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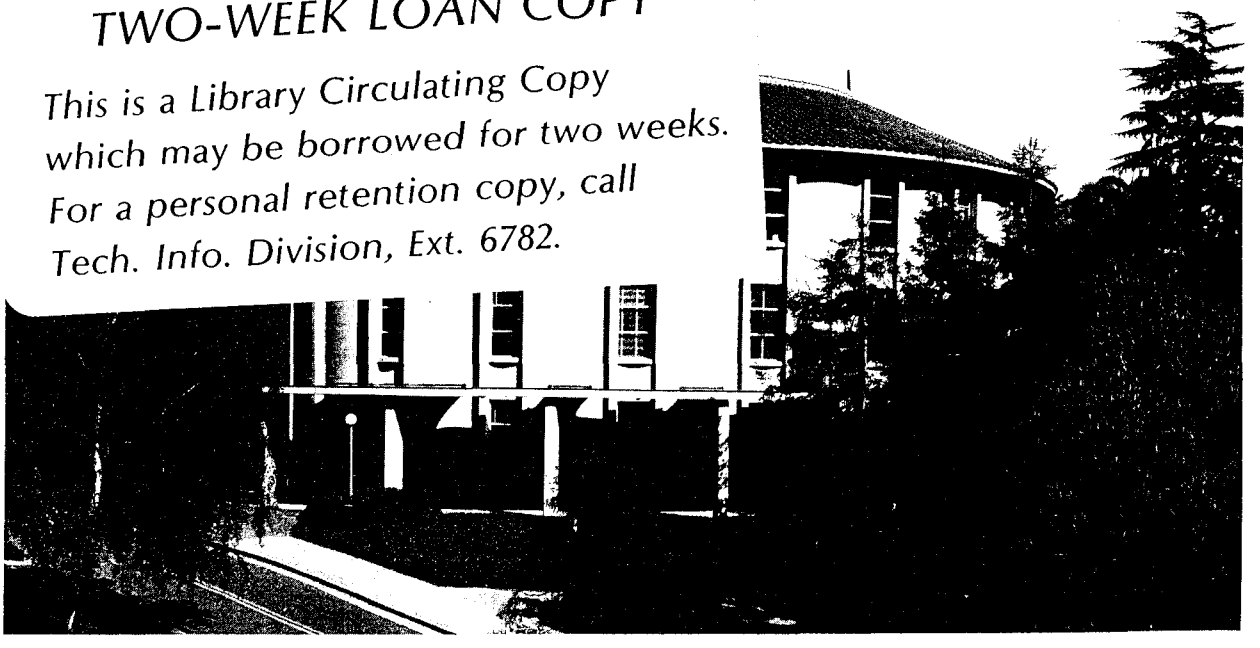

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The Differentiated State of Normal and Malignant Cells or How to Define a "Normal" Cell in Culture

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Summary

If there is one generalization that can be made from all the tissue and cell culture studies with regard to the differentiated state, it is this: since all functions are changed in culture, quantitatively and/or qualitatively, there is little or no "constitutive" regulation in higher organisms; i.e., the differentiated state of normal cells is unstable and the environment regulates gene expression.

Prologue

The search for a biochemical difference between cancer and normal cells is nearly as old as modern biochemistry itself. Despite many advances, as yet there is no single marker that defines a cancer cell. Our failure to identify such a marker stems in part from the fact that this is an absurd goal. Cancer is an exceedingly complex and multifacted disease. Indeed, it may well not be one disease; to hope for a single universal marker is, at best, wishful thinking. However, our failure to define a cancer cell may stem also from our inability to define the normal state. Given that every cell of an eukaryotic organism contains the complete genome of that organism, what causes a cell to become a liver or a muscle cell? And once a cell becomes part of these tissues, what factors regulate the expression of liver-related or muscle-related functions? The former question is beyond the scope of this review. The importance of the latter question lies in the fact that if we could understand what is normal, we would have a chance to understand not only how a cell becomes cancerous and diseased, but also how to prevent and reverse such changes.

To study regulation of gene expression was one of the major aims of tissue culturists during the early part of this century (Paul, 1970; Davidson, 1964). However, the challenge of growing pieces of tissues, and later, single cells proved to be all consuming and function was relegated to a secondary position in such attempts. Tissue-specific traits were altered so rapidly in culture and often in such unexpected ways that it was not always clear what one studied. This did not mean that there were not many attempts to study gene expression in cultured cells, but that the relation of such studies to regulation of function *in vivo* was not often clear. In the last ten years, however, an interest in model systems that have *in vivo* relevance has re-emerged and there are now many attempts to define conditions whereby functions can be retained in culture. For detailed description of various cell types and the kind of functions they express in culture, readers are referred to the following general reviews (Wigley, 1975; Cox and King, 1975; Vasiliev and Gelfand; 1977). For the behavior of malignant cells in culture and the relation of transformation in culture to tumor formation *in vivo*, the excellent reviews by Auersperg (1974), Auersperg and Finnegan (1974), and Ponten (1976) should be consulted. For a very concise history of cell culture, see the introduction to "Cell and Tissue Culture " (Paul, 1970).

An earlier and lucid account of function in cultured cells by Davidson, (1964) is still current. "Nucleus and Cytoplasm," (Harris, 1974) "Cancer a Problem of Developmental Biology," (Pierce, *et al.*, 1978) and various publications from Mintz's laboratory (e.g., Mintz, 1978, a.b.) should be consulted by those interested in the relation of differentiation and malignancy and the underlying mechanisms of gene regulation in higher organisms. There are also excellent reviews of

studies on gene expression using somatic cell hybridizations (Ephrussi, 1972; Davis and Adelberg, 1974; Davidson, 1974, Bernhard, 1976; Ringertz and Savage 1976).

The Scope

One of the purposes of the ambitious title of the present review is to allow me to discuss a number of my prejudices and convictions with regard to the latter part of the title. These are grouped in Part I of this review in the belief that a discussion of the pros and cons of these issues will help clarify some of the current misconceptions about cultured cells and how best to study them. Nevertheless, by necessity the treatment of each category is brief and at times even cursory. To do justice to most of the areas covered in Part I, each would require an additional review. Some of the topics in part I are reintroduced in part II in more detail. Due to the diversity of the topics touched upon in the first section, such an approach appeared justified. In the second part of the review, rather than compiling a catalogue of all the normal, transformed and malignant cells that have been reported to have differentiated functions, I have chosen to limit the discussion to a complex function, collagen, and a complex cell-type, mammary epithelium. Collagen will be discussed as an example of a "single" trait expressed by many cell types, and mammary epithelium will be discussed as a single cell type with many traits. The choice of these two areas is not only a reflection of current research interest in my laboratory, but is further justified as these provide two very different yet representative areas of biological research on gene regulation today. The term 'gene regulation' is used loosely in the review and refers to phenotypic expression. No attempt has been made

to include tissue and organ culture or *in vivo* studies and the relevant literature is cited only in instances where a direct comparison to culture systems was necessary.

I. How to Define "Normal" in Culture

> "In older fields of biology, as in molecular biology, the system was the organism (drosophila, sea urchin, amphibian, E. coli, etc.); but in cell culture, the system has to be created"
>
> (Howard Green, 1978)

A. Terminology

Clear definitions and the correct usage of words are necessary for better communication. Unfortunately, currently, there is little consensus on the usage of terms commonly seen in the cell culture literature. The readers are referred to the appendix at the end of this review for a brief discussion of some of the confusing terminologies especially the usage of the terms "*in vitro*", "*in vivo*", and "in culture" (Appendix 1).

B. Loss of Function upon Culturing: A Liability or an Asset?

The dilemma confronting those scientists who are interested in using cultured cells as model systems for studying gene regulation, is the dramatic alteration in quantity and quality of function when cells are placed in culture (Sato *et al.*, 1960; Davidson, 1964; Wigley, 1975; Schwarz and Bissell, 1977 etc.). The phenomenon is usually viewed as a liability: the very thing you want to study is altered or lost. This has prevented some scientists from seriously considering cultured cells as appropriate model systems. Others basically disregard this fact and study whatever function and regulation that may be expressed in culture, yet others are trying to improve conditions for more accurate reflection of

in vivo situations. Nevertheless, there is a positive and poorly appreciated side to this loss of function by cells in culture. The easy availability of metabolic and regulatory mutants in bacteria led to tremendous advances in our knowledge of gene regulation in prokaryotic systems. There has been no such easy access to mutants in higher organisms. With the exception of recent availablity of cells from patients with genetic disorders, even when mutants are obtained, they are usually derived from cell lines which are many generations removed from the host and which are not normal in the sense defined here (see below).

The modulation of function in culture may not only be "the next best thing to mutants" (Schwarz and Bissell, 1977), but indeed the best tool for studying gene expression in eukaryotes. This is because in animal cells even when the right "mutants" are found, it is not always easy to establish a cause and effect relationship between a lesion in the DNA sequence and the function in question (Davidson, 1974; Harris, 1974). Furthermore, events that accompany the loss of function in culture could be used to advantage in studying regulation of normal physiology (Bissell, D.M. 1976). In the case of rat hepatocytes, for example, the rapid decrease in cytochrome P-450 upon culturing was seen to be associated with a reciprocal increase in microsomal heme oxygenase (Bissell, D.M. *et al.*, 1974) leading to a re-examination of the regulation of microsomal heme oxygenase *in vivo*. Thus, the events that led to loss of function in culture also allowed a more accurate intepretation of the data available from *in vivo* studies. In studies of gene regulation, the most useful situation is when a function can be modulated. It is becoming exceedingly clear that almost all functional changes in primary cultures and even some in cell lines are due to

phenotypic alteration rather than somatic mutation (Davidson, 1974; Auersperg and Finnegan, 1974). It is possible, at least theoretically, to create conditions in culture where the function is restored. Indeed, partial and complete restoration of functions has been demonstrated in many instances (see below and also the reviews cited above). With both the increased availability of truly differentiated cells, and increased recognition of the usefulness of phenotypic changes, primary cultured cells may become model systems of choice in studies of gene regulation and differentiation in higher organisms.

C. The Important Variables

> "The cell in culture is an adaptable organism; otherwise, it would not survive the environmental insults heaped on it by callous investigators"
>
> (Gregg, 1972).

Cell culture can be an ideal system for studying gene regulation, because at least in theory, the environment may be controlled at will. In practice, however, the standard techniques of cell culture do not afford the degree of control necessary for precisely defining the environment. A seemingly single variable is often accompanied by many others which make the interpretation of the data exceedingly difficult. This is best exemplified by population density, an important variable for gene expression in cultured cells. If high and low density population of a single cell type seeded in the same medium are compared, one has to face the fact that the cells at low density will grow much faster than cells in high density, (Stoker and Rubin, 1967) and that they will pick up nutrients and metabolize them at different rates and with a different

pattern (Bissell et al., 1972; Dolberg et al., 1975). These in turn, would lead to a rapid and radical change in medium composition and pH. Furthermore, the cells at high density would form an extracellular matrix which would affect their shape, rate of growth, and other functions. We therefore are faced with a dilemma. If a difference in function is observed, is it due to population density per se or is it a change in growth rate? Is it the pH or the composition of the medium? Is it cell shape or cell-matrix interaction? The situation is even worse when one compares normal cells with their oncogenic counterparts. In addition to the above difficulties, the transformed cells secrete different amounts of extracellular materials (for example see Muscatelli and Rubin, 1976) and "leak" other factors into medium (Bissell et al., 1971; Folkman, 1975). The compounds secreted in the medium or deposited as matrix, in turn, alter the cellular response. These complexities may explain some of the examples of seemingly contradictory results in the literature.

The recent surge of interest in 'defined' culture medium is indicative of the realization that complex and undefined mixtures such as serum, especially at the very high concentrations used by most investigators, is detrimental to a clear analysis of events accompanying functional changes. PH and the composition of the medium are also important variables. Changes in pH lead to large changes in growth rates (Rubin, 1971; Eagle, 1974) and, undoubtedly, many changes in metabolite patterns. These, in turn, alter the pattern of gene expression. Most tissue culture medium require an ambient atmosphere of CO_2 and air. Each time that an incubator door is opened, or each time the cells are removed for experimental manipulations, this atmosphere is radically altered. In trying to overcome some of these obstacles, a few years ago we devised a

"steady state" apparatus where short term experiments could be performed under a constant temperature and pH (Bissell et al., 1973; Bassham et al., 1974). However, most studies of gene regulation require days and not hours, and it is important to be sensitive to the constant changes in the environment of the cultures which would affect the cellular responses.

In addition to a correct and defined medium (Hayashi and Sato, 1976), growth rates and population densities need to be controlled independently and vigorously. High cell density and the substratum on which the cells are grown (see Section IIB 2), in turn, lead to changes in cell shape (or "topography" of the culture) which as recent studies indicate may be instrumental in regulating the rate of growth (Maroudas, 1973; Maroudas et al; 1973; Folkman and Moscona, 1978; Gospodarowicz et al., 1978; Rath and Reddi, 1979; Vlodavsky, et al., 1980), transport properties (Bissell et al., 1977), the nature of m-RNA processing (Benecke, et al., 1978; Farmer, et al., 1978), and the type of function expressed (Allan and Harrison, 1980). In short, it should be appreciated by now, that the multifaceted nature of variables in cultured cells preclude their treatment as the higher organism's substitute for E. coli (Green and Todaro, 1967).

D. What is a Legitimate Differentiated Trait in culture?

1) Morphology

It is no longer acceptable to use gross morphology as the sole criterion of the origin of the cells in culture. Cells in culture even at the primary stages can assume different morphological configurations depending on the culture conditions. The simple generalizations such as "epithelial" or "fibroblastic" morphologies as an indication of the

origin of cells in vivo (from epithelial or connective tissues) is at best misleading and there are countless examples of this in the literature. Nevertheless, morphology, especially at the level of electron microscope remains a legitimate and important differentiated marker. This is especially true for cell types with distinct morphological characteristics such as secretory epithelium. The scope of this review does not allow an in depth documentation of this point. For examples of distinctive morphologies both as a characteristic of the tissue of origin and also as a criterion to distinguish normal from malignant, the readers should refer to individual papers (e. g., see Campbell et al., 1971; Emerman and Pitelka, 1977).

Morphology, even at the level of light microscope remains one of the highly used criteria to distinguish non-transformed and transformed cells in culture. In the case of fibroblasts, non-transformed cells are usually flat and form an organized monolayer, transformed cells pile up in disorganized arrays (e.g., see Bissell et al., 1974 and Schwarz et al., 1978 for examples of virally-transformed morphology). The correlation with other criteria of transformation is generally good and seems to hold true for both viral and chemical transformations in culture. This, however, does not hold true for in vivo derived tumors which are transplanted to culture. Under usual culture conditions, tumor cells often look more "normal" than normal cells in terms of resemblance to the tissue of origin (Auersperg, 1974). This important point (i.e., the relative "normalcy" of tumor cells in culture) merits further discussion and will be considered in more detail below. It should be remembered that histology continues to be the single most accurate criterion of malignancy in vivo.

2. Metabolic Patterns as Differentiated Traits--Luxury vs. Housekeeping Molecules: Is the Distinction Necessary?

These widely used terms, apparently coined by Ephrussi (1972, p. 53) and popularized by Holtzer and his colleagues (Holtzer and Abbott, 1968), until recently have dominated our way of distinguishing tissue-specific (differentiated) from common "ubiquitous" (non-differentiated) traits. By definition then, the molecules that are shared by various cell types and the enzymes involved in their production or catabolism are not considered to be markers for the differentiated state and by inference are assumed not to be regulated in a tissue-specific manner or be markers for the differentiated state (see also Rutter et al.'s, definitions of "primary", "secondary" and "tertiary" proteins (1973)). To my mind, this distinction between the "differentiated" and "non-differentiated" traits is both arbitrary and unnecessary. The distinction may have served a useful purpose in the past. However, at present it causes confusion, and worse, it implies a scientific categorization and truth which simply is not well taken. Recently, the terms are confused even further. The initial "housekeeping molecules" were supposed to refer to intermediary metabolites and functions that were necessary for cell survival (Holtzer and Abbott, 1968). More recently, some investigators refer to cytskeleton elements such as actin and myosin, or even proteins such as collagen as "housekeeping molecules", while others refer to them as "luxury proteins". Indeed, both the initial definitions and the connotations of these words may have kept us from appreciating and understanding the eukaryotic cell in its totality. This confusion may also have contributed to our disregard for culture conditions and our inability to keep cells differentiated in culture.

Furthermore, such a distinction ignores the differentiated nature of metabolic control. Despite the fact that intermediary metabolism is common to all cells, it is well established in vivo that the regulation of such metabolism is unique for each cell type and organ (refer to physiology and biochemistry text books). In culture, because of the trend to define conditions for growth, all cells become similar metabolically; they all become increasingly glycolytic (Paul, 1970).

Nevertheless, in primary cultures, it can be demonstrated that the catabolism of even the most common carbon source, glucose, is regulated in a tissue-specific manner. When primary avian hepatocytes and fibroblasts--derived from the same embryo and maintained under similar conditions--are placed in culture, they have strikingly different metabolic patterns even 48 hr after seeding (Fig. 1). Liver specific metabolism is also observed in primary cultures of rat hepatocytes while "liver" cell lines show metabolite patterns which resemble that of the fibroblast (Fig. 2) (Bissell, et al., 1978). Mammary cells have distinctly different metabolite patterns not only from other tissues, but also during the mammary gland development (Emerman and Bissell, 1979 a,b; see Section II B2), and the list may be extended many fold. The differences observed are not the simple result of varying rates of glucose transport, but reflect instead radically different utilization of various metabolic pathways. While this may be apparent to animal physiologists, it is rarely appreciated by biochemists and cell and molecular biologists who work with cultured cells. I would like to submit that these metabolite "fingerprints" are, in fact, as specific as are production of albumin by liver or type I collagen by bone and tendon. How these patterns change in culture and whether the shifts are the

result or the cause of other functional changes is discussed in Section IF below.

3. Quantity as a Tissue Specific and Developmental Marker

The ability to express a tissue specific gene and not the quantitative expression of the gene has been the traditional definition of a differentiated cell in culture (Green and Todaro, 1967; Holtzer and Abbott, 1968; Kafatos, 1972). This definition, however, has its limitation both *in vivo* and in culture. In order to study the regulation of function in a normal cell in culture, the mere expression of the function is necessary but may not be sufficient. This point, however, does not seem to have been appreciated previously. In the important experiments of pioneers in this field (Holtzer *et al.*, 1960; Whittaker, 1963; Green and Goldberg, 1964; Prockop *et al.*, 1964; Cahn and Cahn, 1966; Lash, 1968, etc.), the absolute level of function, i.e., the level *in vivo* vs. the level in cell culture, was hardly considered. This was understandable in the early studies, but it is time to take a critical look at the question of "quantity" and the traditional definition of a differentiated cell.

To begin with, the earlier definitions assumed that all differentiated functions are unique to one cell type. However, even *in vivo* this definition does not hold true for many functions. For example, bone, cartilage, and tendon all secrete chondroitin sulfate, but to differing degrees. With collagen (see part II A), the situation is even more complex. Many cells in the body have the capacity to synthesize and secrete some collagen; it is the type but also the quantity that distinguishes the various tissues. There are only a few cell types, for

example, tendon or bone, which have the mission and thus the capacity for collagen to be their major cellular product. Collagen comprises 70% of the dry weight of skin and tendon, and 90% of the organic matrix of bone (Grant et al., 1972). In these cells, aside from the type, the quantity of collagen synthesized best defines the cell, i.e., quantity becomes quality.

In cell culture, the previous definition of a differentiated cell is even more inadequate (see Schwarz, 1975). Green et al.,(1966c) demonstrated to their surprise that non-fibroblast cell lines produce collagen. With the more recent demonstrations that some epithelial cells may, in fact, synthesize collagen (Goodfellow, et al., 1969; Dodson and Hay, 1971; Linsenmayer, et al., 1977; Stenn, et al., 1979 etc.; see also Part II A) it can be argued that this is no longer surprising. However, Langness and Udenfriend (1974), used cloned non-fibroblast cell lines to show that cells which were not known for their collagen production in vivo (neuroblasts, kidney) do, indeed, make appreciable amounts of collagen when compared to L-929, a line derived from fibroblastic origin. Therefore, the mere demonstration of collagen biosynthesis would be a poor criterion for differentiation of fibroblasts in cell culture. And this holds true for other functions and other cell types.

The changes in quantitative expression of function in culture as a function of time and subculturing especially when embryonic cells are utilized may yet signify another complexity: the cell which produces less of a given function and can no longer be modulated, may have been switched to the next developmental stage, i.e., it may have "matured" in culture (Schwarz, et al., 1979). When avian tendon cells are placed in culture at low density and under strictly defined conditions (Schwarz and

Bissell, 1977), four distinct periods can be defined in terms of percentage of collagen synthesis (Fig. 3): A, a period lasting less than a day where the tendon cells will produce collagen at the in ovo level regardless of density. During this period, the cells seem to retain the in ovo information and produce 30-35% collagen. B, a period where the cells would produce a low level of collagen even in the presence of ascorbic acid if placed at low density. This drop is easily reversible. As cells approach high density they return to the in ovo level (C period) provided that ascorbic acid is present or is added. And, D., the postconfluent stage, where the collagen begins to fall again after a period of constant production. This latter drop is different from the B period, since subculturing these cells under the identical conditions to B cells will not lead to restoration of function at confluency while subculturing B cells will lead to a repetition of the primary sequence. We have demonstrated (Fig. 4) that if cells are subcultured just before confluency, the B, C and D periods will be repeated. However, when they are subcultured postconfluency, the cells only demonstrate a D period regardless of density and will not be modulated by density or ascorbic acid. For cells to be modulated optimally, there are additional complex requirements such as a "correct" growth rate and exact serum concentrations. But even when these criteria are met, the difference in the B and D periods persists (Schwarz, et al., 1979). Thus, this seems to be a qualitative and not a trivial difference. We have proposed that cells in the D period are analogous to cells in a grown bird: they have "matured" in culture by some as yet unknown signal. The signal could be a critical density itself or a metabolic and/or "positional" consequence of increased density (Bissell et al., 1972, 1977; Folkman and Moscona, 1978). How the

information is "locked" in place so that it gets transmitted in an apparently irreversible fashion to the progenies is, of course, one of the important problems of cellular and developmental biology.

The proposed developmental switch would be easier to accept if the changes were qualitative, e.g., if one could show a switch from a fetal to an adult function similar to isozyme changes that occur in cultured muscle cells (Cardenas, et al., 1979) or hemoglobin changes in sheep red blood cells (Zanjani, et al., 1979) or serum proteins alterations in chick embryo hepatocytes (Grieninger and Granick, 1975.) Nevertheless, it should be appreciated that quantity of function also is strictly regulated and altered after birth and especially by the time the organism matures. For example, in 16-day old avian tendon cells in ovo collagen synthesis is 30% of total protein synthesis (Peterkofsky, 1972; Dehm and Prockop, 1971; Schwarz and Bissell, 1977), upon hatching and while the chicken is still growing it is 18%, and in an old bird (about 5 years) it is less than 1% (Schwarz, et al., 1979). We know very little about the events that bring about such drastic transitions in the level and proportion of a given function. If a critical cell density, or a particular metabolite pattern is responsible for changes in vivo, such change can also be brought about in culture by similar factors. Recent studies in our laboratory (Emerman and Bissell, unpublished) indicate that when mammary epithelial cells from pregnant mice are placed on floating collagen gels, they retain both the qualitative and quantative expression of their various functions including the glucose metabolite patterns. However, the lactating epithelial cells rapidly alter their metabolite patterns and lose their ability to synthesize lactose. On the surface, this loss may be regarded as non-specific. On closer examina-

tion, however, it is observed that the lactating cells appear to have reverted to a "pregnant" state on floating collagen gels as judged by various criteria examined. While the lactose decreases, glycogen synthesis increases and the ratio of glycogen to lactose stabilizes at the level of epithelial cells from the pregnant mice; i.e. the change in the quantity of these functions represents a different stage of mammary gland development (for details, see Section IIB). In short, quantitative changes in culture should not be treated as mere irrelevant artifacts of culture conditions or "dedifferentiation" (see Appendix I). The decrease in quantity of function may be an indication of an altered cell with entirely different regulatory mechanisms than the cell in vivo. Both the absolute and relative (percent) level of function should therefore be consided when a cell in culture is compared to its in vivo counterpart (see also Schwarz, 1975).

E. Should cultured tumor cells and cell lines be used to study normal regulation of function?

Until very recently, most of the studies on gene expression were performed on cultured tumor cells or on permanent cell lines from rodents. There are two reasons for this: 1) Tumor cells and rodent cell lines are easier to establish in culture and many are available, and 2) tumor cells usually retain some tissue specific function while normal cells even when established as a line, lose their function very rapidly. (For an original and precise treatment of this point, see Auersberg, 1974, and Fig. 5). I would like to propose that precisely because tumor cells retain in culture whatever function they were exhibiting in vivo, they should not be used as model systems for studying how genes are regulated in normal tissue. This may sound like a contradiction, but it is not. As Auesperg explains, the

inability of tumor cells to respond to drastic changes imposed on them by culture conditions is, in itself, an indication of the fact that they have become autonomous, and that as such they do not respond to environmental stimuli. Herein lies their liability: by not responding, and/or by being "unpredictable", they would make poor models for understanding normal physiology. While it is widely appreciated that tumor cells have drastically altered growth regulation (no one would think of using tumor cells to study normal growth regulation) it is less appreciated that despite the fact that they may express some tissue specific functions in culture, the regulation of such function could be totally different from normal cells.

