period.

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