This does not mean that tumor cells, especially freshly explanted ones or those from embryonic origin, will not be modulated. Under special *in vivo* or culture conditions, they even can revert to a "normal" phenotype (Schubert *et al.*, 1971; Wylie *et al.*, 1973; Auersperg and Erber, 1976; Pierce *et al.*, 1978; Sachs, 1978; Auersperg, 1978, etc.). Nevertheless, it is difficult to define conditions for modulation, so that the events bear some resemblance to the normal regulation. The unpredictability of the response [e.g., the fact that chick embryo fibroblast infected with Rous sarcoma virus express the globin gene by making globin mRNA (Groudine and Weintraub, 1975)] and our inability to know how the "irrelevant" response, (both those that we can detect and those that we don't suspect), will affect the regulation of the function we are interested in, makes tumor cells entirely unsatisfactory models in studies of regulation of gene expression (see also Bissell, *et al*, 1979b).

Many of the arguments used against tumor cells also apply to cell lines some of which are indeed premalignant. While there may be a significant difference in growth regulation between "normal" cell lines

and their transformed counterparts, in many other respects the cell lines are closer to the transformed cells than they are to the normal tissue from which they were derived. I will discuss only two examples of such differences derived from our own experiments. If one compares the glucose-derived metabolite patterns of primary rat hepatocytes in monolayer culture with that of two commonly used "liver" cell lines, HTC and BRL, one observes both quantitative and qualitative differences (Fig. 2). Utilization of glucose is almost identical in HTC and BRL cells and exceeds that of the monolayer culture by 20-30 folds. In both cell types most of the glucose is converted to lactate (Bissell, D.M. et al., 1978). Thus BRL cells which were derived from normal liver have little in common with the primary hepatocytes and resemble HTC cells which were derived from hepatomas. There are many other metabolic differences between the two cell lines on the one hand and the primary culture on the other. For example, if glucose is removed from the medium and cells are incubated for 24 hrs. in the absence of serum, the level of ATP is retained in the primary cultures while it is reduced to 40% of control in the cell lines (Fig. 6; Bissell, et al., 1978). Furthermore, there are striking differences between the ability of the cell lines to utilize hexoses other than glucose. Sorbitol and fructose are not metabolized further in liver cell lines while the primary cultures utilize both of these sugars even more readily than glucose (Fig. 7; Levine et al., 1978). Undoubtedly, there are additional metabolic differences which affect the physiology of the cells and could alter their responses to environmental effectors.

The response of fibroblasts to ascorbic acid addition is another example of where the "normal" cell lines are more similar to transformed

cells than to the differentiated cells *in vivo*. It has been demonstrated in the past that while cell lines respond to ascorbic acid by increasing the level of hyroxyproline in collagen (Levene and Bates, 1975; see Section II A), the synthesis of collagen itself is totally unaffected by any of the cell lines studied (Peterkofsky, 1972a; Levene and Bates, 1975). This is in contrast to the situation *in vivo* where removal of ascorbic acid (scurvy) leads to appreciable reduction in the rate of synthesis of collagen and its addition leads to restoration of the level of synthesis (Barnes, 1975). It also contrasts with the situation in primary avian tendon cells. Under defined conditions, the presence of ascorbic acid or its removal not only modulates the rate of hydroxylation of proline residues but also the rate of collagen synthesis as would be expected from *in vivo* studies (Fig. 8; Schwarz and Bissell, 1977; Schwarz, R.I. Mandell, R., and Bissell, M.J., in preparation). For a more detailed discussion of this point see Part IIA 3a. In contrast, Rous sarcoma virus transformed tendon cells, while producing fully hdyroxylated collagen in the presence of ascorbic acid (Schwarz, R.I. and Bissell, M.J., unpublished) are similar to cell lines in that they do not modulate the rate of collagen synthesis (Schwarz *et al.*, 1978). To reiterate, the use of permanent cell lines whether from normal or malignant origins as model systems for studies of regulation of gene expression may lead to misleading results. This is especially true for rodent cells (see Ponten, 1976). Cell lines may, nevertheless, give important insight into the nature of malignant and premilignant states especially when used in conjunction with differentiated normal cells.

F. Metabolite Levels, "Positional Control", and Gene Regulation: A Working Hypothesis

The relation of cell shape and extracellular matrix to the regulation of growth and function is a rapidly evolving field. (For simplicity, I will refer to relation of cell shape to gene regulation as "positional control", a phenomenon which may or may not be the same as that described by embryologists in formation of tissues). The importance of such regulation in differentiation has been recognized for at least 30 years (Grobstein, 1953). Others are beginning to emphasize that "positional control" is not only important during embryogenesis and tissue formation (for reviews see Kratochwil, 1972; Grobstein, 1975; and Wessels, 1977), but also important for growth and gene regulation after the organs have been formed (Maroudas, 1973; Folkman and Greenspan, 1975; Folkman and Masocna, 1978; Gospodarowicz, et al., 1978; Benecke, et al., 1978; Farmer, et al., 1978; Emerman et al., 1979; Rath and Reddi,1979; Vlodavsky, et al., 1980, etc.). The importance of cell shape in gene expression is demonstrated strikingly in a series of ingenious fusions between cells capable of erythroid and lymphoid differentiation (Allan and Harrison, 1980). These investigators isolated both adherent and suspended hybrids and demonstrated that only suspended hybrids were inducible for hemoglobin. The importance of cell substrate interaction in regulation of function is discussed further in Section IIB. The importance of metabolite levels in regulation of tissue specific functions in eukaryotes, however, is hardly appreciated.

Any investigator who proposes that metabolites and the enzymes of intermediary metabolism should be considered as an integral part of tissue-specific functions is faced with the extensive literature which in one form or the other assumes that this is not the case. The following

quotation from a theoretical article on the control of development by Caplan and Ordahl (1978) demonstrates the dilemma:

> "These observations can be used to argue that at least two classes of gene products are present: (i) a group that codes for 'housekeeping' proteins or molecules necessary for cell survival and common to all phenotypes and (ii) a group coding for phenotype-specific proteins whose presence in sufficient concentrations dictates the phenotypic properties of a specific cell" (the emphasis is mine).

The dilemma is not that there may indeed be at least two different classes of gene products--that is obvious enough--but that the former is assumed not to be part of the "phenotypic properties" of the cell or worse that the latter "dictates" such a phenotype. The model I would like to present has to first overcome this widespread and, to my mind, misleading notion before it can be put to any critical test. Not only are metabolite patterns tissue-specific but some of the seemingly common enzymes of intermediary metabolism could be used as specific markers for certain tissues or even for sub-populations within tissues. A good example is the case of glycerol-3-phosphate dehydrogenase (GPDH), a soluble enzyme which catalyzes the reversible oxidation of α-glycerol phosphate to dihydroxy-acetone phosphate, and which is easily detectable in cultured fibroblasts (e.g., see Bissell et al., 1976). There are at least two immunological forms of GPDH in human tissues one of which appears to be heart specific (McGinnis and de Vellis, 1979). The brain enzyme is regulated by the adrenal and pituitary glands and its induction by glucocorticoids is specific to central nervous tissue (for review see de Vellis et al, 1978). While the metabolic consequences of GPDH induction in brain still remain

to be determined, both GPDH and its specific induction by steriods can be classified as differentiated markers for brain in general and for oligodendrocytes in particular. It is interesting to note that in general hormones are not known to induce or repress the traditional tissue-specific products directly. Their initial action is usually on the genes responsible for enzymes of metabolic pathways which, in turn, would alter the metabolite levels.

I would like to propose that metabolic pathways such as glycolysis, lipid synthesis, etc. not only do not go their own independent and merry ways in different tissues but that the metabolite levels and ratios indeed are linked to the rest of the cellular functions and at times may actually determine what other genes should be expressed and to what degree. In other words, not only cAMP, but other small metabolites such as ADP, ATP, NAD^+, citrate, lactate, phosphorylated sugars, etc., or their ratios could have the potential to regulate the differentiated state. Additional factors such as hormones that radically alter cellular response could mediate such changes by shifting the balance of metabolic control.

It is usually observed that in animal tissues only the liver and the small intestine have the built in capacity for extreme repression and induction mechanisms (Lehninger, 1975). Such mechanisms are necessary, since in the vertebrates, it is these tissues that are first exposed to the multitude of incoming nutrients. These organs thus maintain the homeostasis of the organism. However, this same filtering mechanism perhaps explains the constancy of gene expression in other tissues. Rather than being incapable of responding to rapid induction and repression mechanisms, other tissues in vivo are exposed to a constant and regulated nutrient supply via the blood and interstitial fluid. However,

as soon as this constant environment is disturbed, these tissues also respond--usually by modulating functions and expressing genes that are not supposed to be modulated or expressed. An example of this phenomenon is the induction of metallothioneins, the binding proteins which are usually found only in liver and kidney in vivo (Wisniewska et al, 1970; Squibb and Cousins, 1977). Yet, regardless of tissue of origin, cells in culture once exposed to Cd^{++} or Zn^{++} are capable of producing the protein (Shaikh and Lukis, 1971; Webb and Daniel, 1955; Failla and Cousins, 1978; Rudd and Herschman, 1979; Hildebrand, et al., 1979). Thus, most cells retain the potential to express uncharacteristic functions or to repress the expected traits. If there is a prolonged disturbance in the homeostasis of a given organ in vivo, the result is a diseased tissue. Culture systems are especially valuable for studying such changes in the "protected" tissues. While it is possible to study regulation of tissue-specific functions in primary targets such as liver in vivo, it is much more difficult to study gene regulation in secondary targets such as tendon. Alterations of diet will elicit rapid response in the former tissue, but not in the latter. To down-regulate the level of collagen with removal of ascorbic acid from the diet, for example, would require 8-10 days before any change is observed in various collagen producing tissues in guinea pigs (Barnes, 1975). Yet, tendon cells in culture once removed from the "protecting" effect of liver and storage suplies, respond to ascorbate addition and removal by modulating the level of collagen synthesis in less than 24 hrs, a kinetic fast enough to relate the two events together, (Fig. 8, Schwarz, Mandell and Bissell, in preparation). It would thus be possible to delineate the sequence of events, metabolic and otherwise, that bring about the "scorbutic" state (see Section IIA).

While there is ample evidence for the tissue specificity of metabolite patterns (see above), the evidence that metabolite levels or ratios may be involved in regulating other functions is scant. There are, however, some individual examples: e.g. it is known that NAD^+ and poly ADP-ribose levels can regulate whether primitive chick limb cells develop into muscle or cartilage (Rosenberg and Caplan, 1974, 1975; Caplan and Ordahl, 1978). This is a developmental process and it could be argued that events that regulate development may be different from those that regulate the differentiated state. However, there are recent results to show that NAD^+ directly influences the rate of cell-free protein synthesis in the lysed rabbit reticulocyte system being stimulatory at 0.16 mM and inhibitory at 4.0mM (Wu et al., 1978). It also has been demonstrated more recently that fructose or glucose-6-phosphate (but not fructose 1,6-diphosphate) reverse the inhibitory activity of double stranded RNA in the same system by presumably preventing synthesis of an inhibitor (Wu et al., 1979).

The most suggestive examples are again derived from cultured cells themselves. Under usual culture conditions (which traditionally have been defined for optimal growth) all cells regardless of their tissue of origin, become similar metabolically: they all become increasingly glycolytic. This occurs quite rapidly and is true for both fibroblasts and epithelial cells. Indeed it has been postulated that survival in culture may require such a glycolytic metabolism (Paul, 1965). Even epithelial cells such as liver which are not known for their glycotic metabolism exhibit this phenomenon (Fig. 9; Bissell, D.M. et al., 1978). Serum is shown to accelerate this event and the process is not due to an outgrowth of fibroblastic cells in the culture. All of the "permanent"

epithelial cell lines we have examined, whether from the liver (Fig. 2) or from the mammary gland (both rodent and human; J.T. Emerman, M. Stampfer and M.J. Bissell, unpublished) demonstrate glucose metabolite patterns which resemble the cultured embryonic fibroblasts (Bissell et al., 1973; Bissell, 1976) rather than the epithelial cells from which they were derived. The examples in this review as well as countless others, draw attention to the rapid alterations of function that occurs upon culturing. The time course of such changes usually coincides with the shift in the metabolic pathways and the increased glycolytic metabolism. It is quite possible that the common metabolic features of cells in culture is partially responsible for the similarity of functions exhibited by cell lines in culture. In this regard it is important to note that when mammary epithelial cells are induced to redifferentiate and express tissue-specific morphology and function by growth on floating collagen gels, they revert to a metabolite pattern that is closer to that of the freshly isolated cells. It is of course important to show that the metabolic changes precede or at least are concomitant with other functional changes. Preliminary results with mouse mammary epithelial cells (J.T. Emerman and M.J. Bissell, unpublished) and rat liver hepatocytes (Bissell, D.M. et al., 1978) indicate that this may be the case. How the metabolite levels directly or indirectly could influence functional differentiation remains entirely speculative.

II. The Differentiated State of Cells in Culture

A. Collagen Producing Cells in Culture

1. Introduction

The choice of collagen as the "single" function to be discussed in this section of the review poses an immediate problem in that clearly collagen is not a single protein. The extent of genetic polymorphism of collagen first observed by Miller and Matukas (1969) is beginning to be fully appreciated only recently. This fact, plus the numerous posttranslational modifications that the molecule has to undergo from its synthesis to its deposition, make collagen a truly complex protein. Nevertheless, to fully appreciate the advantages and shortcomings of using cultured cells as models for studying gene regulation, it is best to use such a molecule as an example. This is especially so because an increasing number of collagen biochemists and chemists are using cultured cells to study this molecule. Additionally, collagen has many other attributes that make it an attractive choice. It is the most abundant and ubiquitous protein in the body, constituting some 30% of total cellular proteins. It is believed to play an important role in morphogenesis, growth and development (Bernfield, 1970; Hay, 1973, 1977), and many diseases are known to involve defects in collagen biosynthesis and regulation (see Section IIA-4). Collagen is also known to play a role in tissue interactions in carcinogenesis (Dodson and Griffin, 1962; Tarin, 1967; Mazzuco, 1972). The purpose of this section of the review is not to discuss the complex biochemistry of this molecule. The recent literature on the chemistry and biochemistry of collagen is enormous and attests to its increased recognition as a key protein involved in growth and development. The molecule is the subject of extensive individual reviews (see below), and a

recent book on its structure and biochemistry (Ramachandron & Reddi, 1976). Collagen is used here as an example of a differentiated function which is modified and modulated by cells in culture.

2. Brief Summary of Known Steps in Collagen Biosynthesis

To appreciate the factors that could affect the regulation of collagen biosynthesis in culture, it is important to have some knowledge of the various steps involved in its biosynthesis. For reviews on collagen biosynthesis see Bornstein, 1974; Gross, 1974; Miller, 1976; Grant & Jackson, 1976; Ramachandran & Reddi (eds.), 1976; and Fessler & Fessler, 1978. A recent review by Prockop et al. (1979) provides a general and readable treatment of both the biosynthesis of collagen and its disorders.

There are at least 7 genetically distinct types of collagen polypeptide chains referred to as α-chains. All collagen molecules have the following characteristics in common: They consist of three α-chains in a triple helix conformation each chain with about 1000 amino acid residues with a sequence $(\text{glycine-X-Y})_n$. The proline content of the chains is about five times that of an average protein and approximately half the proline residues are hydroxylated (usually on the Y position). Table I shows a general summary of the types of collagen and their tissue distribution.

Collagen is made on membrane bound polysomes in a precursor form called procollagen. Each pro α-chain is probably translated from individual mRNA species. The procollagen molecule has an extention of about 1500 molecular weight at the amino terminus and of about 40,000 molecular weight at the carboxy terminus. The "pro" parts are similar to globular proteins, although the amino terminus has a short triple-helical

segment. The amino acid composition of the pro parts are analogous to non-collagen proteins. Recently is has been proposed that collagen, like most other secreted proteins contains a hydrophobic leader sequence at the amino end of the molecule termed "prepro" which is cleaved before intracellular translocation (Palmiter et al., 1979). As the procollagen molecule enters the cisternae of the rough endoplasmic reticulum during the translation process, about half of the proline residues and 20% of the lysine residues are hydroxylated. The hydroxylases required for these reactions are complex enzymes requiring non-helical collagen molecules as substrates and ferrous ions, α-ketoglutarate, ascorbic acid and molecular oxygen as cofactors [for an extensive review of prolylhydroxylase see Cardinale & Udenfriend, (1974)]. As soon as the procollagen is hydroxylated, the hydroxylysine residues are further galactosylated and glucosylated. Concomitant with the synthesis of intrachain and interchain disulfide bonds (depending on the type of collagen) is the formation of collagen triple helix. The protein is then passed through the Golgi complex before leaving the cell. The process of secretion may require a triple helical structure.

The secreted procollagen molecule is then processed by the cleavage of first the amino terminal propart (in the case of type I collagen) and then the carboxy terminal propart by two separate proteases (Leung, et al., 1979) giving rise to the triple helical collagen. The monomer collagen (or small molecular aggregates that may be secreted as such; Bruns, et al.; 1979) are then cross linked to form striated fibrils, and finally the collagen fibers and the characteristic bundles (Tanzer, 1973). The latter steps apply mainly to type I collagen. The banding observed in electron microscope results from the fact that each collagen molecule overlaps the

next neighboring molecule by quarter of its length giving rise to overlap zones which appear light and regions which have fewer collagen molecules and thus appear dark. There is some evidence from cultured studies that newly synthesized collagen is partially degraded intracellularly. (Bienkowski, et al., 1978). It has been suggested that selective degradation of α_2 chain during the biosynthesis of type I collagen may allow the correct pro α_1 to pro α_2 ratio (Parry, et al, 1979). For details and specific references readers are referred to the reviews cited above. Fig. 10 is a schematic and simplified presentation of important steps collagen biosynthesis and processing.

3. Collagen Production by Normal Cells in Culture

a. Stability of the level of collagen synthesis in culture

The important series of papers by Green, Goldberg and Todaro in the 1960's paved the way for a systematic study of collagen synthesis by cultured cells (Todaro et al, 1963; Green and Goldberg, 1963, 1964, 1965; Green et al, 1966, 1968; Green and Todaro, 1967; Goldberg and Green, 1964, 1967). While some of the conclusions drawn form these papers were shown later not to be correct (Peterkofsky, 1972a, see below), these studies, nevertheless, established the fact that almost all cell lines even those of non-fibroblastic origin, produce measurable quantities of collagen in culture. However, the degree of collagen synthesis varied over a wide range (the genetic polymorphism had not been discovered at that time). On the basis of the level of collagen synthesized (expressed as a percent of total protein synthesis), Green et al (1966b) defined 3 classes of cultured cells: i) those cells that produced 1.7-15% collagen; all diploid fibroblast strains and most established fibroblastic lines were in this group; ii) those cells that produced 0.15-1.4% collagen; in this range

were a number of established lines of non-fibroblastic origin (HeLa for example); and iii) those cells that produced less than .002% collagen. These were mainly "primary" cultures which had not been grown in culture for more than a few days and included lymphocytes, reticulocytes and macrophages. The line 3T6 which since then has become one of the prototype cells for study of collagen synthesis and mechanism of action of ascorbic acid was reported to produce about 6.4% collagen (Green and Goldberg, 1965).

Initial studies implicated three factors in regulation of collagen biosynthesis in culture: rate of growth, population density and ascorbic acid. Green and Goldberg (1963) and Goldberg and Green (1964) had shown that immediately after subculture, the percentage of protein synthesis devoted to collagen in 3T6 cells dropped appreciably, and that as the cells reached confluency it rose 15-fold. The general conclusion was that growth was detrimental to expression of function because growing cells were cytologically and chemically undifferentiated. The repetition of these experiments in the presence of ascorbic acid, which is known to affect the activity of prolyl hydroxylase (for review see Cardinale and Udenfriend, 1974) indicated that the percentage of collagen synthesis during exponential growth was still much lower than stationary cells (Green and Goldberg, 1965). A yet later study showed the increase at confluency to be still present, but it was only 2-fold in the presence of ascorbic acid (Green et al., 1966). Similar observations were made by Priest and Bublitz (1967). Despite the discrepancy in the magnitude of increase of collagen synthesis at confluency, the general conclusion for 3T6 and later for L-929 cells (Gribble et al., 1970) was that cell density and growth rate did affect the actual rate of collagen synthesis.

All of these studies utilized an assay where the level of hydroxy- proline in collagen was used as a measure of collagen synthesis. However, as described in the summary, collagen polypeptide is first synthesized and then hydroxylated. Therefore, underhydroxylated collagen - if such existed under the conditions used by the above investigators - would have gone undetected. In 1972, Peterkofsky (1972a) repeated these experiments using an assay which separates the process of collagen polypeptide synthesis from its hydroxylation (Peterkofsky and Diegelmann, 1971). In the presence of ascorbate, where the collagen molecule is expected to be fully hydroxylated, she found no increase in collagen polypeptide synthesis when cells reached confluency in both 3T6 and L-929. In the absence of ascorbate she found only a shift in proline/hydroxyproline ratios which would have been interpreted as a shift in collagen biosynthesis in the assay used by Green, et al. In addition she showed that the level of collagen synthesis by 3T6 was only 0.9% of total protein synthesis (as opposed to 6.4% reported previously by Green and Goldberg). The latter result confirmed the observation by Margolis and Lukens (1971). Peterkofsky also showed that ascorbic acid was unstable under the usual culture conditions. This paper demonstrated a number of points rather nicely: i) it is difficult to draw conclusions about collagen "synthesis" using an assay which measures the degree of hydroxylation of the collagen polypeptide. The older results in the literature, therefore, need to be viewed with this in mind; ii) that 3T6 cells, with repeated passage had lost their collagen synthetic ability to a large extent; and iii) that it was necessary to add fresh ascorbate to culture medium daily to ensure its continuous presence (see also Peterkofsky, 1976, and Bissell et al., 1980).

Peterkofsky thus concluded that the density effect observed by Green and others, even in the presence of ascorbate, was due to increased hydroxylation of collagen and not due to increased synthesis of collagen polypeptide. More recently, Levinson, et al., (1975), also, could not demonstrate a density effect with chick embryo fibroblasts. Levene, Bates and their colleagues, have conducted a series of experiments on the role of ascorbic acid in collagen synthesis in 3T6 cells (Levene and Bates, 1970, 1975; Levene, et al., 1972a,b, 1974; Bates and Levene, 1971; Bates, et al., 1972 a,b,c) and concluded that ascorbate has no effect on the synthesis of collagen polypeptide chain in culture. It was concluded, therefore, that the positive effect of both density and ascorbate on collagen synthesis in culture is confined to their effect on hydroxylation rather than on synthesis of collagen polypeptide chain.

These results contrast with in vivo findings. It has been shown in guinea pig skin that acute scurvy not only results in a slightly under hydroxylated collagen, but also in a marked reduction in synthesis of collagen polypeptide (Barnes et al., 1970). This is also true in scorbutic catfish (Wilson and Poe, 1973). Our results with primary cultures of avian tendon, using Peterkofsky and Dieglemann's assay to measure the rate of collagen polypeptide synthesis, indicates that density and especially ascorbate are, indeed, strong modulators of the rate of collagen polypeptide synthesis (Schwarz and Bissell, 1977; Fig. 8). Why then does the synthesis of collagen polypeptide in the cell lines not respond to ascorbic acid or density? The answer may lie again in the nature of established cell cultures, and whether or not they are appropriate models for study of factors that regulate function. Despite the inaccuracy of the assay, it is possible that studies of Green and Goldberg in 1965

indeed measured the ability of 3T6 cells to respond to ascorbate by increased synthesis of collagen polypeptide at confluency. But just as the percent of collagen synthesized in these cells has dropped progressively from 6.4% to about 1%, their ability to respond to "modulation" by population density and ascorbate has been lost also. It may be argued, therefore, that these cells are no longer appropriate models for collagen biosynthesis by the fibroblast.

With regard to primary cultures and collagen synthesis, an important experiment was performed again by Peterkofsky (1972b). Frontal bones of chick embryos are known to devote 60% of their total protein synthesis toward the synthesis of collagen _in ovo_. After only 3 passages in culture, the synthesis dropped to 3%. Addition of ascorbate raised it to 8%, only 1/7th of that _in ovo_. A comparison of some of the results found in the literature with regard to the quantitative expression of collagen biosynthesis in culture is summarized in Table 2. In a later paper Peterkofsky and Prather (1974) summarized their findings as follows: "Differentiated fibroblasts in connective tissue synthesize large amounts of collagen, but cultured cells lose much of this capacity...Therefore, the question arises as to whether this property truly reflects the state of differentiation of cells in culture." Even with primary cultures, therefore, the requirements for the expression of _in vivo_ level of function needs to be defined for each cell type as has been defined for primary avian tendon cells (Schwarz and Bissell 1977; Schwarz, _et al_, 1979).

b. The Phenotypic Alteration of Collagen Types in Culture

It is clear from current literature that in addition to defining the quantity of collagen, one also needs to define the type. This is doubly

important because not only do different tissues synthesize different types of collagen, the various proportions of which are altered in culture (Table 1), but also drastic type changes (referred to perhaps erroneously as "type switching") may occur under some conditions. Again, the number of publications in the last few years which have dealt with this phenomenon is a testimony to the interest of investigators in this field. The factors responsible for both the decrease in the level of collagen and the changes in type have yet to be identified and studied.

Table 3 is a summary of some of the literature on only one cell type (chondrocytes) for which extensive type changes have been reported. Layman et al., (1972) demonstrated that as rabbit chondrocytes (known to make only type II collagen) are placed in culture, they begin to synthesize type I while the cutaneous fibrocytes which start with type I continue to make type I (with no appearance of type II). Removal of serum from the chondrocyte cultures for 24 hr did not cause reversion to type II. The whole cartilage, on the other hand, retained type II specificity. Deshmukh and Kline (1976) and Deshmukh and Sawyer (1977) showed the importance of cell shape and $CaCl_2$ on the type of collagen synthesized. If chondrocytes were placed in suspension and no $CaCl_2$ was present in the medium, the cells reverted to synthesis of Type II collagen. They suggest that elevated levels of cAMP is secondary to internal calcium pools and that increased cAMP causes a switch to type I collagen by mobilizing the intracellular calcium. Addition of prostaglandins E_2 and F_2 had a very slight effect on collagen synthesis. Deshmukh and Nimni (1978) also demonstrated that addition of liver lysosomal enzymes to bovine cartilage slices causes an induction of

synthesis of type I collagen - a process which may play a role in osteoarthritis.

Benya et al, in a series of elegant experiments, showed that when rabbit articular chondrocytes were grown in monolayer culture, not only did new species of collagen appear (types I, III, type I trimer and a new species termed X_2Y) but also the proportion of these changed during subsequent subculture (Chung et al., 1976; Benya et al., 1977; Benya et al., 1978). Their data (see Fig. 11) demonstrates both the modulation in total level of collagen when cells are placed in culture and a lack of coordinate regulation for the different types. It is interesting to note that the synthesis of the small level of type III collagen seems to be insensitive to culture conditions and to subculturing (Benya et al., 1978). Benya and Nimni (1979) have shown recently that organ culture of the cartilage continue to make predominantly type II collagen even during proliferation. While some type III, X_2Y and eventually type I synthesis could be demonstrated, these are a small portion of the total collagen synthesized under organ culture conditions. Mayne et al., (1976) showed that growth in BrdUrd or continued passage ("senscence") leads to appearance of type I and type I trimer. Embryo extract, however, seems to cause the same type changes in younger chondrocytes (cited in Mayne, et al., 1976), Thus the relation of "senscence" to changes in type may be incidental to other factors such as injury (Deshmkh and Nimni, 1973) or metabolic alterations.

Collagen type changes also occur in cells other than chondrocytes, during development (Linsenmayer, 1974; Newsome, et al., 1976; Smith et al., 1976), in some connective tissue diseases, and after viral transformation (see below). Endothelial cells which are believed to synthesize type IV

collagen in vivo (Howard, et al., 1976, Barnes, et al., 1978), synthesize appreciable quantities of type III and I in culture (Barnes, et al., Smooth muscle cells (from rabbit, pig, human and monkey aorta) show varying proportion of types III and I with type III about 70% in vivo and only about 30% in culture (McCullough and Balian, 1975; Burke et al., 1977; Leung, et al, 1976, Barnes, et al, 1976, Mayne, et al., 1977). In the case of human fetal smooth muscle cells only type I was detected in culture (Layman and Titus, 1975).

Since some tissues are handled and kept in culture before the collagen types are determined, it is not clear at times if the tissue-specific types have remained unaltered. For example, three kinds of human glomerular cells have been described: those with an epitheloid and circular morphology which synthesize a single size type IV with a high ratio of hydroxyproline and hydroxylysine to proline and lysine, and 11-17% of hydroxyproline as the 3-isomer; the smooth muscle-like cells that synthesize types III and I, and small ovoid glomerular cells that are morphologically and biochemically intermediate between the other two (Scheinman, et al., 1978). The question remains as to which one of these (or any of them) indeed would represent the cell in vivo? This is also true of endothelial cells (Barnes, et al., 1978) and other type of cells. In other words, while an altered type of collagen in culture may say something about the cellular origin in vivo, it is important to be cautious in over interpreting the "typing" when data is derived mainly from cultured cells. Goldberg (1977), for example, has argued recently that because 3T3 cells synthesize only types I and III collagen in culture, they must be from fibroblastic rather than vascular endothelial origin. Hata and Peterkofsky (1977) using also clone A31 of Balb 3T3 demonstrated synthesis

of type I and another type designated X. However, they did not detect type III in untransformed 3T3 cells. It is, therefore, difficult to determine exactly the type specificity of collagen synthesized by 3T3 cells. This is especially so because by now there are many different clones of 3T3 cells each with their own charateristics. Furthermore, appreciable synthesis of types I and III can also be detected in endothelial cells in culture as mentioned before (Barnes, et al., 1978). By the time endothelial cells are subcultured as often as 3T3 cells, they could well lose all their capacity to synthesize type IV collagen (in a situation analogous to the loss of type II collagen in chondrocytes (see Fig. 11).

4. Collagen Production by "Malignant" and Diseased Cells in Culture.

Control studies on collagen production by "malignant" cells in culture so far have been limited mainly to instances where cells are transformed by oncogenic viruses. There are many reports of decrease (rhabdosarcoma) and increase (chondrosarcomas and fibrosarcomas) in collagen synthesis and degradation by the various tumor cells in vivo. The development of epidermal tumors in the skin of mice is shown to be preceded by an appreciable decrease in collagen levels (Mazzuco, 1972). A reduction in the tensile strength of the collagen in the tissue matrix (caused by the use of lathyrogens) has been shown to lead to a higher incidence of metastasis in tumor bearing animals (Gordon, et al., 1972). However, it is often difficult to know what cell type is producing the collagen in vivo and how it should be compared to its normal counterpart. Just as defining the type of collagen in cell lines by attempting to trace their tissue of origin could be misleading (IIA, 3) so are attempts to

define the origin of the tumor by the kinds and the amount of collagen it makes (Moro and Smith, 1977). A tumor in vivo by definition is released from growth regulation, and "positional" and metabolic controls. It is therefore quite likely that the make up of the collagen synthesized by the tumor would be unpredictable both in vivo and in culture. Given the degree of type changing that occurs in normal cells in culture and in diseased cells in general (see below), it is not surprising that the collagen produced by passaged tumor cells bears no resemblance to either the tissue of origin or the histological manifestation of the tumor tissue. For example, Moro and Smith (1977) studied collagen production by a tumor that initially arose from transformation of epithelial cells of the salivary gland in culture followed by subctuaneous injection of the cells into mice. Tumors were maintained by serial passage, and histologically were shown to contain intercellular bone, cartilage matrix and epithelial components. Thus the investigators expected to find not only type I collagen, but also types II and III. Instead, they found that the tumor contained type I and the more unusual type I trimer. It can be concluded from this study that determining the type of collagen in a tumor will say very little about the tissue of origin or the present state of the tumor. Of course, if type I trimer or another unusual collagen type were found to be associated only with tumors, this could serve as a marker for the malignant state. However, this does not appear to be the case (see Table I).

Transformation of cells with tumor viruses or other agents can provide a means of following early changes in the amount or type of collagen to determine whether such changes are the direct result of malignant transformation. These studies are not numerous, and the earlier

results were at times contradictory. Todaro, et al., (1964) reported an increase in collagen synthesis after polyoma or simian virus 40 transformation of 3T3 cells. Since these investigators used the level of hydroxyproline as a measure of collagen synthesis, their observation can, perhaps, be explained in the light of the ascorbate-independent hydroxylation observed in virus-transformed cells or cells in high density (Peterkofsky., et al, 1980). In a later study, Green, et al., (1966a) observed a decrease when ascorbate was present as might be expected from above interpretation. Temin (1965) had reported that while acid mucopolysaccharide synthesis increased appreciably after Rous-transformation of chick cells, collagen synthesis was either unaffected or decreased slightly. Levinson, et al., (1975) using the same system showed a decrease in collagen synthesis. However, they reported an unexplained 4-fold induction of prolyl hydroxylase after transformation. Peterkofsky and Prather (1974) reported a reduction of collagen synthesis in Kirsten sarcoma virus transformed 3T3 cells and restoration of this function by dibutyryl cyclic AMP (dbcAMP). Levinson, et al.,(1975) had found no evidence of restoration of collagen synthesis in virus-transformed chick fibroblasts by dbcAMP. The apparent contradictions perhaps reflect the insensitivity of the earlier techniques (see II3), variations in culture conditions and probably the low level of collagen synthesized by most of the cells used. In studies of Levinson, et al., (1975) for example, chick embryo fibroblast were shown to synthesize collagen at only 1.2% of the total protein synthesis, and 3T3 cells even at high density produce collagen at 1.9% (at low density the level is about 1.1%; Hata and Peterkofsky, 1977).

In general, more recent studies using RSV-transformation of both chicks embryo fibroblasts and chick embryo tendons have confirmed the studies by Levinson, et al., (1975) in terms of a drop in collagen synthesis (Schwarz et al., 1977; Soo, et al., 1977; Kamine and Rubin, 1977; Arbogast, et al., 1977; Adams, et al., 1977; Schwarz, et al., 1978; Rowe, et al., 1978; Sandmeyer and Bornstein, 1979). Further questions to be addressed are the following:

i. Is the change in collagen synthesis due to viral replication or transformation?

ii. Is the drop in collagen synthesis an early event after transformation?

iii. Are the initial regulatory steps transcriptional, translational, or post-translational?

iv. Does transformation necessarily lead to a change in the type of collagen synthesized and if so, how is this brought about and how fast?

The first three questions have been addressed only with cells transformed with Rous sarcoma virus (RSV). The following detailed analysis of the rather scant data in this area is meant to emphasize the fact that summary decisions based on sophisticated biochemistry and molecular biology performed on poorly defined cell systems may, indeed, be erroneous. If the behavior of the "normal" cells is unpredictable and poorly defined, it is difficult to draw meaningful conclusions about the mechanism of transformation. The first question can be answered with the use of transformation-defective mutants or those temperature sensitive for transformation (for review of retroviruses see Vogt, 1977, and Bishop, 1978). This approach has been tried in our laboratory with transformation-defective RSV (Schwarz, et al., 1977; Schwarz, et al., 1978) and with

RSV temperature sensitive for transformation (Soo, et al., 1977, and Soo, 1979) on primary avian tendon cells, and by others (Kamine and Rubin, 1977, and Arbogast, et al., 1977) on chick embryo fibroblasts. The conclusion is that the transformation event itself is necessary for a maximum decrease in collagen synthesis. Nevertheless, viral replication or some other event at non-permissive temperature may have some additional effect on collagen synthesis as judged by the fact that infected cells at the nonpermissive temperature do not have an entirely normal level of collagen synthesis (for example see Fig. 4(a) in Kamine and Rubin, 1977; and legend to Fig. 12). This may be attributed to the "leakiness" of temperature sensitive mutants at the non-permissive temperature. However, using LA-24 (a temperature sensitive mutant derived from Prague A subgroup) we see almost no leakiness in other transformation parameters at the non-permissive temperatures (41.5%) (Bissell, et al., 1979a; Soo, 1979). Recently it has been shown that inhibition of limb bud chondrogenesis after infection with retroviruses in culture is independent of the final transformation event (i.e. the activation of the src gene) since viruses that lack the src gene (transformation-defective), or temperature sensitive mutants at the non-permissive temperature, also inhibit chondrogenesis (Gross and Rifkin, 1979). C-type RNA virus production has also been shown recently to play a role in myeloid leukemia cell differentiation (Libermann and Sachs, 1978). On the basis of available evidence, therefore, it is immature to accept that virus infection and/or virus reproduction per se play no role in the decrease of collagen synthesis after infection with transforming viruses.

The second question, i.e. the time course of this event, has been addressed at our laboratory (see Fig. 12, and Soo, et al., 1977; Soo, 1979). Kamine and Rubin, (1977) did present 2 time points (4 hr and 20 hr) after temperature shift with chick embryo cells infected with temperature sensitive mutants of RSV. But they were not concerned with the time course of this event, and it is not possible to draw conclusions from their data concerning the kinetics of the change in collagen synthesis. Normal cells at zero time or 4 hrs after shift were shown to synthesize collagen at a rate 4 times higher than RSV-infected cells at either temperature. The rate of synthesis of collagen in normal cells themselves changed about 4-fold during the course of the experiment, and the kinetic of shift down of RSV-infected cells indicated no change at 4 hrs and only a 25% decrease after 20 hrs in the precent of collagen synthesized. The reason for these discrepancies is not clear. Our data with tendon cells where the rate of collagen synthesis is high, clearly indicates that the drop in the rate of collagen synthesis after a shift to the permissive temperature is an early and rapid event after onset of transformation. The decrease is 50% complete by 4 hrs, and 100% complete by 8 hrs (Fig. 12), while the recovery, after a shift to the non-permissive temperature is much slower and requires at least 12 hrs for a 50% rise to the control levels.

The third question has been addressed by Adams, et al., (1977), Howard, et al., (1978), Rowe, et al., (1978), and Sandmeyer and Bornstein, (1979). While it is clear that the level of mRNA for collagen is lower in RSV-transformed cells, the first three studies are mainly concerned with the steady-state levels of such messages in normal cells and those transformed with wild type viruses. As mentioned above, such studies

cannot distinguish between the initial and later events. Sandmeyer and Bornstein, (1979) have presented kinetic evidence for a concomitant decrease in mRNA for collagen and the rate of collagen synthesis when avian tendon cells are infected with wild type RSV. While the data clearly indicates a drop in both total and translatable message after transformation, it also demonstrates the need for a careful reevaluation of some of the conclusions drawn. Since temperature sensitive mutants were not used, the data shows the time course of RNA virus infection and not that of transformation. Furthermore, the time points were given for 12 hr intervals. It is clear from our data (Fig. 12) that a 12 hr time point would obscure the difference between the early events (shift down, 4 hrs) and later events (shift up, 12 hrs). Additionally, there is an unexplained and clear uncoupling between the rate of procollagen synthesis and the level of hybridizable message in the normal cells (see Fig. 2 in Sandmeyer and Bornstein, 1979). Similar discrepancies are observed also in the other studies on the comparison of mRNA levels and the rate of collagen synthesis in normal and RSV-transformed cells. The relative change in the amount of mRNA for normal cells in culture in studies of Rowe, et al., (1978) was higher than the relative drop in the rate of procollagen synthesis. For example, while procollagen synthesis dropped to 80% of the control, the mRNA level dropped to either 30% or 45% of the control depending on the method of determination. A drop to 42% of the control (tendon fibroblasts at high cell density) was accompanied by mRNA levels of 19% and 29% of the control. With transformed cells, almost the reverse was true, i.e. the synthesis dropped faster than the available mRNA level. It can be argued that at this stage of our expertise it is not possible to get perfect correlation between the mRNA levels and the

rate of synthesis. But by the same token, at this stage of our knowledge, it becomes difficult to draw clear conclusions about transcriptional vs. translational controls when the data fits only half the time. It is just as important to define the correlation between the level of message and the rate of synthesis for normal cells under various culture conditions.

Recent studies in chick embryo fibroblasts infected with temperature sensitive mutants of RSV and shifted to the permissive temperature indicate that while the total message drops to the level of wild type transformed cells by 12 hrs, there is only a 10% drop by 5 hrs (Sobel, M., personal communication). We have shown for chick tendon fibroblasts that the drop in collagen synthesis is more than 50% complete by 4 hrs (Fig. 12). The rapidity of loss of collagen synthesis, and lack of symmetry in the shift down and shift up experiments would indicate to us that initial decrease in collagen may be post-transcriptional (rapid message degradation, elaboration of collagenolytic activity, etc.), followed by a slower decrease in the synthesis of messenger RNA for collagen. The longer time required for a return to control levels after a shift up would then be an indication of the need to synthesize and mobilize new message. Until a careful analysis of the absolute rate of collagen synthesis vs. the mRNA levels is performed early after shift down of cells with temperature sensitive mutants, no conclusion about the direct relation of viral transformation and regulation of collagen biosynthesis can be drawn.

It should also be mentioned that a drop in messenger RNA levels after transformation under usual culture conditions does not necessarily establish a cause and effect relationship between the transforming event and the expression of a particular gene. For example, Olden and Yamada

(1977) had shown that RSV-transformation of chick embryo fibroblasts leads to a 5-6-fold decrease in the rate of biosynthesis of firbonectin. Adams, et al., (1977) determined that translatable levels of mRNA for both collagen and fibronectin in these cells were also reduced 5-fold after RSV-transformation. They concluded that oncogenic viruses modulate the rate of fibronectin and collagen synthesis by regulating the level of available mRNA in avian cells. However, we have shown recently (Parry, et al., 1979) that RSV-transformation of differentiated avian tendon cells does not alter the rate of fibronectin synthesis, but only its subsequent processing. While the two systems are not strictly identical, they are fibroblastic, embryonic and avian. Thus RSV-transformation of avian cells does not inevitably lead to decreased fibronectin synthesis.

The results by Hata and Peterkofsky (1977) demonstrate clearly the varied and complex nature of cellular response to transformation events in terms of collagen types. They showed that BALB-3T3 cells change to a new pattern of collagen synthesis after transformation but that different transforming agents cause different changes. This means that there may not be such a thing as a single transformation phenotype with respect to the type of collagen synthesized. All chemically and virally transformed cells had a reduced rate of collagen synthesis, but the type varied. RNA virus transformation led to increased rate of what was then described as type "Y" collagen (and now is known to be type III; B. Peterkofsky, personal communication). SV40 virus transformation did not alter the type of collagen synthesis, while chemically transformed cells exhibited entirely different types of collagen. These cells contained no α_1(I) and α_2 components and exhibited two main components which eluted slightly behind α_2 (now known to be types A and B; B. Peterkofsky, personal communications).

We have found (Soo, 1979; W.J. Soo, R.I. Schwarz, J.A.Bassham, and M.J. Bissell, unpublished) that transformation of avian tendon cells by RNA tumor viruses does not change the type of collagen synthesized. The absolute level of collagen synthesis is reduced by 3-fold (Parry, et al., 1979) and the relative rate by 5-10-fold (Soo, et al., 1977); but the type remains essentially unchanged. In our studies, a very small amount of a high molecular weight component is detected in transformed cells. This component disappears upon reduction. This may be viewed as induction of a new type of collagen (type III ?) in transformed cells. However, it is important to consider the possibility that a small amount of both type III and type I trimer are present in tendon cells and most other cell types. Since type I collagen is reduced drastically after transformation, the relative proportion of these minor components relative to type I collagen would increase, leading to the detection of the minor components. In any case, it is apparent that the type "switching" after malignant transformation, in vivo or in culture, would require much systematic analysis if it is to provide a possible tool for diagnostic purposes.

Collagen changes due to diseases other than malignancy may be simpler to study in culture. This is especially true for cultures derived from patients (and animals) with genetic disorders. Aside from giving information as to the nature of the disease, such cultured cells also illuminate the importance of various steps in biosynthesis and processing of collagen. For this reason, a brief description of some disease related studies is given below.

Diseased cells fall into two categories: Cells that manifest an inborn error of collagen metabolism and those that acquire the abnormality later in life. Table 4 shows a summary of some of the changes observed

using cultured cells from normal and diseased individuals. Some of these such as lysyl hydroxylase deficiency disease (Ehlers-Danlos syndrome VI), other Ehler-Danlos syndromes and osteogenesis imperfecta are examples of inborn errors. Others such as osteoarthritis or scleroderma or keloids have as yet unknown ethiologies. While genetic susceptibility may play a role in the latter diseases, it is not the primary factor. Table 4 does not include any examples of changes in collagen synthesis in epithelial cells. It is being recognized recently that epithelial cells not only synthesize collagen, but in some cases may be responsible for the collagen found in the basement membranes in vivo. Endothelial cells, for example, have been known for some time to synthesize basement-membrane collagen (Howard, et al., 1976; Jaffee, et al., 1976). As discussed above, they also have been shown to synthesize the interstitial collagens (types I and III; Barnes , et al., 1978). Liotta, et al., (1979) have demonstrated that rat mammary epithelial cells deposit their own basement membrane collagen in culture and Guzelian and Diegelmann (1979) have demonstrated collagen synthesis in rat hepatocytes, and there are more recent examples. It is thus quite possible that changes in collagen synthesis which accompany a number of pathological disorders in liver or mammary gland may be due to altered collagen synthesis by the epithelial components.

There are undoubtedly many changes in the rate of collagen synthesis and in other properties of collagen as cells age. Culture systems are obviously useful tools to determine whether such changes are the intrinsic property of aging cells or whether they can be modulated if the conditions are altered. Age related changes have been reported for enzymes involved in collagen biosynthesis, prolyl hydroxylase (Uitto, et al., 1969) lysyl hydroxylase (Anttinen, et al., 1973) and galactosyl and glucosyl-trans-

ferases (Anttinen, et al., 1979). Recently, Basler, et al., (1979) have indicated that ultrastructural changes and fiber production by "aging" human fibroblasts is similar to that of fibroblasts from Werner's syndrome (a premature aging syndrome). These cells produce a type of collagen fiber with high subunit molecular weight and altered characteristics. Given the abundance and the importance of the collagen molecule, it is expected that the list for disease related and age related changes in collagen metabolism will grow rapidly in the next few years with cell culture playing a crucial role in determining which events are primary and which are incidental.

5. Summary of Factors that Modulate Collagen Synthesis in Culture.

The literature discussed above implicates ascorbic acid and population density as positive modulators, and serum and transformation by oncogenic viruses or chemicals as negative modulators of collagen synthesis. Tumor promoters have been shown also to reduce collagen synthesis appreciably (Bissell, et al., 1979a; Delcos and Blumberg, 1979). There is no evidence, however, that these factors work through a common mechanism. With regard to the negative modulators, the kinetics of reduction of collagen are entirely different with different agents. Activation of the src gene of RSV, e.g., or addition of tumor promotors, lead to a rapid and specific drop in collagen synthesis, detectable in 2 hrs or less. On the other hand, the serum effect is much slower and requires about 24 hrs for detection. However, since serum rapidly stimulates non-collagen protein synthesis, the decrease in collagen, relative to total protein synthesis is detectable by 3 hrs (R. I. Schwarz, C. Hatie, and M. J. Bissell, unpublished). Earlier literature has implicated lactic acid, acidosis and CO_2 as positive modulators of both prolyl

hydroxylase and collagen synthesis (Green and Goldberg, 1964; Comstock, et al., 1970; Langness and Udenfriend, 1973; Schwarz, et al., 1976) and there is evidence of hormonal modulation of collagen levels. The mechanism by which these factors increase collagen synthesis is not well understood. Table 5 summarizes some of the factors that have been implicated in regulation of collagen synthesis in cultured cells.

IIB Mammary Epithelial Cells in Culture

We will now shift emphasis from collagen and mesenchymal cells to milk components and mammary epithelium. The choice of the mammary epithelial cell as the "single" cell type to be discussed in this section is complicated by the fact that unlike chondrocytes or tendon cells described in Section IIA, mammary cells in vivo do not exist as homogeneous populations. The mammary epithelium is an integral part of the mammary gland which is composed of at least 4 different cell types (epithelial, myoepithelial, fat and fibroblasts). The structure of the gland is exceedingly complex (Bloom and Faucett, 1975). Both the spatial and the quantitative relations of these cell populations change constantly in vivo during the life cycle, and during the reproductive cycle of the female mammal (the estrus or menstrual cycle, pregnancy, lactation and involution). The functioning of the gland is dependent on the interplay of a network of endocrine and nervous factors, the composition of which is different at different stages of the gland's development. However, it is precisely these same complexities again which make the mammary epithelial cells an attractive model for culture studies. To sort out such interactions and regulations, it is critical to be able to understand each component separately and then in combination.

1. The Monolayer Culture of Mammary Epithelial Cells from Normal and Tumor Tissues

The mammary gland clearly is one of the most versatile models for studying the role of hormones in gene regulation (for reviews see Denamur, 1971; Rivera, 1974, and Banerjee, 1976). The mammary epithelial cells also provide attractive models for the relation of cell shape to function and the influence of both endogenously synthesized and exogenously added matrix (usually collagen) on regulation of tissue-specific functions (see below). The mammary gland and mammary epithelium are attractive also from the point of view of the hypothesis presented in the first part of this review. The intermediary metabolism and mammary-specific functions are intimately related. Glucose is both the energy source and the substrate for tissue-specific milk components, and as will be seen later, by studying glucose metabolite patterns we have gained new insights into how lactose synthesis may be regulated prior to parturition.

Most of the studies on mammary cells in culture have utilized either the whole gland or pieces of mammary tissue ("fragment culture"; Banerjee, 1976). This is understandable in the light of the complexity of the gland and the requirement for intact architecture. As discussed initially, these studies, despite their abundance and importance, will not be included here. However, there are a surprising number of earlier attempts to culture mammary epithelial cells in a "sheet" or monolayer culture. The gland itself probably provided one of the earliest examples of tissue culture cultivation (Maximow, 1924). The early attempts to culture the mammary epithelium were frustrated by the overgrowth of the fibroblasts or obstruction of growth by fatty tissues (Santesson, 1935, Hardy, 1950). Lasfargues (1957a) reported some success with the use of collagenase to

remove the fatty and collagenous tissues. He soon observed that the presence of some fibroblasts actually helped to orient the epithelium and allow some functional activity (Lasfargues, 1957b). Ebner et al., in Larson's laboratory succeeded in maintaining bovine mammary cells in mixed epithelial-fibroblast cultures in presence of 20% bovine serum (Ebner et al., 1961a,b.). They studied milk specific products and enzymes, and found--as expected--that these functions are diminished or lost in culture. They also noted that such loss does not occur simultaneously for all functions under study. The "secretory" appearance could persist for many months, while lactose synthesis disappeared entirely after one day and UDP Gal-4-epimerase was undetectable by day 10. Since then, there have been additional attempts at culturing mammary epithelium of many species from mice to men where some functional differentiation has been retained (Schingoethe, et al., 1967; Blanco, et al., 1967; Kinsella, 1968, 1972; Kinsella and McCarthy, 1968a,b; Larson, 1969; Castor, 1969; McGrath and Blair, 1970; Wiepjes and Prop, 1970, Lasfargues and Moore, 1971; Owens and Hackett, 1972; Owens, et al., 1974; Das, et al., 1974; Feldman, 1974; Pickett, et al., 1975). Ceriani (1976) demonstrated that post confluent cultures of mammary epithelial cells from midpregnant mice could be induced by hormones to synthesize increased levels of casein-like material. Nonetheless, adequate morphological and functional retention or induction were not achieved in any of these studies. Two-dimensional substrates (plastic culture dishes) and/or high serum concentrations perhaps were responsible for lack of success in maintaining or inducing function. Furthermore, culture of mammary tumor cells resembled those of normal cells when grown on plastic in both ultrastructure and function (Santesson, 1935; Das, et al., 1974; Pickett, et al., 1975). It should be

recognized, however, that many of these culture systems were (and are) useful in elucidating the mechanisms of mammary tumor virus induction and replication (for review see Nandi and McGrath, 1973). In the light of many attempts, but small successes, to retain function by isolated mammary epithelial cells in culture and the much higher level of success in inducing or maintaining function in organ culture (see Banerjee, 1976), the question could be asked as to whether or not the mammary epithelial cells in monolayer culture somehow irreversibly lost their ability to remain differentiated, or whether additional factors needed to be supplied. In this regard, an intriguing and original study was performed many years ago by Daniel and Deome (1965). Cultured mammary epithelial cells which were not displaying mammary specific characteristics were transplanted into gland-free mammary fat pads (DeOme, et al., 1959). While some of the subsequent outgrowths in vivo indicated partial abnormality, most of the outgrowths appeared to be similar to normal mammary glands. They had the characteristic normal morphology and during pregnancy responded in a predictable manner and were capable of milk secretion after birth. This clearly proved that the cells in culture had not changed irreversibly. Furthermore, it also indicated that if culture conditions were altered and an adequate substrate and environment (including other cell types) were to be found, the mammary cells in culture should be able to regain or retain their mammary specific traits.

2. The Expression of Function by Normal and Malignant Cells:
The Importance of the Substrate

The fact that it is difficult to maintain functional epithelial cells in culture under the same conditions where mesenchymal cells retain function tells us that the minimum requirements for these cells exceeds a flat plastic surface and/or the conventional culture medium. Thus, growth in three-dimension, extremely high densities, or other conditions may be a prerequisite for a functional epithelium. The stringency of the requirements perhaps is dependent on the state of the given epithelium *in vivo*. Gospodarowicz *et al.*, (1978), for example, have demonstrated that corneal epithelial cells when grown on plastic flaten out, lose their *in vivo* characteristics and respond only to fibroblast growth factor. When maintained on a collagen substrate, however, these cells alter their morphology by becoming tall and columnar and respond to epidermal growth factor. Interestingly, they point out that endothelial cells, which in *vivo* grow in a single layer or in "two-dimensions" adapt to regular culture conditions readily, synthesize their own basement membrane and retain their differentiated state (Gospodarsowicz *et al.*, 1978).

The introduction of reconstituted rat-tail collagen as a substrate for cultured cells, (Ehrmann and Guy, 1956; Bornstein, 1958) and the identification of collagen as the active component of "conditioned medium" for growth and differentiation of single muscle colonies (Hauschka and Konigsberg, 1965) led to a new phase in the studies of extracellular matrix and its influences on gene expression. The extensive literature in this field is beyond the scope of the present review. The individual

papers at least are as numerous as the literature on collagen. For review see Epithelial-Mesenchymal Interactions (Eds., Fleischmajer and Billingham, 1968; Slavkin, 1974); Extracellular Matrix Influences on Gene Expression (Eds., Slavkin and Greulich, 1975), and Cell Interactions in Differentiation (Eds., Saxen and Weiss, 1977). It is appropriate, however, to mention that the work of early pioneers who drew attention to the importance of mesenchymal-epithelial interations in embryogenesis and tissue modeling (Grobstein, 1967; Grobstein and Slavkin in the above reviews) are very relevant to the more recent studies where cellular configuration is shown to play an important role in growth regulation, m-RNA processing and differentiation (Maroudas, 1973; Michalopoulas and Pitot, 1975; Emerman et al., 1977, 1979; Folkman and Mascona, 1978; Gospodarowicz, et al., 1978; Farmer, et al., 1978; Allan and Harrison, 1980, etc.). Most recently, Gospodarowicz and his colleagues (Gospodarowicz and Ills, 1980; Vlodavsky et al., 1980) have demonstrated that if corneal endothelial and lens epithelial cells or human tumors from various origins are plated on an extracellular matrix derived from endothelial cells, the normal cells will no longer need growth factors and would proliferate at maximum rates, and the tumor cells exhibit lower serum requirements and demonstrate epitheliod morphology.

The discovery that primary culture of rat liver hepatocytes retain morphological and functional differentiation when grown on a floating collagen gel (Michalopoulos and Pitot, 1975) paved the way for a series of important experiments on mammary epithelial cells. Emerman (1977), Emerman and Pitelka (1977), and Emerman et al., (1977, 1978, 1979) performed a series of morphological and functional studies on mammary epithelial cells of pregnant mice on various substrates. When plated on a

floating collagen gel, in the presence of appropriate hormones (insulin, cortisol and prolactin) these cells display characteristic mammary morphology (compare Figs. 13 and 14). If the same cells were plated on plastic or glass, or on attached gels, they lost many of their differentiated characteristics (Fig. 15). Mammary specific morphology was regained if cells were removed from the plastic and replated on the floating gels (Emerman and Pitelka, 1977), indicating again the reversible nature of loss of function at the early stages of culturing. The cells on the floating gels were shown to be also biochemically responsive to lactogenic hormones by accumulating and secreting increased amounts of casein (Emerman et al., 1977). Previous studies (Feldman and Deome, 1975) had indicated that on a monolayer culture of mammary epithelium, the cells on the center produced "casein" (this was not identified biochemically) and fat droplets and synthesized little DNA, while those on the edges synthesized DNA actively, and contained very little casein and fat. This, of course, is the familiar and classical observation with dense cultures. The relation of growth to expression of differentiated function and the importance of high density in gene expression are complex topics that cannot be dispensed with summarily as already discussed in Section IC. Nevertheless, the observation is relevant here in terms of deciding what the cells on a floating gel may have in common with the cells on the center of a mammary colony grown on a flat surface. Emerman et al., (1979) have discussed four factors that distinguish the cells on a flat surface from those on a floating gel: access of nutrients to the basolateral cell surfaces, close proximity of cells to the medium surface and gas phase, interaction of epithelial cells with stromal elements, and

substrate flexibility leading to changes in cell shape. They concluded that the first and the fourth were the most important. I would like to propose that the latter, i.e., cell shape, is probably the feature that cells in high density have in common with cells on a floating gel. It can be envisioned that if in crowded cultures cells are not growing at the top of each other, they would become more "columnar" than those at low density. Indeed, Folkman and Moscona (1978) have shown that the "height" of a Balb/3T3 cell in a crowded monolayer was about 14.8 nm, while growing cells on a cleared wound area where there was room for migration, were flat and had a height of 6.8 nm. Thus the relation of height to width is undoubtedly different in cells at differing densities. It would be important (and simple) to verify this for epithelial cells at the level of electron microscope.

One additional factor that needs to be considered is the change in metabolic patterns with increasing density in the center of the epithelial colony and on the floating gels. It has been shown previously that chick embryo fibroblasts show density dependent (as well as growth-dependent) changes in their metabolic patterns (Bissell, et al., 1972; 1973). Consistent with the argument put forward in Section ID.2 and IF, one may expect mammary-specific metabolite patterns in vivo during different stages of mammary development and in culture on floating gels. This, indeed, was found to be the case. The glucose metabolite patterns of the mammary tissue pieces from the virgin and the lactating mice are shown in Fig. 16 (Emerman and Bissell, 1979a). Pieces of both glands were in the presence of lactogenic hormones and as can be seen readily, the difference in the pattern of metabolites far exceed the presence or the absence of lactose. This may not be surprising because the ratio of fat cells

to epithelial cells are entirely reversed in the virgin and lactating glands. Nevertheless, the dramatic difference confirms the tissue-specificity of metabolite patterns. Measurement of glycogen levels at different stages of mammary gland development indicated that it was greatly down modulated during the transition from pregnancy to lactation (Emerman and Bissell, 1979b). Freshly isolated mammary epithelial cells from the gland of virgin, pregnant and lactating mice also showed strikingly different patterns of metabolites (see below). The detailed analysis of the metabolic levels derived from catabolism of uniformly labeled ^{14}C-glucose revealed unexpectedly that in addition to the fat cells, mammary epithelial cells were also synthesizing much glycogen (Fig. 17). A comparison of glycogen and lactose synthesis revealed a reciprocal relationship at parturition: As glycogen levels fell rapidly, lactose synthesis rose sharply. This led us to propose that since the two pathways share common intermediates, they may be modulated at the expense of each other. An analysis of the enzymes involved in glycogen synthesis and breakdown has confirmed the interrelationship of the two pathways and the possibility that glycogen synthesis during pregnancy is used by the cells to prevent increased lactose synthesis before parturition (Bartley, et al., 1979; Emerman, J.T., Bartley J. and Bissell, M.J. submitted). Thus high ratios of glycogen to lactose can be used as a marker for the pregnant state: When ^{14}C-glucose is the carbon source, a ratio of ^{14}C-glycogen to ^{14}C-lactose of greater than one would signal late pregnancy; ratios smaller than one would signal lactation. In mice, an additional marker for the pregnant state was the demonstration that the the glucose-derived alanine pool was extremely large in comparison to virgin or the lactating cells (Fig. 18) However, since alanine is an

intermediate and not an end product, the size of the pool by itself cannot be used as a measure of the state of mammary gland development.

A further insight into the differentiated state of mammary epithelium could be gained by studying metabolite patterns of cells plated on plastic or on floating gels. We now conclude that such patterns provide a refined device for measurement of the differentiated state of cells in culture when compared to the patterns obtained immediately after cell isolation. Mammary epithelial cells from pregnant mice maintained on the floating gel appear similar to late pregnant (or early lactation) by the criteria of morphology and casein levels (Emerman and Pitelka, 1977; Emerman, et al., 1977), and in pregnant rabbit by α-lactalbumin synthesis (Häuptle, et al., 1979). The rate of glycogen synthesis and the low level of lactose synthesis (Fig. 17) and the metabolite patterns are similar to the cells at the time of isolation and are maintained for several days (J.T. Emerman, and M.J. Bissell, unpublished). This indicates that conditions for "normalcy" of mammary epithelial cells from pregnant mice have been almost achieved, although the presence of the lactogenic hormones at the concentrations used perhaps are not necessary and other factors may be required to bring about lower casein secretion and a morphology more analogous to the original state of the freshly isolated cells. Recently, epithelial cells from lactating mice have been shown also to retain morphological differentiation on the floating gels (Burwin and Pitelka, 1980). These cells, however, synthesize low levels of lactose and do not have maintain milk-specific components and a lactating-specific metabolite patterns J.T. Emerman and M.J. Bissell, unpublished). The rate of lactose synthesis drops rapidly and consistent with our model for regulation of lactose synthesis discussed above, glycogen synthesis

increases in a reciprocal fashion (Fig. 19). Such changes do not appear to be random. While the decrease in lactose synthesis by itself could have been taken as "dedifferentiation", the quantitative change in the rates of glycogen and lactose synthesis perhaps indicates a modulation to the pregnant state with the cross over point indicating a reversal of lactation (Fig. 19). In other words, the floating gels, the added hormones and the medium used, provide an environment which is more like the environment of the gland during pregnancy rather than lactation. Thus, regardless of whether the epithelial cells are derived from the glands of pregnant or lactating mice, they respond in an analogous fashion to the identical environment: they both appear at mid to late stages of pregnancy. This can be seen readily in Fig. 20, where the rapid increase in glycogen synthesis levels off at the pregnant stage. Other metabolite markers for the epithelial cells from pregnant mice, such as the increased glucose-derived alanine pool (Fig. 18) are also observed in this conversion of lactation to pregnancy (J.T. Emerman and M.J. Bissell, unpublished). One could use the rates of glycogen and lactose synthesis and the metabolite patterns to adjust or to "titrate" the medium and the matrix until the lactating epithelial cells retain the specific function of lactating gland. It should be mentioned that human mammary cell lines such as HBL-100 and MCF734B which are reported to have some mammary specific functions (Polanowoki, et al., 1976; Soule, et al., 1973) have metabolite patterns which are analogous to fibroblasts (Bissell, et al., 1973, 1976; Bissell, 1976) just as are liver cell lines (Fig. 2, Section I; J. T. Emerman, M. Stampfer and M. J. Bissell, unpublished). This, of course, would be expected from our results with the mouse cells grown on plastic. It would be of interest to compare the behavior of these human

cell lines on the floating collagen gels and on plastic dishes to determine whether or not additional mammary-specific traits can be elicited if the cells are maintained on floating gels.

It has recently been shown that mammary epithelial cells deposit their own basement membrane collagen in culture (Liotta, et al., 1979a) and that these cells show a preference for type IV collagen for attachment and growth (Wicha, et al., 1979 a,b) similar to that observed for epidermal cells (Murray, et al., 1979). Mammary fibroblasts and dermal fibroblasts, however, are shown to attach and grow equally well on all types of collagen substrates. Most recently, Wicha et al., (1980) have demonstrated that interference with basement collagen deposition through the use of cis-hydroxyproline, interferes with mammary gland development. It would be important to grow mammary epithelial cells on floating gels made of type IV collagen to see whether or not mammary specific funtions are enhanced further or retained longer.

We are only beginning to appreciate the complexities of the factors that regulate gene expression in higher organisms. The significance of the discovery of the different types of collagen in different tissues (see section IIA), the fact that other proteins such as fibronectin may mediate cellular attachment to collagen (Klebe, 1974; Kleinman, et al., 1978), the specificity of epithelial cells for specific types of collagen (Murray et al., 1979) or total matrix (Rojkind, et al., 1980), modulation of collagen types by the presence of glycoproteins such as keratan sulfate (Conrad, et al., 1980) etc., will all have to be sorted out in terms of a sequence of signaling from "outside" to "inside". There is every reason to believe that the extracellular matrix is contiguous with the cytoskeleton (e.g., see Singer, 1979) and the nuclear membrane, and that a disruption in the

overall structure (brought about mechanically or biochemically) could have profound effects on the expression of the differentiated traits in culture (and in vivo).

III. Concluding Remarks: Towards a New Definition of Normal

> "An unflinching determination to take the whole evidence into account is the only method of preservation against the fluctuating extremes of fashionable opinion".
>
> Alfred North Whitehead

It is perhaps important at this point to ask the question: how far should (or could) a cell type be defined before it can be pronounced an appropriate model for studies of differentiated functions? It may be argued that if tissue-specificity is dependent so totally on the cellular environment, a cell in culture by definition could never be entirely normal. This may be especially true for epithelial cells from complex tissues where not only the organization is disrupted completely in culture but also the cells are deprived from interacting with other cell types. The very first step of cell separation (trypsinization, collagenase treatment, etc.), by removing membraneous receptors and matrix elements and structures, produces a discontinuity between the cell and its environment. Thus, if the "outside" indeed directs what (and how much) the cell should or should not produce, the flow of information may never be exactly the same. Nevertheless, in this paradox lies the challenge of cell culture.

In our attempts to "create" model systems in culture, we are constantly increasing the numbers of criteria by which we define the differentiated state. Under defined conditions, most of the functions we

have measured in primary avian tendon cells appear to be comparable to the tendon *in ovo* (Table 6). There are, however, at least two exceptions. The extent and the composition of the extracellular matrix is an important differentiated trait of tendon cells. As can be seen in Fig. 21, the tendon *in vivo* produces an extensive and organized matrix consisting primarily of collagen bundles. In the presence of ascorbic acid, these cells produce an extensive matrix also in culture (Tung *et al.*, 1977). The organization of the matrix, however, is not analogous to the tendon *in vivo*. Both in scanning (Fig. 22) and transmission (not shown) electron micrographs, the matrix appears as a mesh-like network where collagen bundles have a smaller diameter than the bundles observed in intact tendon. This may be because tendon cells *in vivo* are lined up in orderly arrays (see Bloom and Faucet, 1975), while in culture they have little or no orientation especially at subconfluent stages. If cells in culture could be made to line up by providing them with a preformed collagenous matrix (Schwarz and Bissell, 1977), it may be possible to achieve an organized deposition of a *de novo* synthesized matrix. The metabolite patterns of these cells in culture, especially at low densities, also are not totally similar to the intact tendon (Bissell, M.J., Schwarz, R.I., and Hatie, J., unpublished) and would require further modifications of the cellular environment. The degree of differentiation of mammary epithelial cells on the floating gel has been discussed in detail in section IIB and the results are summarized in Table 7.

I have tried in this review to point out that our knowledge of how gene expression is regulated in eukaryotic systems is very meager, indeed. We know too little about development, too little about tissue formation, too little about gene regulation in general to dismiss any one function

or area as unimportant in shaping and maintaining the differentiated state. Cell culture will occupy a more central role in all future studies on gene regulation and malignant transformation. To avoid pitfalls and to gain relevant information, however, the investigators can no longer afford the luxury of using these cells without appreciating the complexities involved and without understanding the physiology and the pathology of normal and malignant cells in vivo. I have tried also to emphasize that all of our knowledge from cell culture studies indicate that very few, if any, cells are locked in a predetermined pattern of gene expression. The cell in culture, indeed, is an adaptable organism. Unless conditions are strictly defined, the answers will have little relevance to the questions asked. In his comments at the Third Decennial Review Conference on Cell, Tissue and Organ Culture, Howard Green (1978) pointed out that the fields of molecular biology and cell culture "are moving together rapidly, and it is hardly possible to do molecular biology of eukaryotic cells without being something of a cell culturist". While that "something" is a science unto itself, it is important to remember that the reverse is also true: it is no longer sufficient to do cell culture for its own sake. One must characterize the cells biochemically and functionally. The biochemistry and molecular biology performed on poorly defined cell systems, in turn, need to be reevaluated using cultures with in vivo reference points. There is one point, however, that cannot be emphasized enough: While it is true that the usefulness of a culture system is increased by how far it is developed to mimic the in vivo situation, we use cultured cells because the in vivo events, in fact, are not well understood. It cannot a priori be decided that a system or a set of data are "irrevelant" because they do not conform to the in vivo situation. Some apparent culture

"artifacts"--if evaluated correctly--will give us insight into important mechanisms in gene regulation.

We use cultured cells because we can simplify the milieu to understand normal physiology. We also use them to learn how to manipulate gene expression. While the molecular biologists reorder the genes, the cell and developmental biologists, by defining the cellular environment, may call the shots in the long run.

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APPENDIX I

Terminology

1. In vitro, In vivo, In culture: Cultured cells are used by investigators from diverse disciplines--molecular biology, virology, biochemistry, clinical medicine, etc. In the former disciplines, an animal cell in culture is considered to be analogous to a bacterium in that it is alive and it can function and/or reproduce, thus the term in vivo is used often. Biochemical reactions in cell-free systems are referred to as in vitro reactions. On the other hand, to clinicians and to some biologists, in vivo refers only to the intact animal. They thus refer to cultured cells as being in vitro. This is not a trivial matter; the same cell system is referred to as in vivo or in vitro depending on the bias of the investigtors. Given the fact that the Journal of Tissue Culture Association is called In Vitro, this may seem like a losing battle. Nevertheless, I have proposed many times in the past, and I will reiterate again, that this confusion need not exist if a third term is introduced. We can reserve in vivo for whole organisms only (whether a bacteria or a mammal), in vitro for cell-free systems where the integrity of the cell has been disturbed and where the cell is no longer alive (see also Banerjee, 1976), and in culture for cultured cells which, indeed, are alive and no longer grown "on glass". (The word in vitro is derived from Latin meaning "in glass".)

2. Transformation, tumorigenicity and malignancy: Again, the dichotomy between the disciplines as well as lack of clear cut definitions lead to the confusing usage of these three terms. The word

"transformation" has a complex history. Aside from its specific connotation in microbiology (transfer of genetic material),it initially referred to a cell population that had undergone some change in culture , (referred to as "crisis") and had become a permanent cell line. More recently, however, the term refers to cells which have acquired oncogenic potential by tumor viruses and chemical and physical carcinogens. Clinicians usually are disturbed when "transformed" cells in culture are referred to as "malignant." Undoubtedly, in many cases, transformation in culture is not equivalent to malignancy. The latter term should be reserved, perhaps, to denote "invasive" tumors, although even in vivo there are many exceptions (Ponten, 1976). Nevertheless, to refer to oncogenically transformed cells in culture as malignant may be excused if the cultured cells in question have been shown to be malignant in vivo. Thus "normal" 3T3 cells, while "transformed" by the old definition, are tumorigenic when imbedded on special three-dimensional substrates (Boone, 1976) but are not considered malignant, while Rous sarcoma virus-transformed cells are "transformed" by the new definition of the word and are also malignant in vivo. In fact, that was how Peyton Rous discovered the virus initially (1916). Reinnoculation of the virus, or virally transformed cells in chicken leads to tumor formation, progression, and death in most cases. I suggest, therefore, that we reserve the word malignant for those cultured cells where the malignancy in vivo has been demonstrated under acceptable and routine testing conditions. The current

3. Dedifferentiation; modulation; mammalian: There are a few words in the literature of cell culture which are used as a convenient catch all. The best example is the word "dedifferentiation". Whenever a cell appears to lose its tissue specific functions, it is referred to as a dedifferentiated cell. Thus tumor cells in vivo and cultured cells, whether from normal or malignant origin, are thought to be "dedifferentiated." The word dedifferentiation, however, carries the connotation of irreversibility and total loss of function (Ephrussi, 1972). In reality, there is no firm experimental basis for the process of dedifferentiation. While in practice a lot of cultured cells, especially cell lines, do not produce the kind and the quantity of functions they expressed in vivo, by no means does this indicate that they have lost the capacity to respond if they were put in an appropriate environment. The very fact that cultured cells are rapidly altered may be indicative of the fact that they are adapting--in a normal fashion--to the radically altered environment. The numerous unexpected appearances of gene products in various cultured cells, the ability to induce differentition in culture where the environment of tumor cells is altered (e.g., Schubert et al., 1971, Flaxman, 1972), the ability of cultured "undifferentiated" mammary cells to form mammary outgrowth and secrete milk once reimplanted in the clear fat pad of the mammary gland (Daniel and Deome, 1965), the unequivocal demonstration that embryonal carcinoma cells (albeit a special type of tumor cells) could differentiate and lose their tumorigenicity (Pierce et al., 1978), or even the demonstration of synthesis of m-RNA for hemoglobin in chick embryo fibroblast cells infected with Rous sarcoma virus (Groudine and Weintraub, 1975), or the complete modulation of collagen synthesis to the in vivo level after an initial drop in avian

tendon cells in culture (Schwarz and Bissell, 1977) are all testimony to the reversibility of both differentiated and "dedifferentiated" cells. As we have stated before, (Schwarz and Bissell, 1977), it is important to ask whether or not "terminal dedifferentiation" need ever occur. Indeed, even the seemingly "irreversible" changes in culture may signal a new developmental stage rather than a defifferentiated cell (see Section ID-3). It is thus preferable not to equate loss or altered function with dedifferentiation. I would propose a moratorium on the use of this term and would substitute it with the word "modulation" until we know more about the process of differentiation and regulation of gene expression.

One last minor point: those who review the literature on cultured cells often refer to all animal cells as mammalian (to distinguish them from bacteria, invertebrates and plants). At least until recently, much work on gene expression and viral transformation has been done with avian cells and these too are usually included under a mammalian heading. Since birds are not mammals, it is more appropriate to refer to animal cell culture or vertebrate cell culture when research on avian cells are cited.

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Table I The Genetic Polymorphism of Collagen and Its Tissue Distribution

Type Collagen	Molecular Form	Tissue Distribution
I	$[\alpha_1\ (I)]_2\alpha_2$	Bone, dentin, tendon, skin, arteries, uterous, cornea (almost ubiquitous)
II	$[\alpha_1(II)]_3$	Hyaline cartilage, intervertibral disc, nucleous pulposus, etc.
$III_{(1)}$	$[\alpha_1(III)]_3$	Skin, arteries, lung, GI tract, uterine wall, etc.
IV(2)	$[\alpha_1(IV)]_3$	Basement membranes (lens capsule, kidney glomerula, etc.
$V_{(3)}$	$\alpha A(\alpha B)_2$ A & B chains	Basement Membranes (placenta, migrating epithelium, synovial membrane, etc.
Type I $Trimer_{(4)}$	$[\alpha_1(I)]_3$	Chondrocytes in culture embryonic cells, tumor cells, rat dentin, etc.
Others (5)		

(1) Type III probably occurs in most type I containing connective tissue (with the exception of bone; it is low in tendon and cartilage). It can be distinguished from Type I by its interchain disulfide bridges at the carboxy end of the collagen helix. It is similar to type I in its

Table 1 (cont'd)

low content of hydroxylysine (type II has a high content of hydroxylysine). Types II and III make small fibrils as opposed to the broad banded fibrils of type I. For review see Miller, 1976.

(2) There are still many questions as to the nature of type IV collagen. The globular procollagen extentions are retained. The molecule(s) have high 3-hydroxyproline and low alanine and arginine content. For reviews see Kefalides, 1973, 1978; for individual reports see Kefalides, 1971, 1978 Hudson and Spiro, 1972; Daniels and Chu, 1975; Dehm and Kefalides, 1978; Timpl et al., 1978; Dixit, 1978; Roll et al., 1980).

(3) These are found as minor components in pepsin digests of many tissues and were first described by Burgeson et al. (1976) and Chung et al. (1976). They are similar to type IV collagen in high content of hydroxylysine, low alanine and almost full glycosylation. For other reports see Jimenez et al. (1978); Rhodes and Miller, (1978); Madri and Furthmayer, 1979; Roll et al, 1980).

(4) These have increased content of 3- and 4-hydroxproline and hydroxylysine. The chain appears to be identical to α_1(I) by peptide mapping. For individual reports see Müller et al. (1974); Mayne et al. (1975); Benya et al. (1977); Moro and Smith (1977); Newsome et al. (1976); Jimenez and Bashey, (1977); Jimenez et al, (1977); Uitto, (1979).

(5) A few additional forms of collagen have been reported and more are expected. Some may be related to the above forms already described, e.g. "TSD4" specific collagen from a teratocarcinoma cell line may be related to $[\alpha_1(I)]_3$ (Little et al, 1977), and X_2Y from rabbit chondrocytes (Benya et al, 1977; 1978) are probably related to A and B. Two α chains called C and

Table 1 (cont'd)

D have been isolated from human placenta, human glomeruli and human and porcine kidney cortices (Bailey et al, 1979; Kresina and Miller, 1979; and Dixit, 1979).

Table 2 Comparison of the Level of Collagen Biosynthesis of Various Cells *in Vivo* and in Culture

	Radioactive proline incorported into collagen (% of total protein)	Collagen synthesis (% of total protein)
Avian Cells		
Frontal bone of 16 day embryo:		
a) *in ovo*[1]	89	60
b) after 3 passages in culture[2]	14	3
c) after 3 passages + ascorbic acid [2]	30	8
Tendon: 16-17 day embryo:		
a) undissociated tendon[3]	54-68	20-30
b) freshly isolated tendon cells[3]	63-73	25-35
c) in culture with 5% fetal calf serum, 1 day after plating (high density), no ascorbic acid [4]	5	1.0
d) 6 day after plating (high density) (no ascorbic acid)	38	10.0
e) in 10% fetal calf serum (hvigh density?*) (5) + ascorbic acid	41*	12*
Embryo mix:		
a) secondary chick embryo fibroblasts[6]	7.2	1.3

Table 2 (cont'd)

Mammalian Cells

Cell strains (fibroblasts)(7)	37-52	10-17
3T3 (8,9)	2-19	0.5-4.0
3T6 (8)	1-5	0.2-1.0
L-929 (8)	5	1.0

The data for this table is derived from a number of sources. Almost all studies involving collagen synthesis use proline as the radioactive label. This is both because some assays used for collagen rely on the radioactive proline being specifically converted to hydroxyproline, and because proline is found 5.2 times more often in collagen than in the average non-collagen protein, giving the collagen assay a greater sensitivity. The first column shows the data in terms of the percentage of radioactive proline in collagen vs. total protein. To convert the hydroxyproline data it was assumed that it constitutes 47% of the proline content of collagen. To remove the "bias" due to the high proline content of collagen, these values were converted using the following formula: (Diegelmann and Peterkofsky, 1972):

$$\text{Corrected \% collagen} = R/(21.4/4.1)/1-[R-(R/21.4/4.1)]$$

$$= 0.19R/(1-0.81R)$$

where R = % radioactive proline in collagen vs. total protein

21.4 = mole % proline in collagen (Bornstein et al, 1972)

4.1 = mole % proline in the average protein (Diegelmann & Peterkofsky, 1972)

Table 2 (cont'd)

This corrected percent collagen is shown in the second column and is abbreviated "collagen synthesis" as a percentage of total protein synthesis. In the text,only the corrected percent of collagen synthesis is used to avoid confusion. It should be recognized, however, that some investigators do not correct for the bias in the level of proline incorporation when they report percent collagen synthesis.

1) Diegelmann & Peterkofsky (1972)
2) Peterkofsky (1972a)
3) Dehm & Prockop (1971)
4) Schwarz et al. (1976); Schwarz & Bissell (1977)
5) Kruse & Bornstein (1975)
6) Levinson et al. (1975)
7) Green et al. (1966)
8) Peterkofsky (1972b)
9) Green & Meuth (1974)

*Kruse & Bornstein, (1975) did not indicate at what day or what denisty they did their experiments. Judging by the data in 4, where even 3% serum is very inhibitory unless the density is high, we would expect that their experiments were performed at high densities. (Modified from Schwarz, 1975).

Table 3. Alteration in Collagen Types in Chondrocyte Culture

Tissue & Cell Type	Type In Vivo	Collagen Types in culture	Culture Conditions	Reference
Rabbit cartilage (chondrocytes)	II	II	Whole cartilage in culture	Layman et al., 1972
		I,(II)[1]	F12, Monolayer (19% FCS)[2]	"
		I,(II)	Monolayer, F-12, 10% FCS (Confluency) $\pm CaCl_2$	Deshmukh & Kline, 1976
		II	Suspension, Dulbecco's complete or special medium, 10% FCS (no $CaCl_2$)	"
		I,(II)	Suspension + 1.8 mM $CaCl_2$	Deshmukh & Sawyer, 1977
		I,(II)	Increased endogenous cAMP or addition of dibutyryl-cAMP	"

Table 3 (cont'd)

		I,III $[\alpha_1(I)]_3$ & X_2Y (A&B?)	Monolayer culture-- The ratios change as subcultured (see Fig. 11)	Benya et al, 1977,1978
		II,(III,I)	Organ Culture	Benya & Nimni, 1979
Chick embryo cartilage (chondrocytes)	II	I,$[\alpha_1(I)]_3$	With subculture & aging (10% FCS)	Mayne et al, 1976
		I,$[\alpha_1(I)]_3$	Grown in BrdUdr (10% FCS)	Mayne, et al, 1975
		II,I,III (relation to fibronectin)	F-12, 10% FCS	Dessau et al, 1978
Bovine articular cartilage	II	I,(II)	Slices; in presence of liver lysozomal enzymes	Deshmukh & Nimni 1973

(1) The parenthesis indicates that the given type was synthesized but in much smaller quantities.

(2) FCS = Fetal Calf Serum

Table 4. Changes in Levels and Types of Collagen in Cultured Cells from Normal Individuals and Those with some of the Connective Tissue Diseases.

Origin of Control Cells	Collagen Type & Level in Control Cells(1)	Origin of Diseased Cells	Collagen Type &/or Amount in Diseased cells(2)	Reference
Human Skin fibroblast	I, III; (IX)(1)	Osteogenesis Imperfecta	(↓) I/III ratio; (↓) amount of synthesis	Steinmann et al, 1979
Human skin fibroblast (or skin from scar tissue)	(IX)	Keloid fibroblast	(↑) amount of collagen synthesis	Diegelmann et al, 1979
Human articular cartilage	II	Osteoarthritic cartilage	II,I	Deshmukh & Nimni, 1979
Human skin fibroblast	I,III	Ehlers-Danlos Syndrome, Type V	I	Gay et al, 1976

Table 4 (cont'd)

Human skin fibroblast	(IX)	Hydroxyl lysine deficiency disease (Ehlers-Danlos syndrome, Type VI)	(↓) 10-14% of control lysyl-hydroxylase-- (↑) soluble collagen, (↑) degradation of collagen	Krane et al, 1972; Eyre & Glimcher, 1972
Human skin & tendons	(IX)	Ehlers-Danlos syndrome, type IV	(↑) procollagen synthesis, (↓) procollagen peptidase	Lichtenstein et al, 1973
Human skin fibroblast	(IX)	Ehlers-Danlos Syndrome V	(↓) lysyl oxidase (↓) soluble collagen	Di Ferrante et al, 1975

Table 5. Factors that Modulate The Level of Collagen Synthesis in Culture

A - Positive Modulators

Factors	Collagen	Non-Collagen	Synergistic Agents	Cell Types
Ascorbic acid	↑↑↑	-	High density	Avian tendon fibroblasts (1)
Ascorbic acid	↑↑↑	↓	"old cultures"	WI-38 human fibroblasts (2)
Population density (high)	↑	↓	-	Avian tendon fibroblasts (1,3)
Lactic acid	↑↑	-	High density	Avian tendon fibroblasts (3); L-929 (4)
CO_2	↑↑	?	High density	Avian tendon fibroblasts (3)
O_2 partial tissue	↑↑	-	Organ culture	Mature peridontal mouse tissue (5)
dbcAMP	↑	?	Viral transformation by MSV	3T3 (6)
Insulin	↑↑	↑	Serum albumin?	Calvaria from fetal rats (7)
Insulin	-	-	Ascorbic acid? High serum?	Human skin fibroblasts (8)
Glucocorticords	↑↑ (matrix)	↑	SV-40 transformation	Human skin fibroblasts (9)

Table 5 (cont'd)

A - Positive Modulators

Factors	Collagen	Non-Colalgen	Synergistic Agents	Cell Types
(Insulin, prolactin, hydrocortisone, progestrone, estradiol)	↑↑↑ (prevents degradation)	?	All hormones?	Mouse mammary ducts and alveoli (10)
Platelet factor(s)	↑↑	↑↑	Ascorbic acid?	Monkey arterial smooth muscle cells (11)
Increased glucose	↑↑	-	?	Human skin fibroblasts (8)

B - Negative Modulators

Population density (sparse)	↓↓	↓	-	Avian tendon fibroblasts (1,2)
Ascorbic acid(b)	↓↓	↓	"Young cultures"	WI-38 human fibroblasts (2)
Serum	↓	↑↑	?	Tendon fibroblasts (3,12)
Glucocorticoids	↓	↓	?	Mouse sponge granulomar and mouse fibroblasts (13)
cAMP	↓↓	-	?	Human foreskin fibroblasts (14)
Active product of *src* gene	↓↓↓	↑↑	Other viral factors?	(for references see section IIA,4)

Table 5 (cont'd)

B - Negative Modulators

Factors	Collagen	Non-Collagen	Synergistic Agents	Cell Types
Other viral and chemical agents	↓↓↓	↑↑	?	3T3 (15)
Tumor promoters	↓	↑	?	Avian tendon fibroblasts (16 and chick embryo fibroblasts (17)
Fraction I of embryo extract	↓↓	↓	?	Chondrocytes and other chick cells (18)

Table 5 (cont'd)

A and B References and Footnotes

(a) Arrows indicate an increase or decrease in the level of collagen. The "level" must be distinguished from "synthesis", since in most of these studies, collagen degradation was not measured. Three arrows indicates three-fold or more increase. When the number of arrows are the same in the collagen and non-collagen columns and in the same direction, the effect is non-specific.

(b) If this effect is not due to selective toxicity, to my knowledge this is the only report in the literature where addition of ascorbic acid leads to a decrease in the rate of collagen synthesis.

(1) Schwarz and Bissell (1977).

(2) Paz and Gallop (1975).

(3) Schwarz, et al, (1976).

(4) Langness and Udenfriend (1974).

(5) Yen, et al, (1979).

(6) Peterkofsky and Prather (1974).

(7) Canalis et al, (1977)

(8) Villee and Powers (1977)

(9) Furcht, et al, (1979).

(10) Liotta, et al, (1979)a

(11) Burke and Ross (1977).

(12) Schwarz, et al, (1979).

Table 5 (cont'd)

(13) Kruse, et al, (1978).

(14) Baun, et al, (1978)

(15) Hata and Peterkofsky (1977).

(16) Bissell, et al, (1979)a

(17) Delclos and Blumberg (1979).

(18) Schlitz and Ward (1980).

Table 6. How Normal are Primary Avian Tendon Cells in Culture?

	Intact Tendon or Freshly Isolated Cells (16-17 day old embryos)	In Culture (7 days)
% Collagen	25-35%(1,2)	22-31% (3)
Rate of Synthesis	0.63 μg/10^6 cells/hr(4)	0.6 μg/10^6 cells/hr(5)
Collagen Type	Mainly Type I (>95%)	Mainly Type I (>95%)(6)
Response to Ascorbate		
1) Hydroxylation	+ (by inference)	+(5)
2) Synthesis	+ (by inference)	+(3)
Fiber Formation		
1) Spacing	670 Å(7)	670 Å(7)
2) Diameter	370-560 Å(7)	150-250 Å(7)
Glucose Metabolite Patterns	"Normal"(8)	"Altered"(8)

(1) Dehm & Prockop (1972)

(2) Schwarz, Farson & Bissell (1979)

(3) Schwarz & Bissell (1977)

(4) Kao et al. (1977)

(5) Schwarz & Bissell (1979); Schwarz, R.I., Mandell, B.R. and Bissell, M.J. (unpublished)

(6) Soo (1979); Soo, W.-J., Schwarz, R., Bassham, J.A., and Bissell, M.J. (unpublished)

(7) Tung et al. (1977); Tung, S.A., Schwarz, R.I. and Bissell, M.J. (unpublished)

(8) Bissell, M.J., Schwarz, R.I. and Hatie, C. (unpublished); by "normal" we mean the pattern observed for intact tendon or freshly isolated cells.

Table 7. How Normal are Mammary Epithelial Cells in Culture?

Parameter	Mid to Late Pregnant		Lactating	
	Freshly isolated epithelial cells (or tissue pieces)[1]	In Culture (floating gel)	Freshly isolated epithelial cells (or tissue pieces)[1]	In Culture (floating gel)
Morphology	secretory epithelium	secretory epithelium[2]	secretory epithelium	secretory epithelium[3]
Casein synthesis	++	++[4]	++++	?
Casein secretion	±	++[4]	++++	?
Lactose synthesis	+	+[5]	++++	+[5]
Lactose synthase	present	present[6]	present, high	?
Medium chain fatty acids	+	±[7]	++++	?
Glycogen synthesis	++++[8-10]	+++[8-10,5]	+[8-10]	+++[8-10,5]
Alanine pool	++++[5]	++++[5]	+[5]	++++[5]
Glucose metabolite patterns	"Normal"	"Normal"[5]	"Normal"[11]	Pattern similar to cells from the gland of pregnant mice[5]

Table 7 (cont'd)

(1) Tissue pieces from the gland of the pregnant mice include appreciable amount of fat cells and other cell types; thus the freshly isolated cells and tissue pieces are not equivalent. The predominant cell type in the lactating gland is the epithelila cell; thus tissue pieces and freshly isolated cells are comparable. For review of mammary gland functions *in vivo*, tissues, and isolated cells see Banerjee (1976).

(2) Emerman & Pitelka (1977).

(3) Burwen & Pitelka (1980).

(4) Emerman. *et al.*, (1977).

(5) Emerman, J.T., Bartley, J. & Bissell, M.J. (unpublished).

(6) Häuptle, *et al.*, (1979).

(7) Bartley, J. (Personal Communication).

(8) Emerman & Bissell (1979b).

(9) Emerman, *et al.*, (1979).

(10) Bartley, *et al.*, (1979).

(11) Emerman & Bissell (1979a); by "normal" we mean the pattern observed for isolated cells immediately after the gland's removal from the animals. In the case of lactating gland, these patterns are similar in the tissue pieces and the isolated cells (see 1).

LEGENDS TO FIGURES

Fig. 1. Comparison of the metabolite patterns of chick embryo fibroblasts and chick embryo liver. Chick embryo fibroblasts and hepatocytes were isolated from 16 day old embryo. Two days after seeding in medium 199, cells were incubated with [U-^{14}C]-glucose (5.5 mM) for 1 hr and processed for paper chromatography and autoradiography as described (Bissell, et al., 1973; Bassham, et al., 1974). O, origin (contains glycogen and other macromolecules); 6PGA, 6-phosphogluconate; 3PGA, 3-phosphoglycerate; PEP phosphoenol pyruvate; GP α-glycerol phosphate; Asp, asparate; Cit, critrate; Mal, malate; Fum, fumerate; Glut, glutamate; Gluc, glucose; Gln, glutamine; Ala, alamine; Lac, lactate (M.-W. Teng and M. J. Bissell, 1976, unpublished).

Fig. 2. Comparison of labeled glucose from primary BRL (Buffalo rat liver cells, a "normal" cell line) and HTC (a hepatoma cell line). UDPGA; UDP glucaronic acid; UDPG, UDP-glucose; UDP Gal; UDP-galactose; F6P, fructose-6-phosphate; FDP, fructose 1,6-diphosphate; G6P, glucose-6-phosphate; GA1P, glucuronic acid-1-phosphate;GA, glucuronic acid; PMP, pentose monophosphate (intermediates); Fruc + Sorb,, fructose + sorbitol; other legends as in Fig. 1 except cells were exposed to ^{14}C-glucose for 30 min. (From D.M. Bissell, et al., 1978; reproduced with permission). Note the absence of GA and GA1P from liver cell lines and decreased Lac, Ala and GP in primary cultures.

Fig. 3. Four stages of collagen synthesis in primary avian tendon cells in culture. Cells were seeded at 1.2×10^5 in medium F12 supplemented with 0.5% fetal calf serum and 50 ug/ml ascorbic acid. The left ordinate shows the percentage of ^3H proline sensitive to collagenase; the right ordinate is the corrected value for the ratio 5.2 of proline content in collagen to that in other cellular proteins (see Table 2). Only the corrected value is referred to in the text. See text for description of A, B, C and D periods. (Schwarz, et al., 1979 reproduced with permission).

Fig. 4. The influence of the stage of subculturing on the subsequent rate of collagen synthesis. Primary avian tendon cells seeded as in Fig. (3),(●) secondary cultures prepared before confluence on day 5 (○) and after confluence on day 7 (□). (Schwarz, et al., 1979 reproduced with permission).

Fig. 5. Upper Figure (1): Primary culture of normal rat adrenal cortex. Most cells have assumed a spindle-shaped 'fibroblastic' form. There is little resemblance to the histologic appearance of the tissue. Lower Figure (2): Primary culture of a moderately well-differentiated rat adrenocortical carcinoma. The cells have maintained the epithelial form and growth pattern characteristic of the in vivo state. Cytoplasmic granularity is due to lipid inclusions, a tissue-specific feature of adrenal cortex. These cultured malignant cells resemble the normal tissue of origin more than do the cultured normal cells shown above. (From Auersperg, 1974; reproduced with permission). X224. (Original photo courtesy of N. Auersperg).

Fig. 6. Effect of glucose-free medium on ATP levels in liver cell cultures. Primary cultures, 24 h after plating, and BRL and HTC cells, at confluency, were changed to serum-free modified 199 medium with or without 5.5 mM glucose, at time 0. At time points indicated, cultures incubated with glucose ("control") or without glucose were anaalyzed for ATP content. (From D. M. Bissell, et al., 1978; reproduced with permission).

Fig. 7. Metabolism of [U-^{14}C] sorbitol by liver-derived cells in culture. Cultured primary hepatocytes and liver cell lines were exposed to 30 μCi/ml ^{14}C-sorbitol for 1 hr. Other legends as in Fig. 1 and 2. (From Levine, et al., 1978; reproduced with permission).

Fig. 8. Modulation of collagen synthesis in primary avian tendon cells by ascorbic acid. Cells were grown in medium F12 plus 0.5% serum with (●) or without (▲) ascorbic acid (50 μg/ml). Ascorbic acid was added on day 4 to scorbutic cultures (△) and removed on day 5 from cultures containing ascorbic acid (○). Other legend as in Fig. 3. (From Schwarz and Bissell, 1977).

Fig. 9. Effect of incubation period and serum on lactate production by primary hepatocytes in monolayer culture. When serum was present, it was added at time of cell plating and maintained throughout the indicated incubation period. (From D. M. Bissell, et al., 1978; reproduced with permission).

Fig. 10. Schematic and simplified presentation of steps involved in collagen biosynthesis and processing.

Fig. 11. Changes in the types of collagen synthesized as a function of subculturing of chondrocytes. (From Benya, et al., 1978; reproduced with permission).

Fig. 12. The change in the rate of collagen biosynthesis as a function of viral-transformation. Primary avian tendon cells were plated at a density of 8×10^5 per 25 cm^2 flask. They were infected with a Rous sarcoma virus mutant, temperature sensitive in the src gene (LA-24) at a multiplicity of 10. Infected cells were kept at 39° for the first two days and then switched to either 41° or 35°. Ascorbic acid 950 μg was added daily after day 4. On the 7th day, half the plates at either temperature were shifted to the other temperature. Collagen synthesis was measured at 0, 4, 8, 12, and 24 hrs after the temperature shifts. (Soo, et al., 1977; W. J. Soo, R. I. Schwarz, J. A. Bassham, and M. J. Bissell, submitted). The percent level of collagen in temperature sensitive infected cells at the non-permissive cells is much higher than cells at the permissive temperature as can be seen. However, the level is not entirely 'normal'. While uninfected primary avian cells at 39°C could synthesize collagen at 25-30% of total protein synthesis (Schwarz and Bissell, 1977), ts-infected cells at the non-permissive temperature synthesize collagen at 12-17% (see text).

Fig. 13. Electron micrograph of cells in an alveolus of a gland from a lactating mouse. Tight junctions (TJ) joining adjacent cells and microvilli (Mv) are found at the apical (luminal) surface of the gland. A basal lamina (BL) is present at the basal cell surface. The cytoplasm has abundant endoplasmic reticulum (ER), a large Golgi apparatus (G), secretory vesicles (arrowhead), and part of a fat droplet (F). The nucleus (Nu) is locted toward the basal end of the cell. Courtesy of Dr. Dorothy Pitelka. X7360.

Fig. 14. Electron micrograph of a 5-day culture of epithelial cells dissociated from midpregnant mouse mammary gland and plated on a floating collagen gel (FG). The pavement cells have microvilli (Mv) and tight junctions (TJ) at their apical surface. A basal lamina (BL) separates the epithelium from the gel and cells below. The cytoplasm contains an extensive network of distended rough endoplasmic reticulum (ER) Golgi apparatus (G), secretory vesicles containing dense glandular material (arrowheads) and fat droplets (F). Nuclei (Nu) are central or basal. Embedded in the collagen matrix at the bottom of the picture is a stromal cell, probably a fibroblast, also with distended rough endoplasmic reticulum. The content of the cisternae is distinctly different from that in the epithelial cell. (From Emerman and Pitekla, 1977; reproduced with permission; the original courtesy of Dr. Joanne Emerman. X9000.

Fig. 15. Electron micrograph of a 7-day culture of epithelial cells dissociated from midpregnant mouse and plated on plastic substrate. Mammary specific differentiation is lacking (compare with Fig. 14). However, the epithelial origin of these cells is identifiable by the presence of tight junctions linking adjacent cells at their apical borders in a continuous mosaic. Some microvilli (Mv) are present. (From Emerman, et al., 1979; reproduced with permission; the original courtesy of Dr. Joanne Emerman).

Fig. 16. Autoradiograms of labeled glucose metabolites form lactating and virgin mice. Mammary tissue from lactating and non-lactating mice were incubated in 0.5 ml of 5.5 mM [U-^{14}C-glucose). F + S, fructose and sorbitol. Other legands as in Figs. 1 and 2. Note the presence of lactose spot in (A) and its absence in (B). (From Emerman and Bissell, 1979; reproduced with permission).

Fig. 17. Glycogen and lactose synthesis by mammary epithelial cells from mice (8 days pregant to 10 days lactating). Cells were isolated and incubated in 11 mM [U-^{14}C]-glucose. After 1 hr incubation, lactose and glycogen (origin of the chromatograms) were isolated by procedures described (Figs. 1, 2, 16) ●-●, rate of glycogen synthesis; o-o, rate of lactose synthesis. Arrow indicates the time of parturition. (J. T. Emerman, J. Bartley, and M. J. Bissell, submitted).

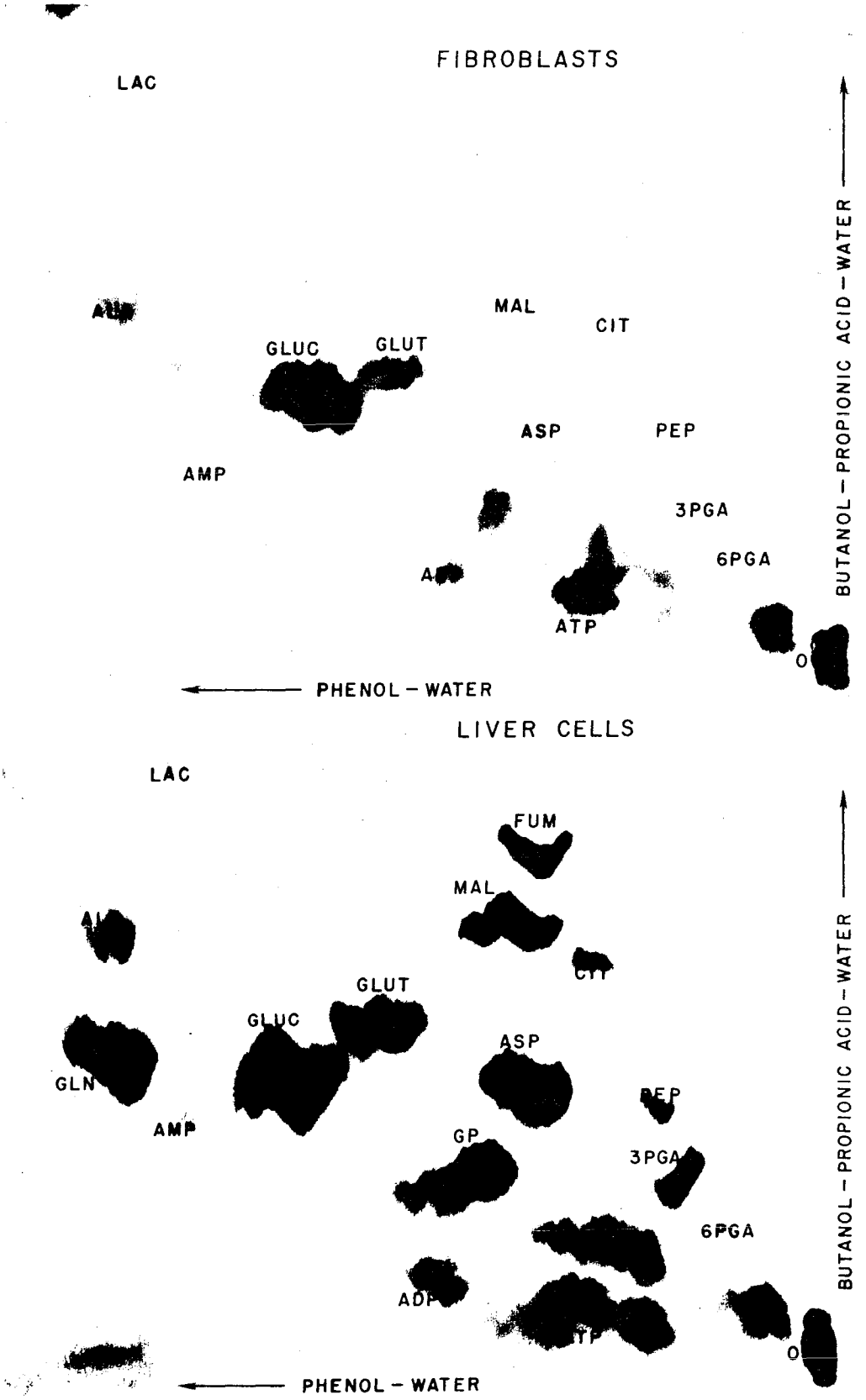
Fig. 18. Glucose derived alanine pool during different stages of mammary gland development. Cells were isolated and labelled as described in Fig. 17. Labelled alanine pools were from chromatograms as described in Fig. 1.

Fig. 19. Formation of glycogen and lactose from [U-^{14}C]glucose in mammary epithelial cells from lactating mice cultured on floating collagen gels. Cells were incubated in medium 199 containing 5 µg each of insulin, cortisol and prolactin. Each point represents the amount of ^{14}C-glycogen or ^{14}C lactose synthesized after a one hr exposure to [U-^{14}C] glucose on the days indicated. (J. T. Emerman and M. J. Bissell, unpublished).

Fig. 20. Glycogen synthesis by mammary epithelial cells on floating gels. Cells from pregnant and lactating mice were seeded on collagen gels as described (Emerman and Pitelka, 1977). Glycogen synthesis was measured from the origin of paper chromatograms as described (Bissell, et al., 1973).

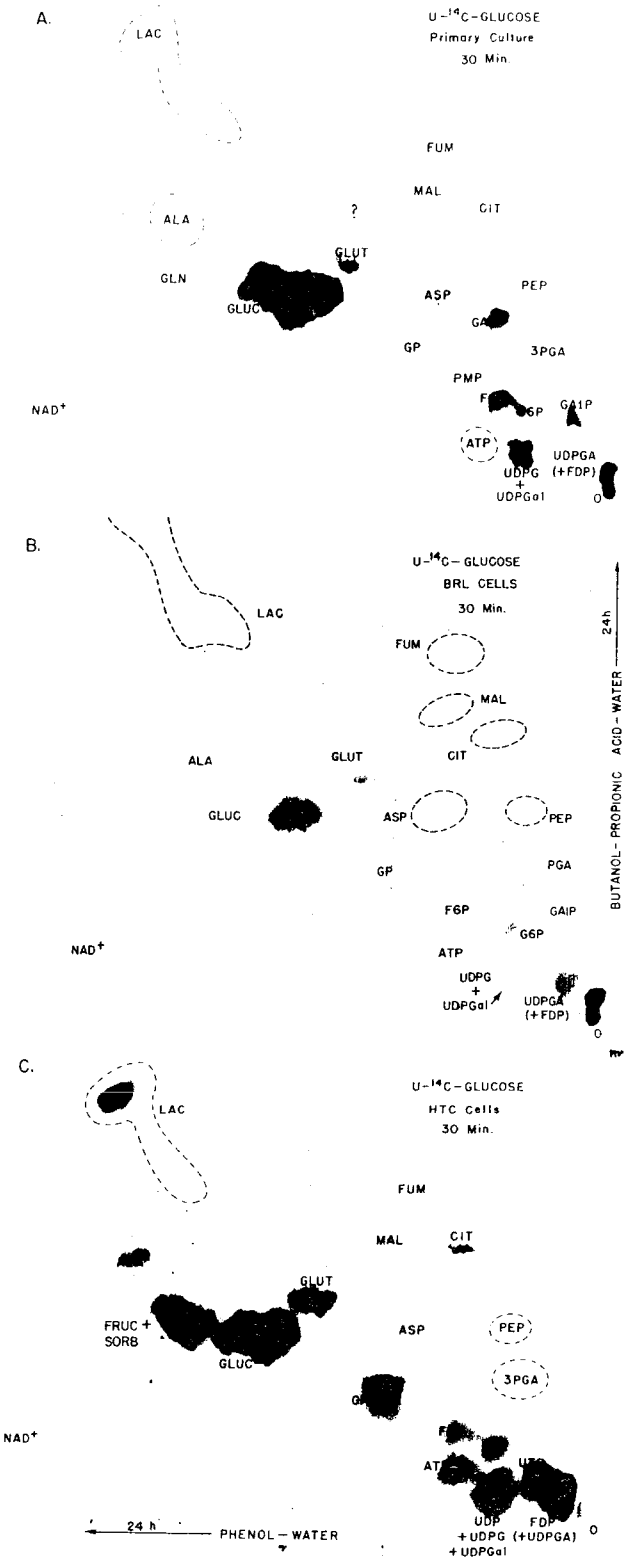
Fig. 21. Transmission electron micrograph of cross section of 16-day old chick embryo tendon. (Tung, S.-A., Schwarz, R. I. and Bissell, M.J., unpublished).

Fig. 22. Scanning electron micrograph of primary avian tendon cells in culture. Picture was taken of confluent monolayer of tendon cells after one week in culture(medium F-12, 50 µg/ml ascorbic acid, 0.15% fetal calf serum; Tung. S.-A., Schwarz, R. I. and Bissell, M.J., unpublished).



XBB 765-4020A

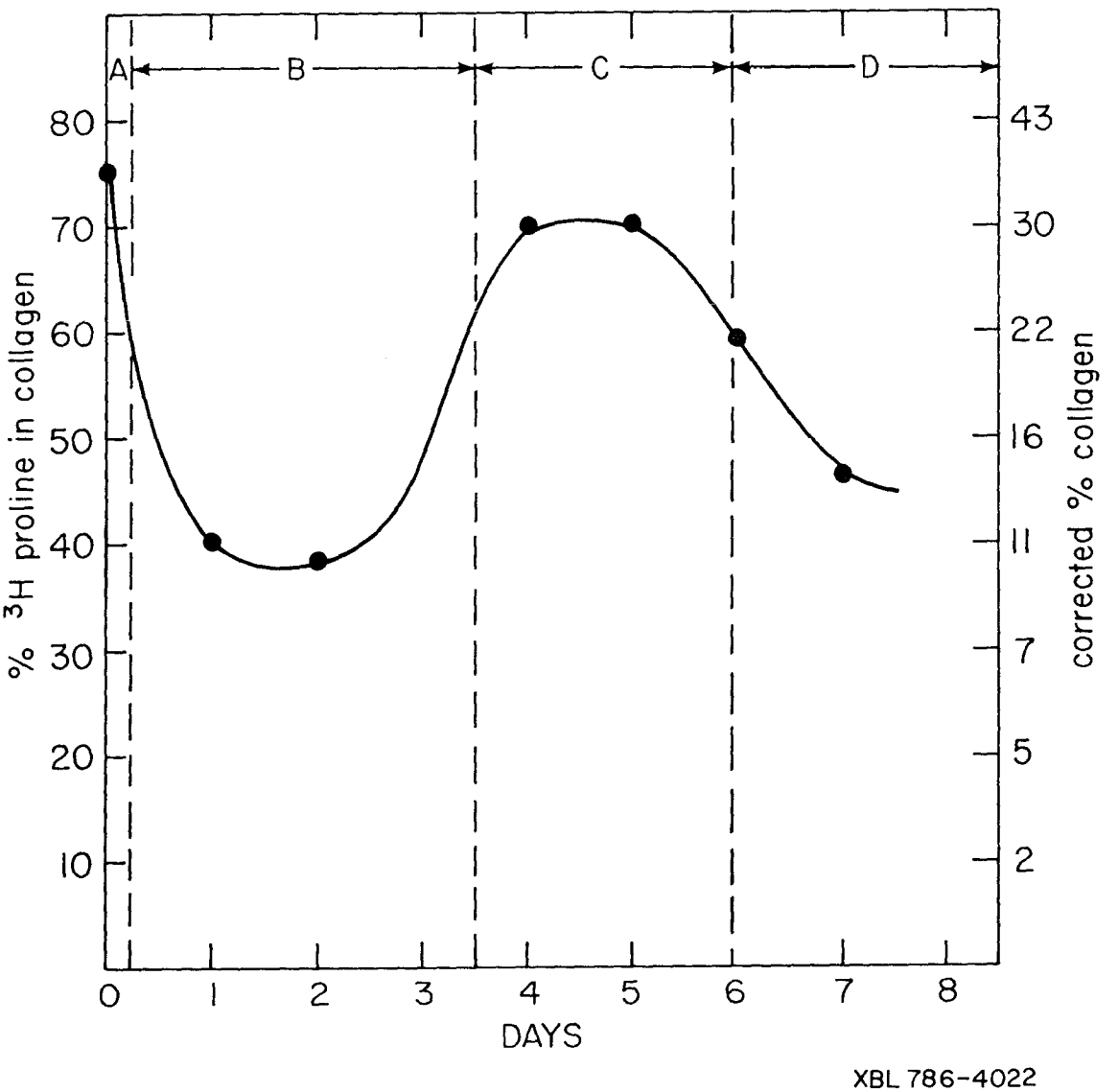
Bissell, M.J.
Fig. 1



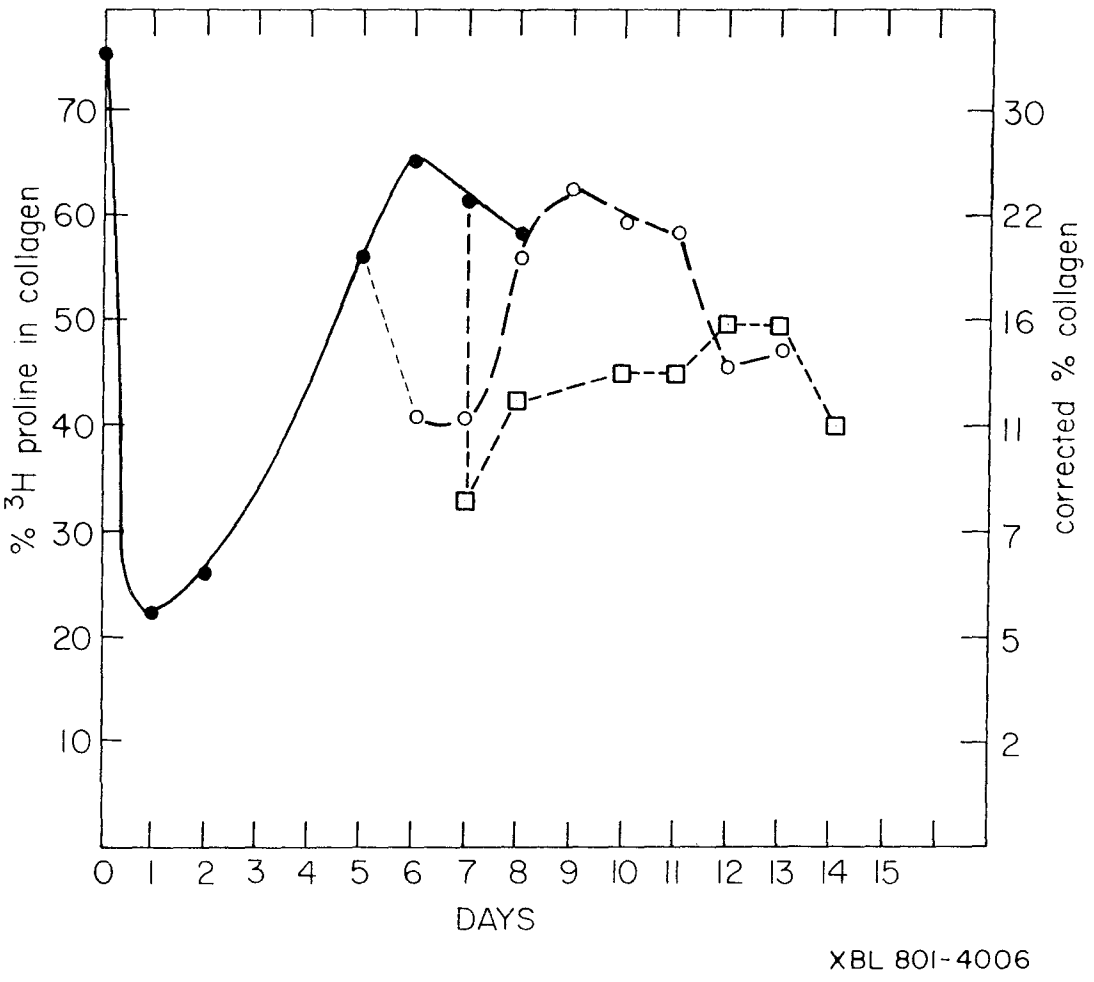
XBB 767-6538A

Bissell, M.J.

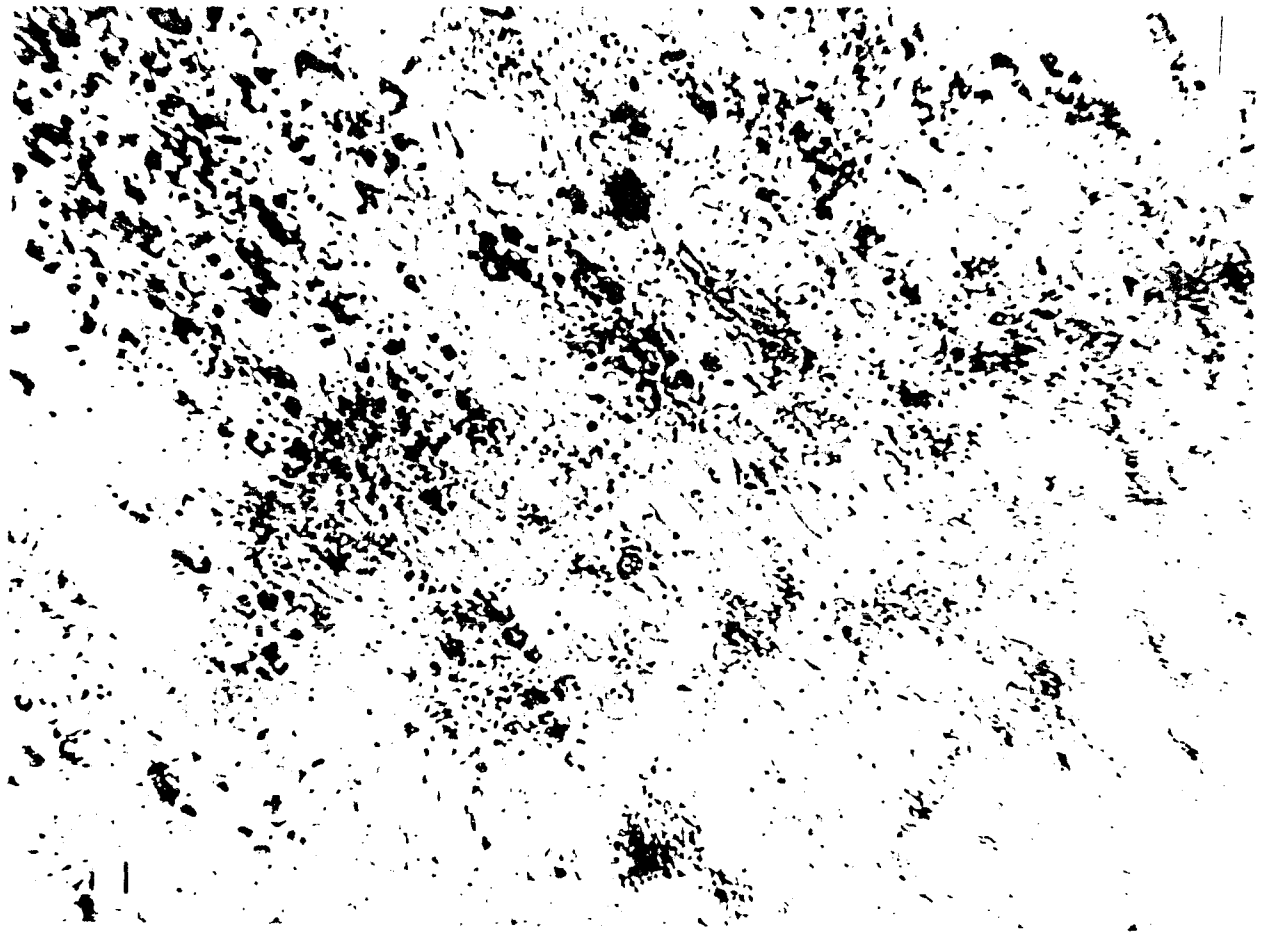
Fig. 2



Bissell, M.J.
Fig. 3



Bissell, M.J.
Fig. 4



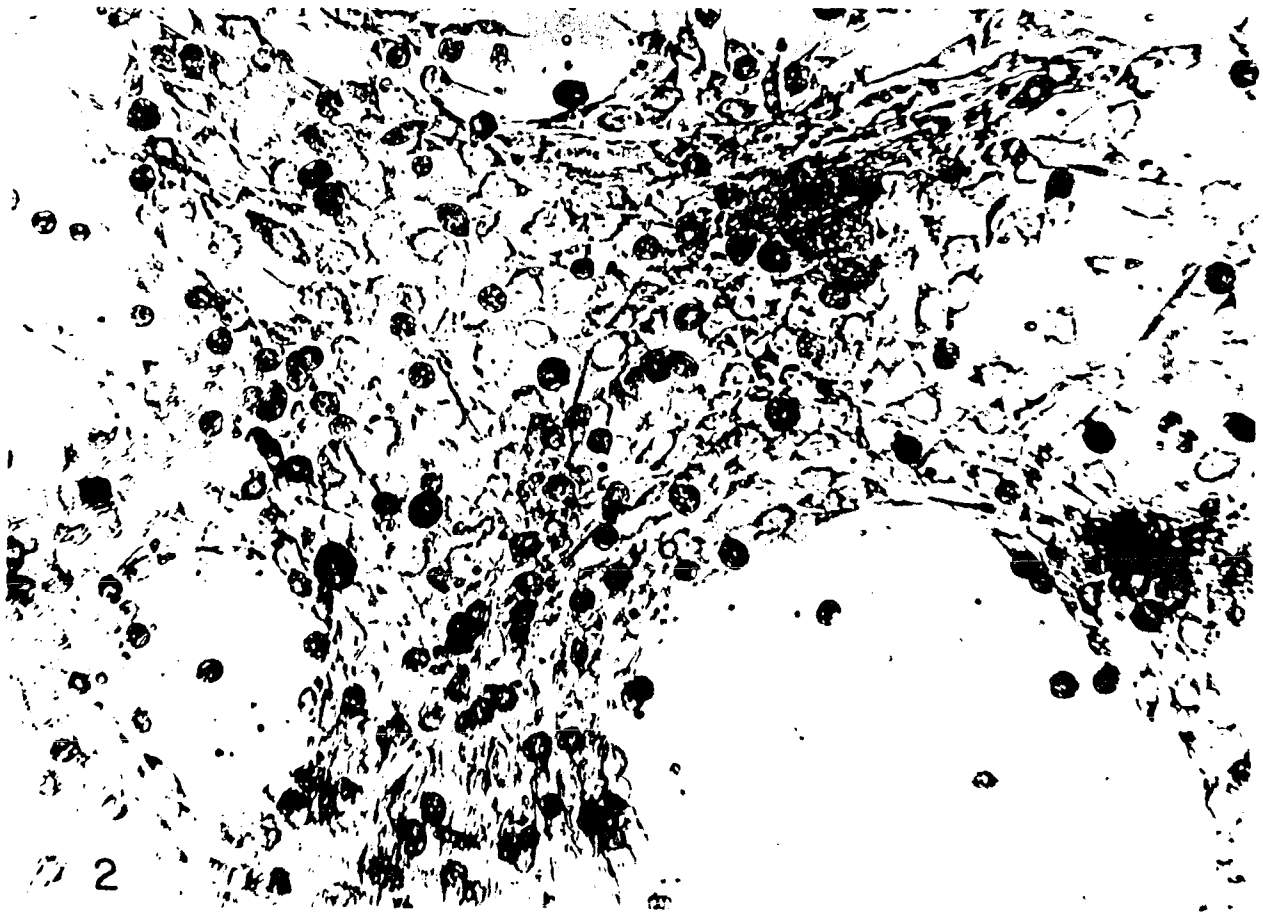
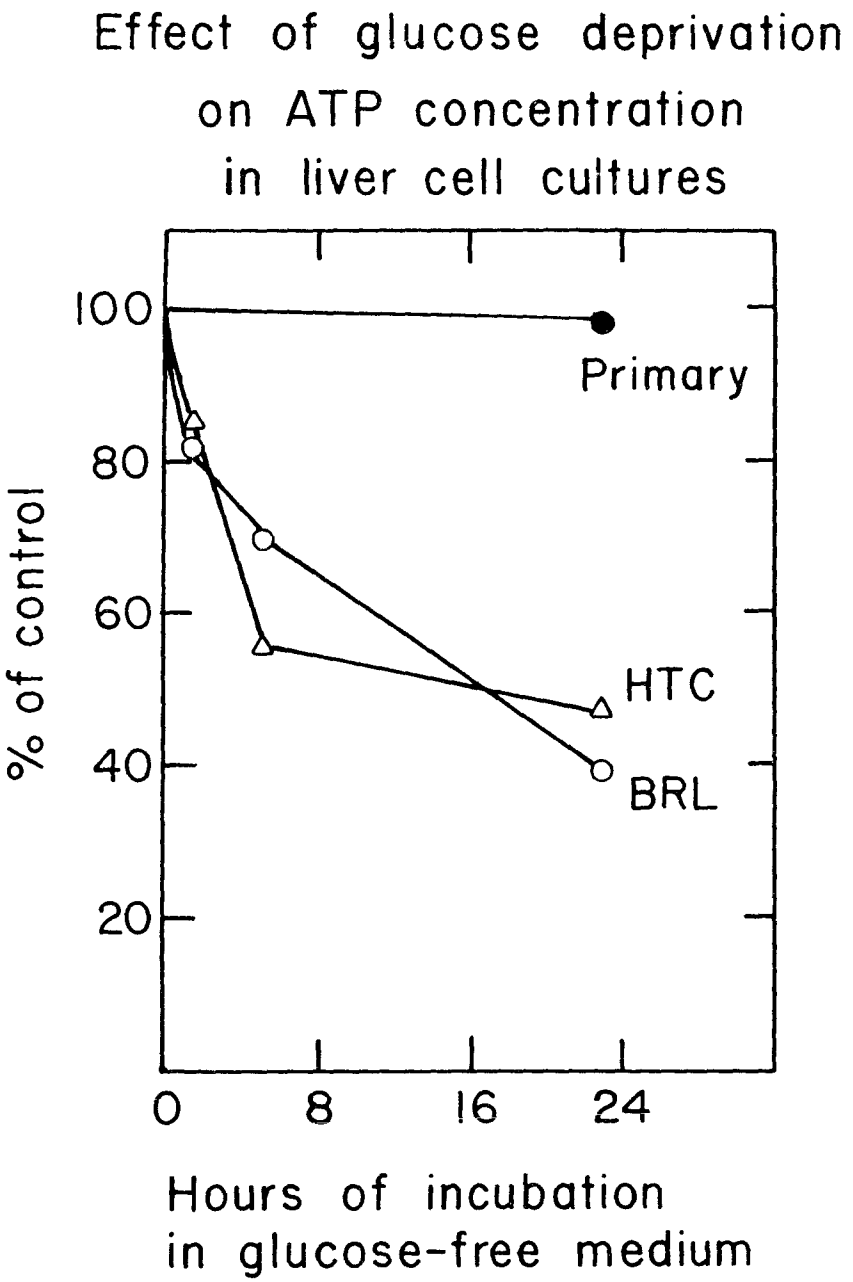


Fig. 5

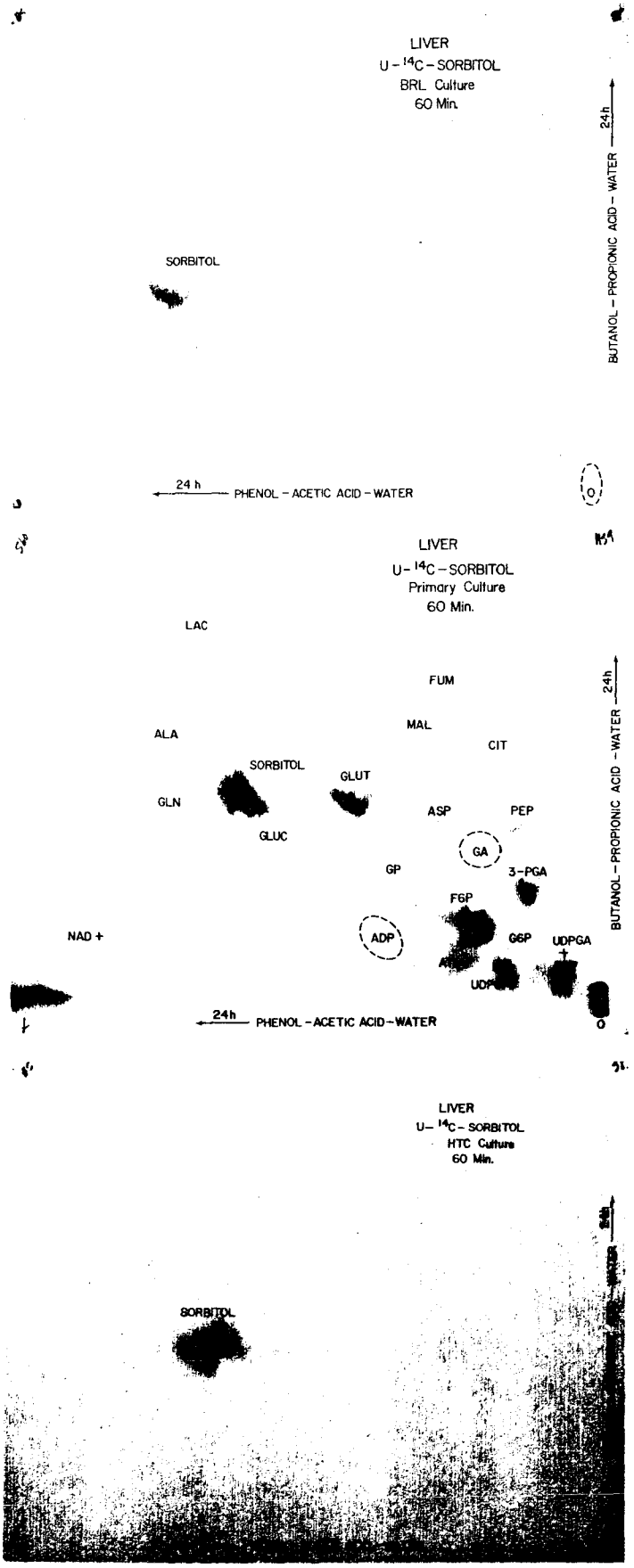
Bissell, M.J.

XBB 801-838



XBL 7612 – 10829A

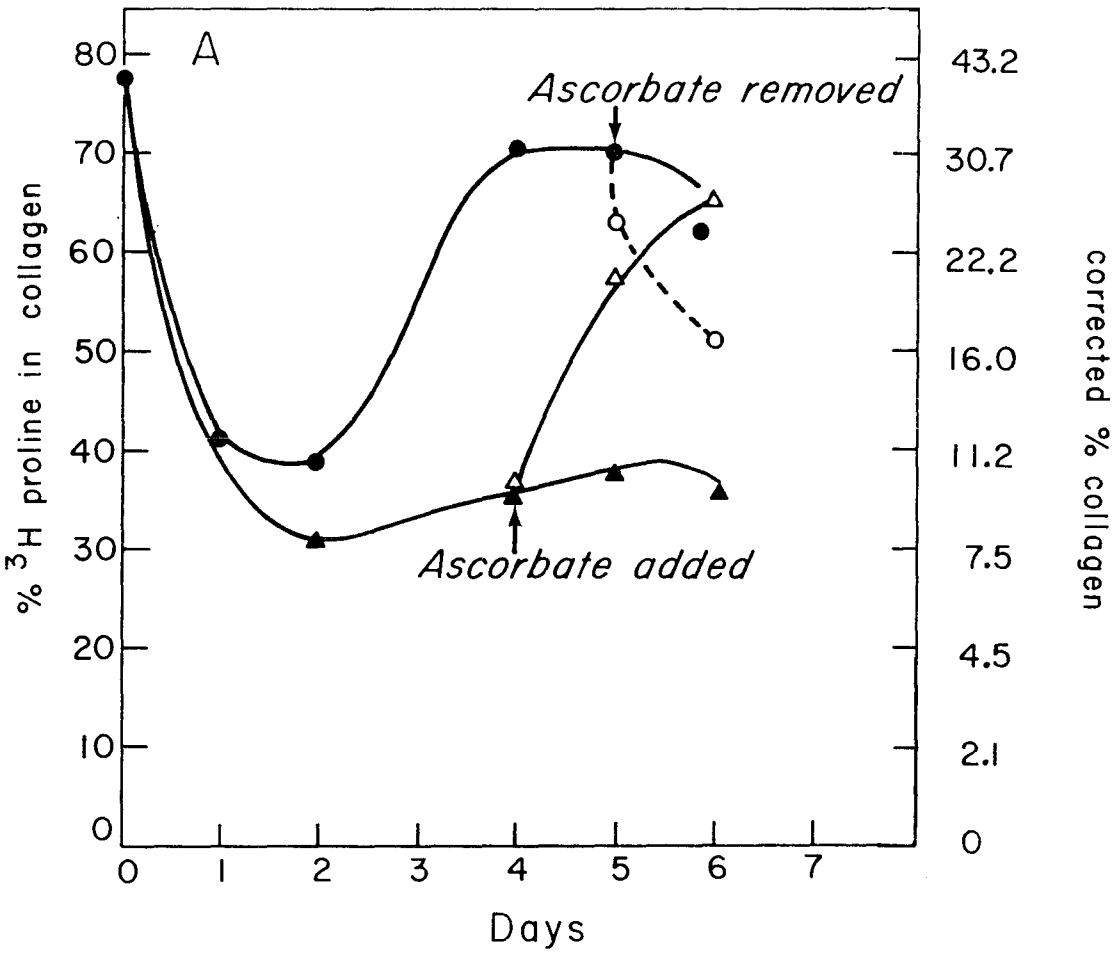
Bissell, M.J.
Fig. 6



XBB 777-7169

Bissell, M.J.
Fig. 7

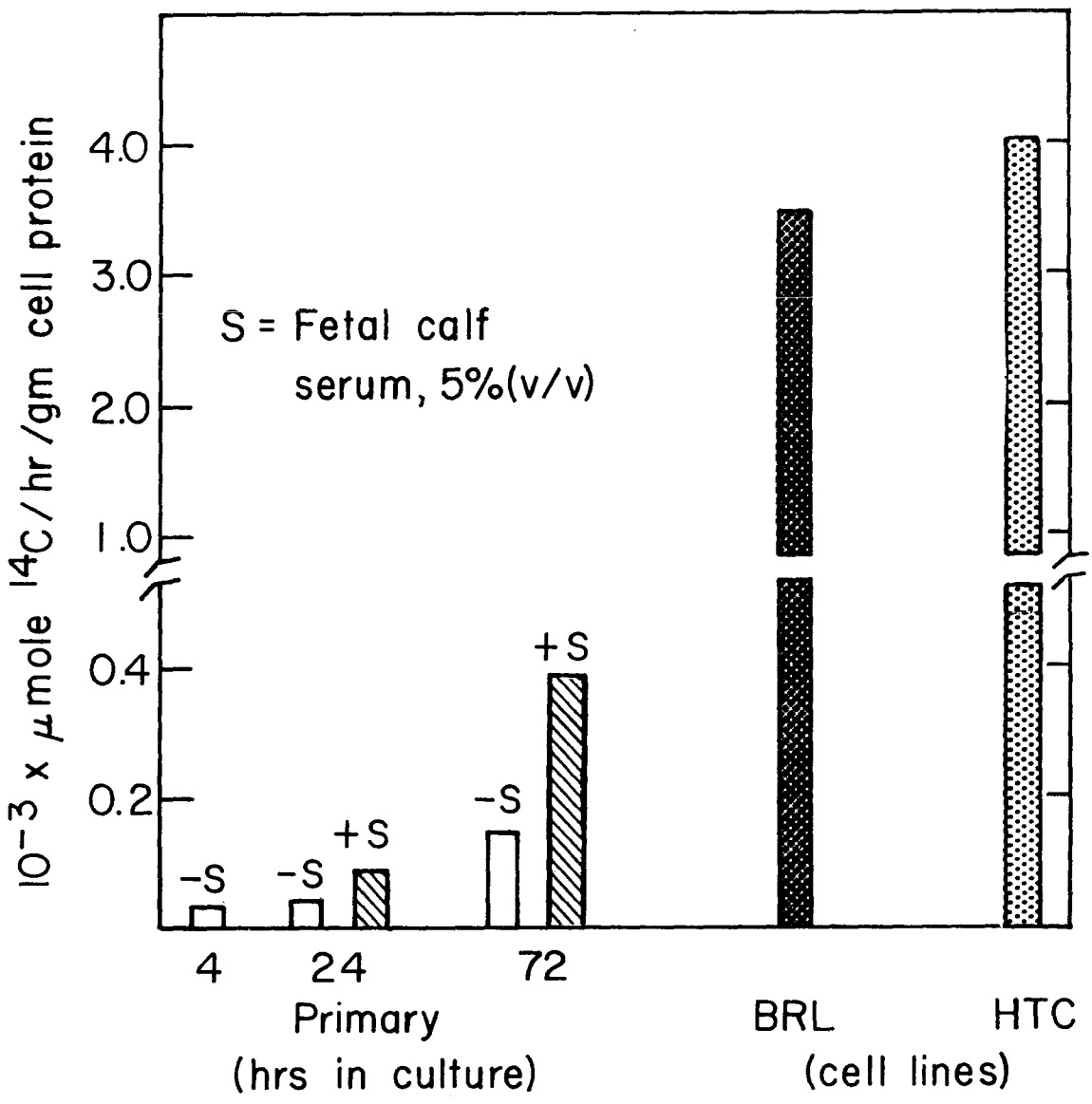
Ascorbate modulation of collagen synthesis in primary avian tendon cells



XBL 771-4131A

Bissell, M.J.
Fig. 8

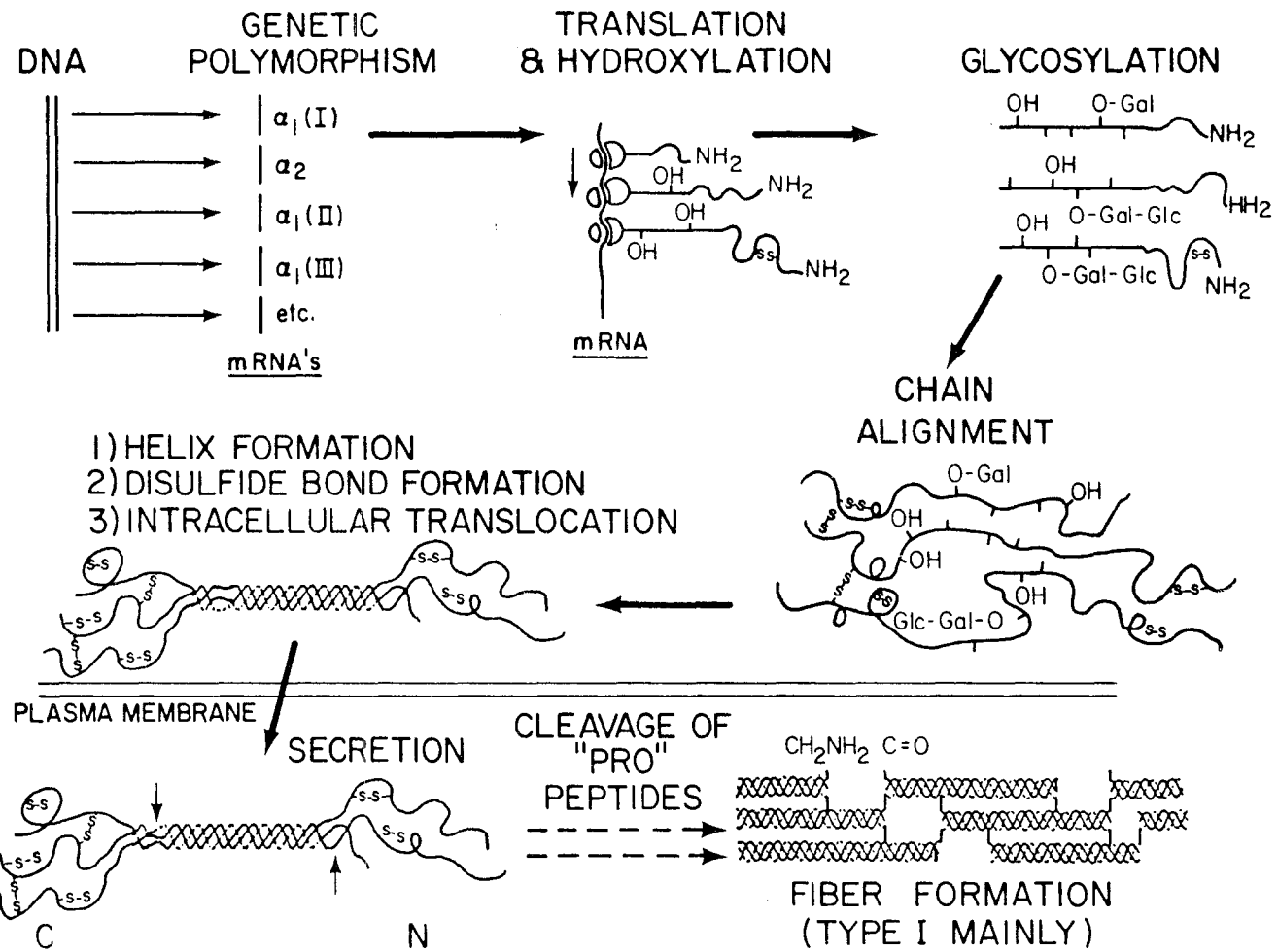
Production of lactate by liver cell cultures



XBL 7612 - 10833A

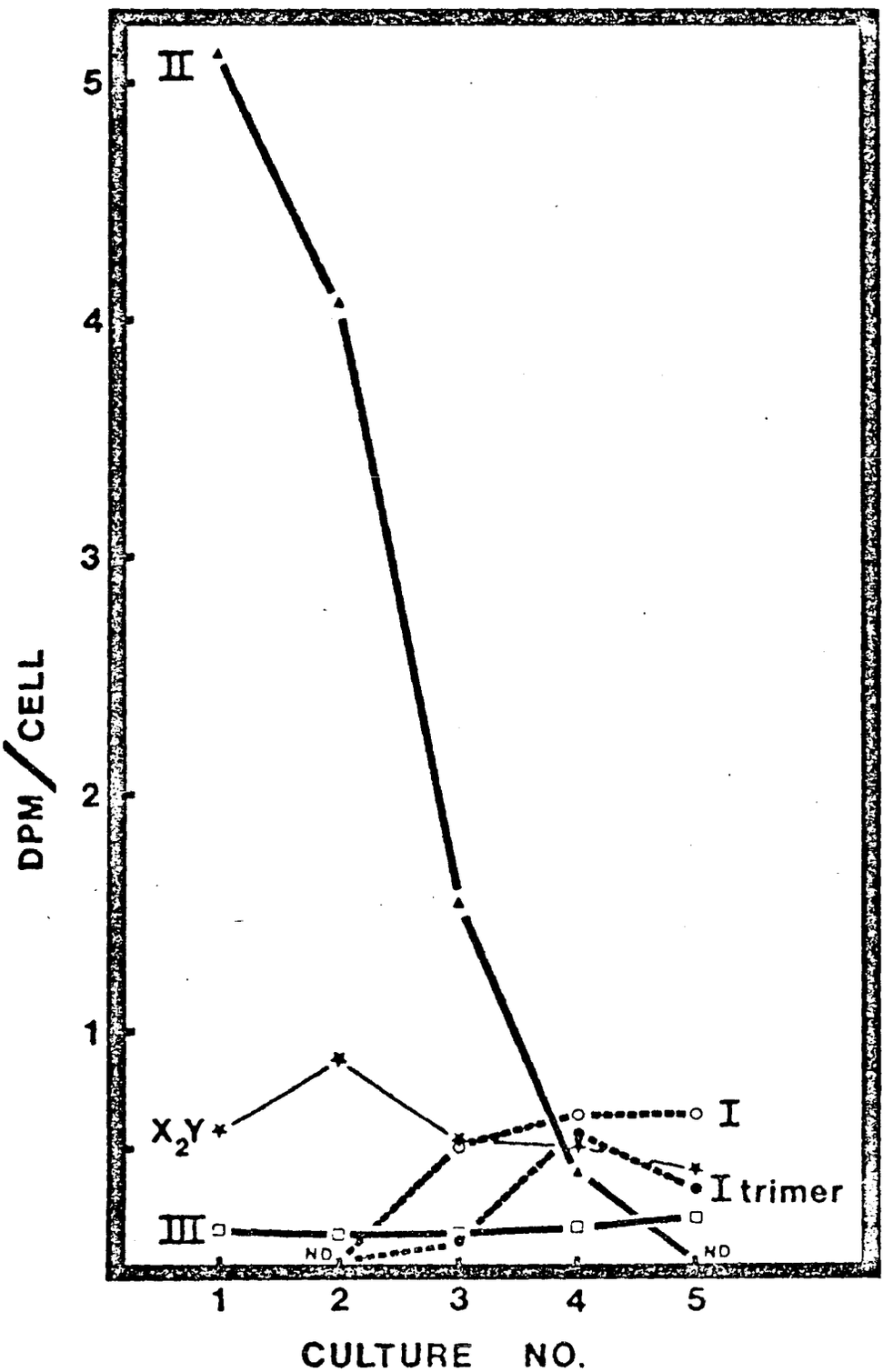
Bissell, M.J.
Fig. 9

Summary of Steps Involved in Collagen Synthesis and Processing



XBL 802-4052A

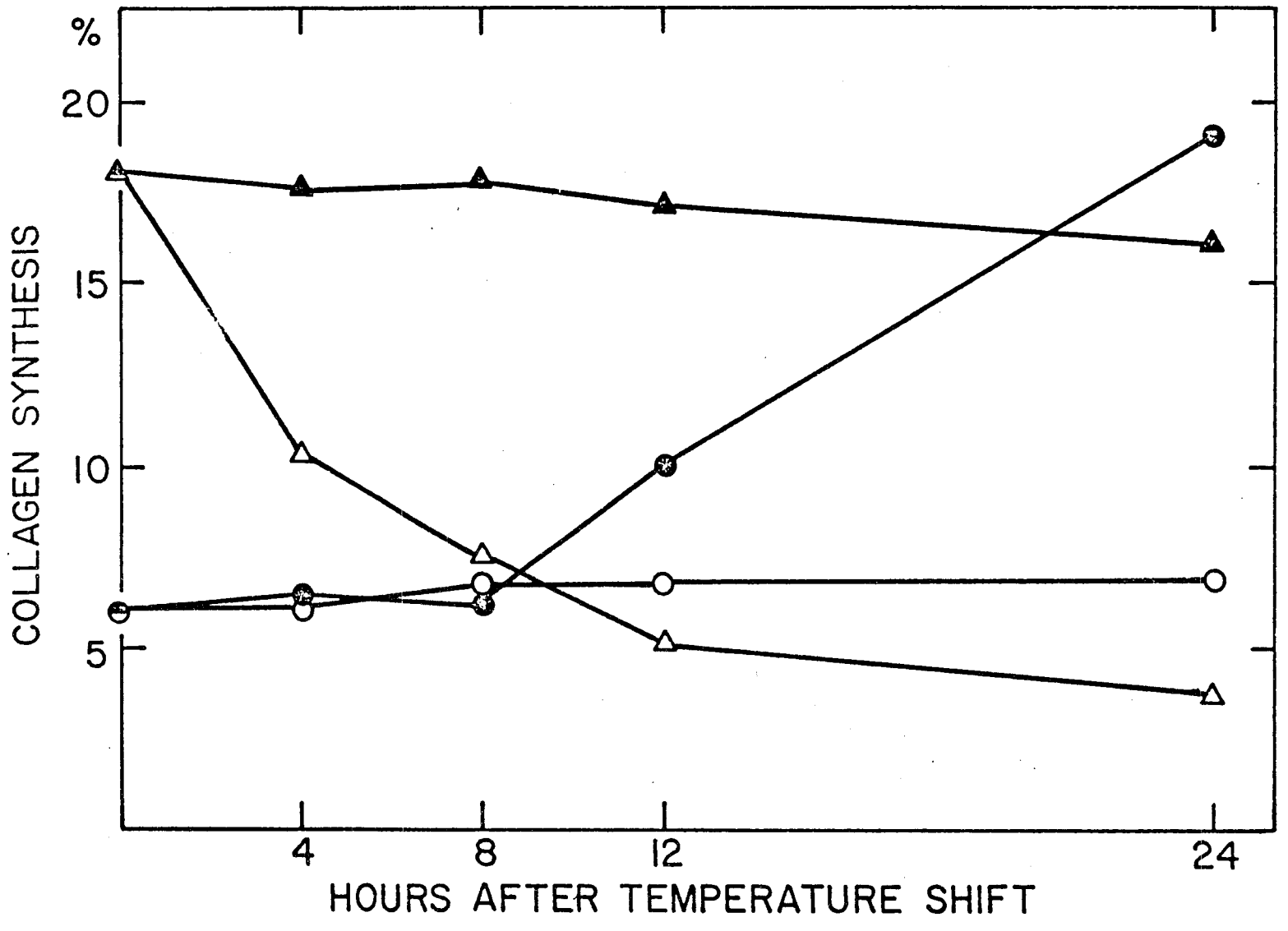
Bissell, M.J.
Fig. 10



XBL 802-8033

I. J. Bissell

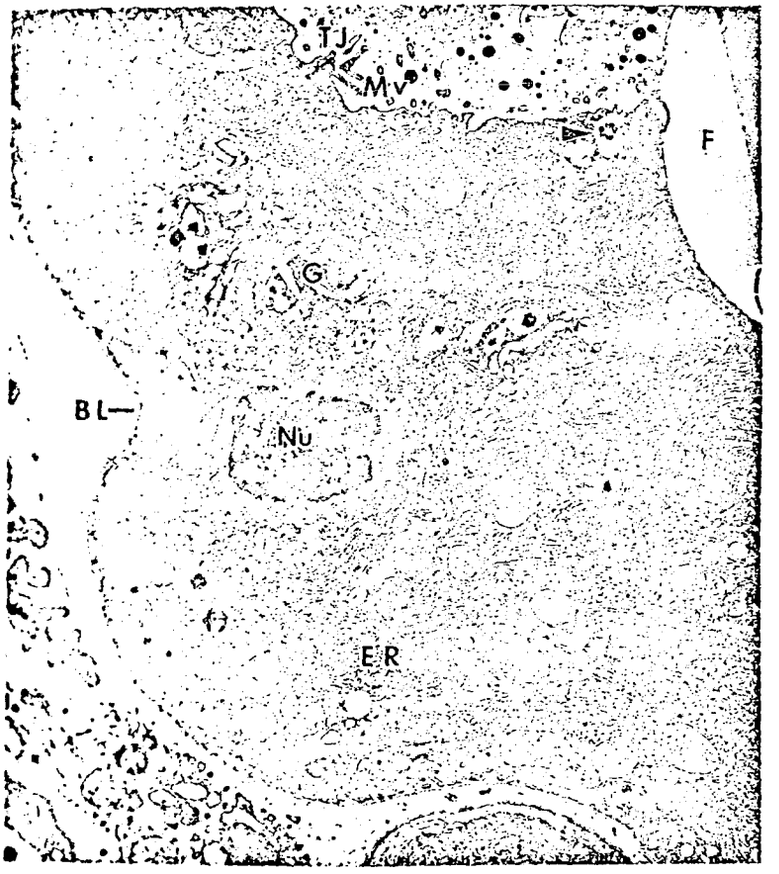
Figure 11



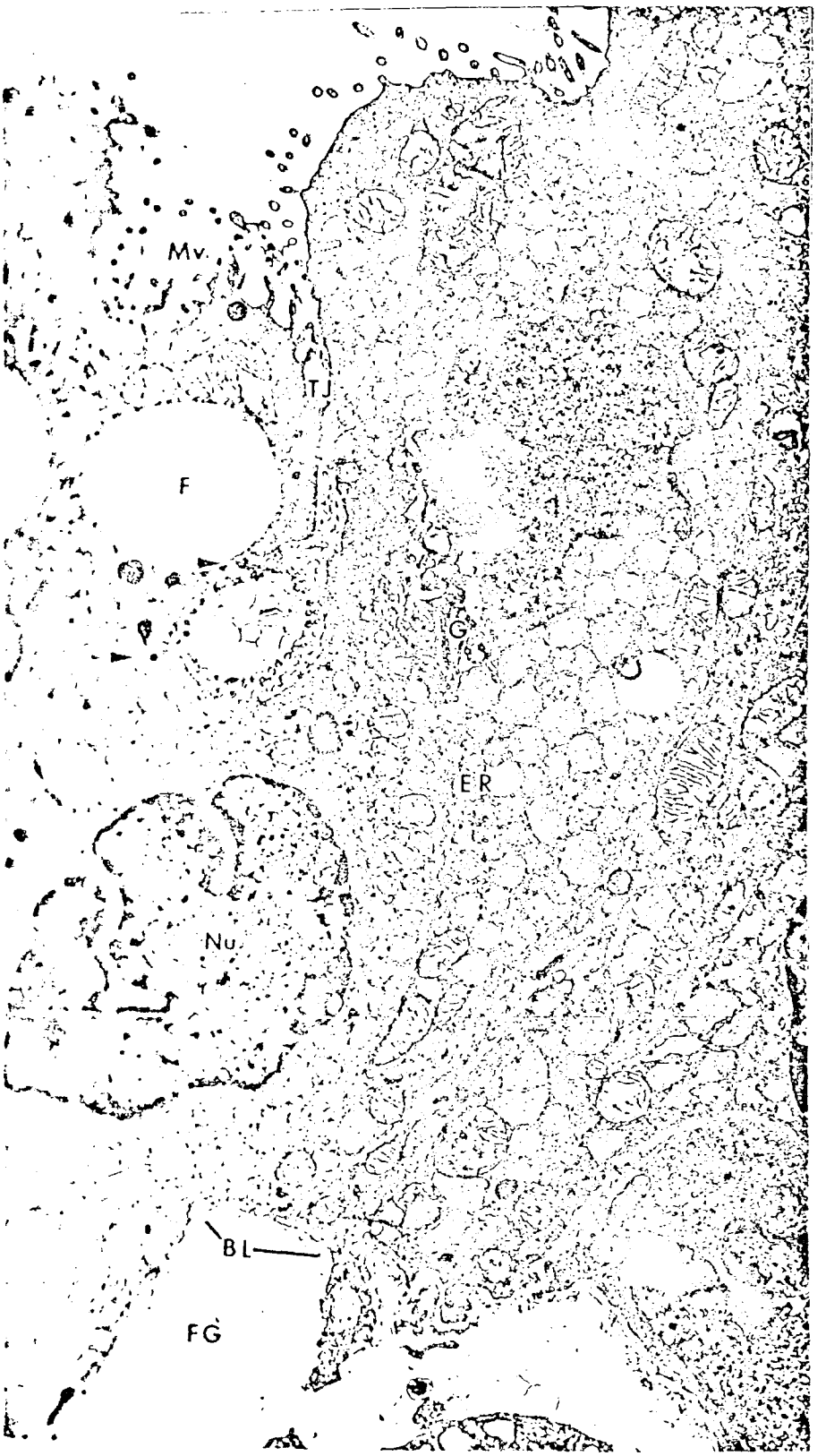
XEL 7711-4745

1. J. Bissell

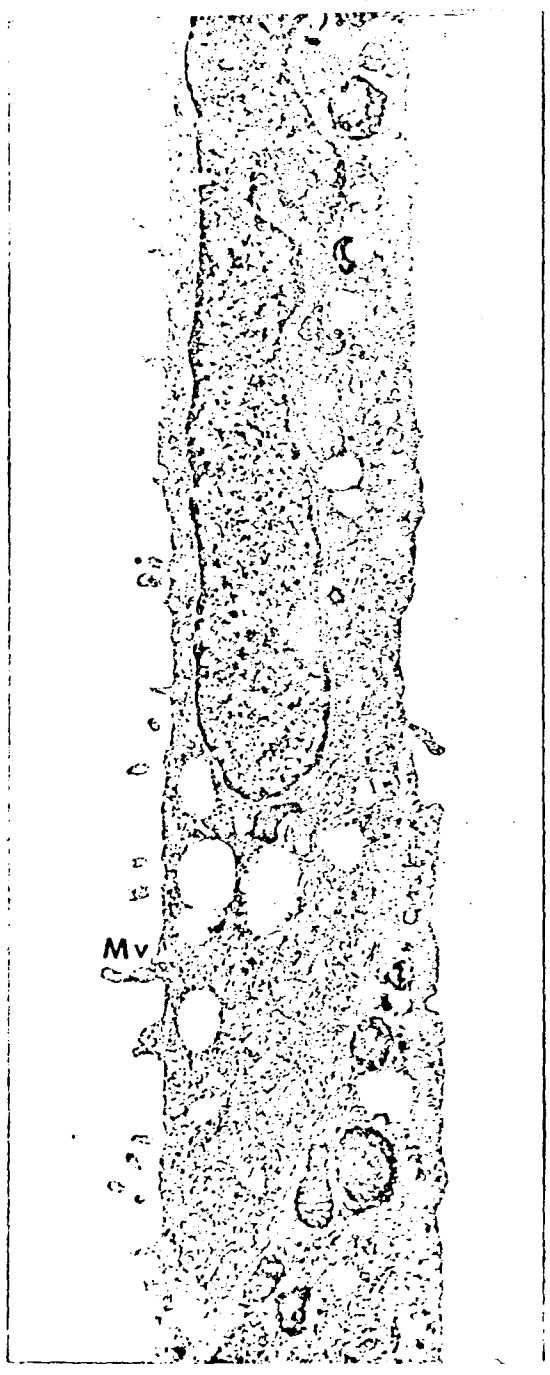
Figure 12



Bissell, M.J.
Fig. 13

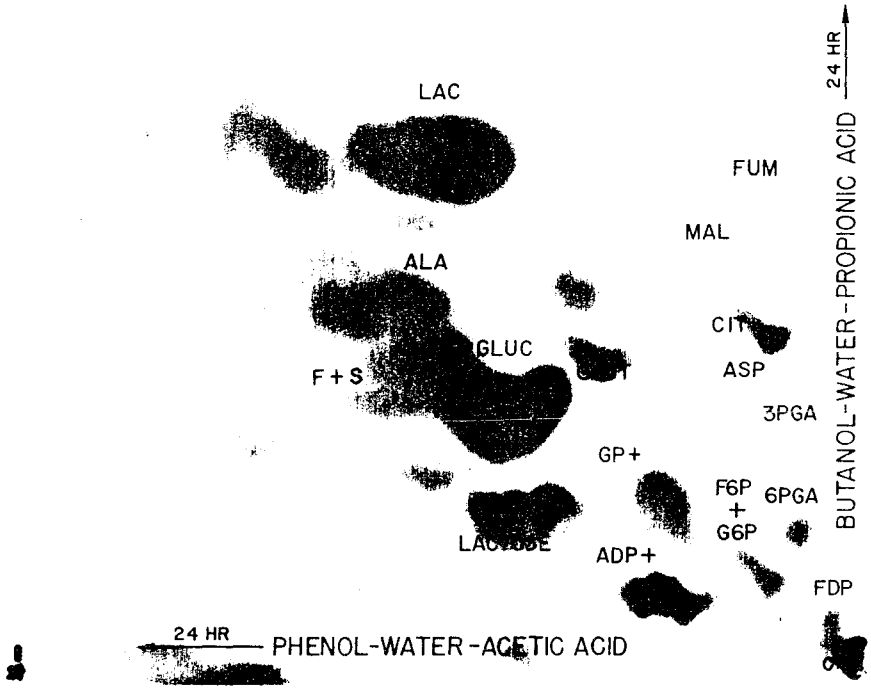


Bissell. M.J.
Fig. 14

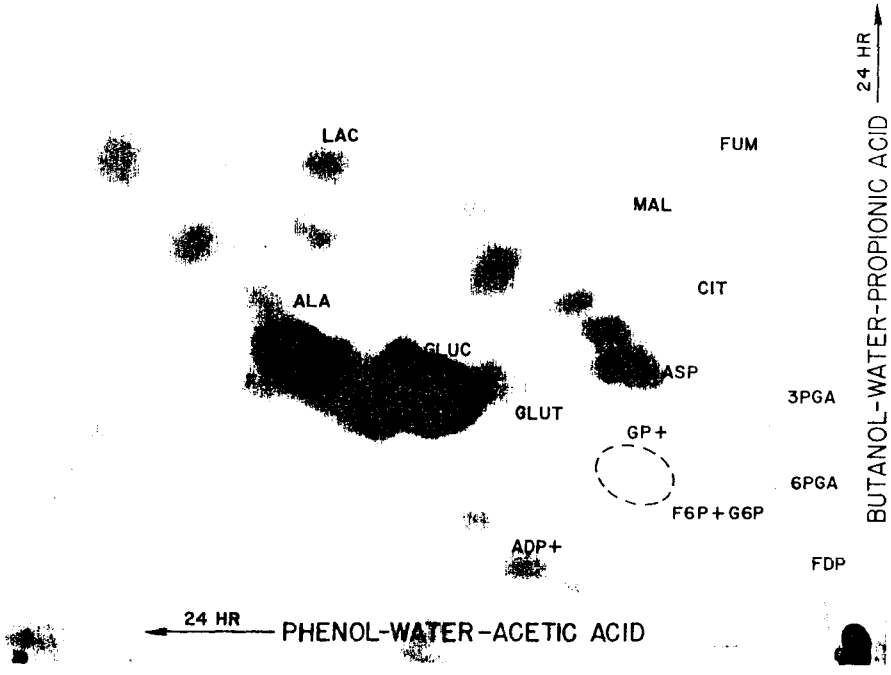


H. J. Bissell
Figure 15

(A) MAMMARY TISSUE OF LACTATING MICE JE15



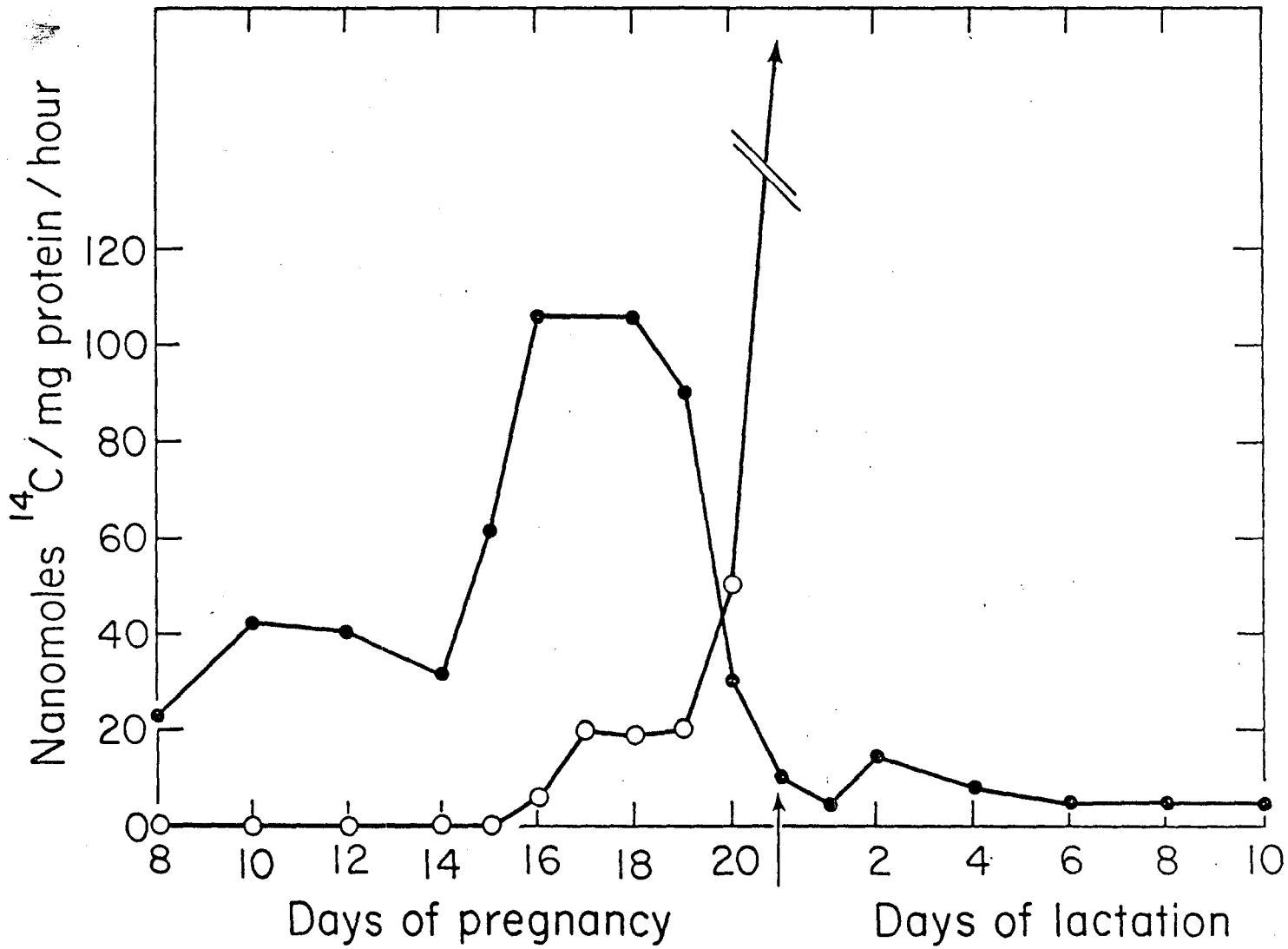
(B) MAMMARY TISSUE OF NONLACTATING MICE JE15



XBB 788-9491A

Bissell, M.J.

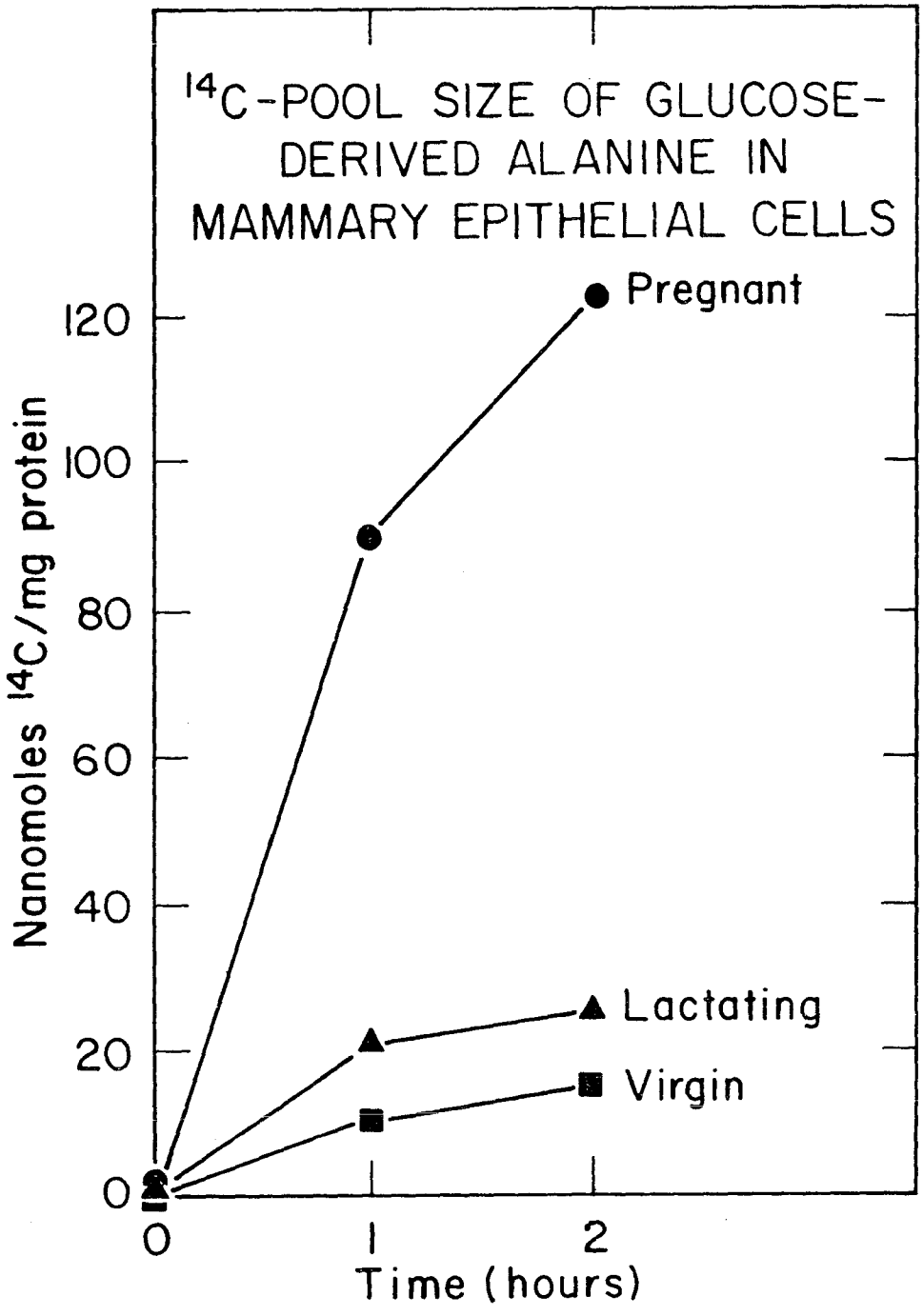
Fig. 16



XBL 799-4509

M. J. Bissell

Figure 17



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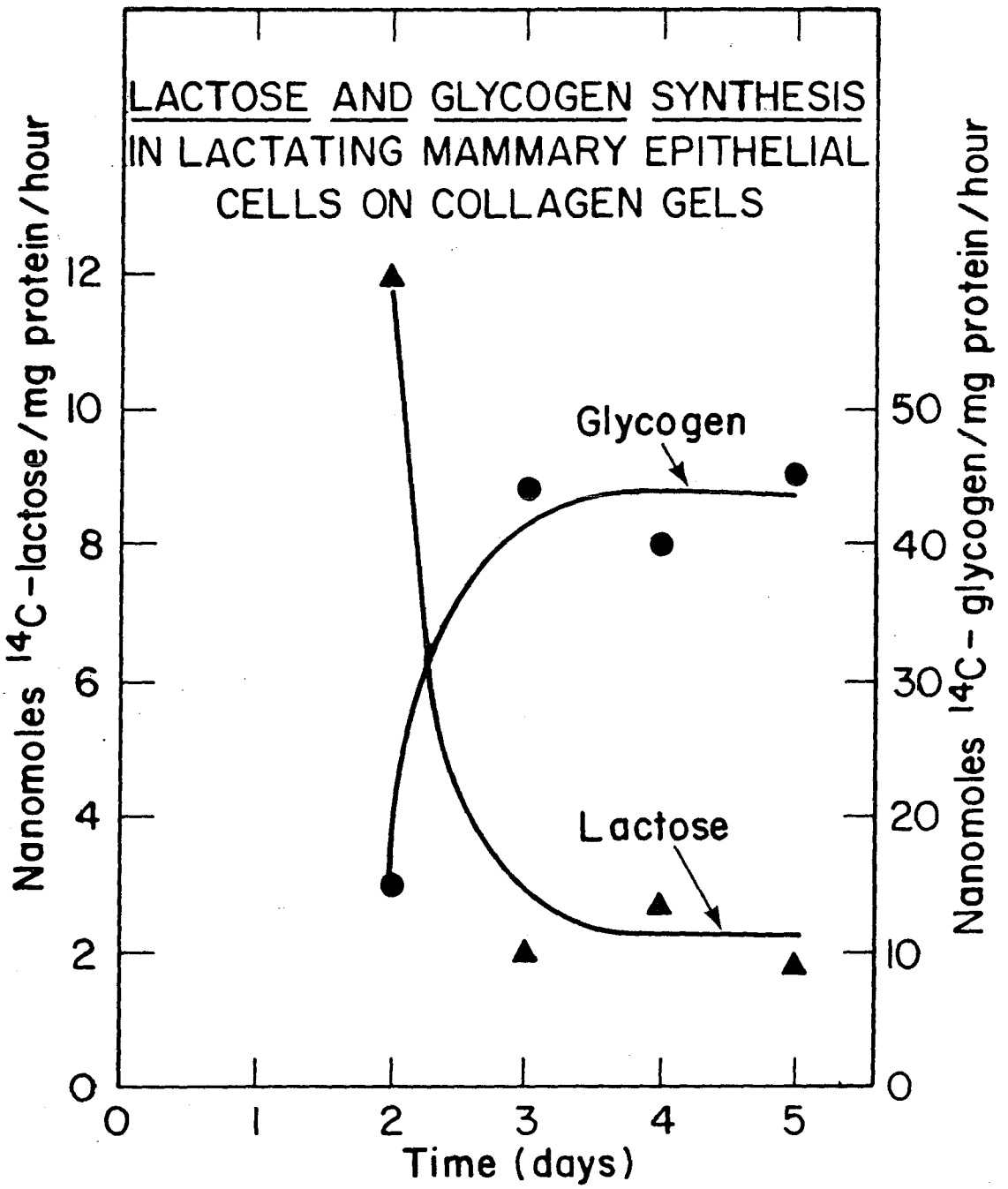
Bissell, M.J.
Fig. 18



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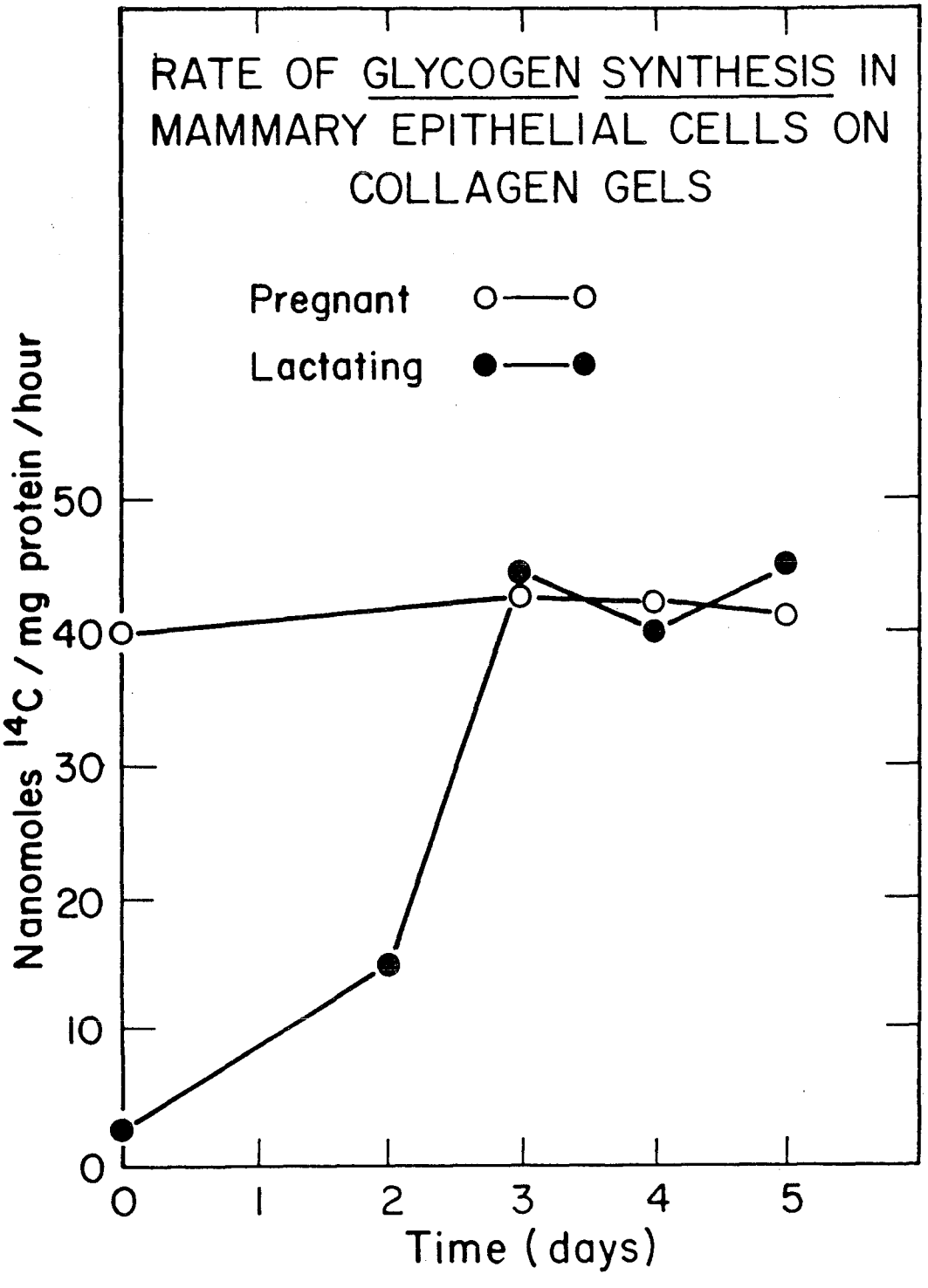
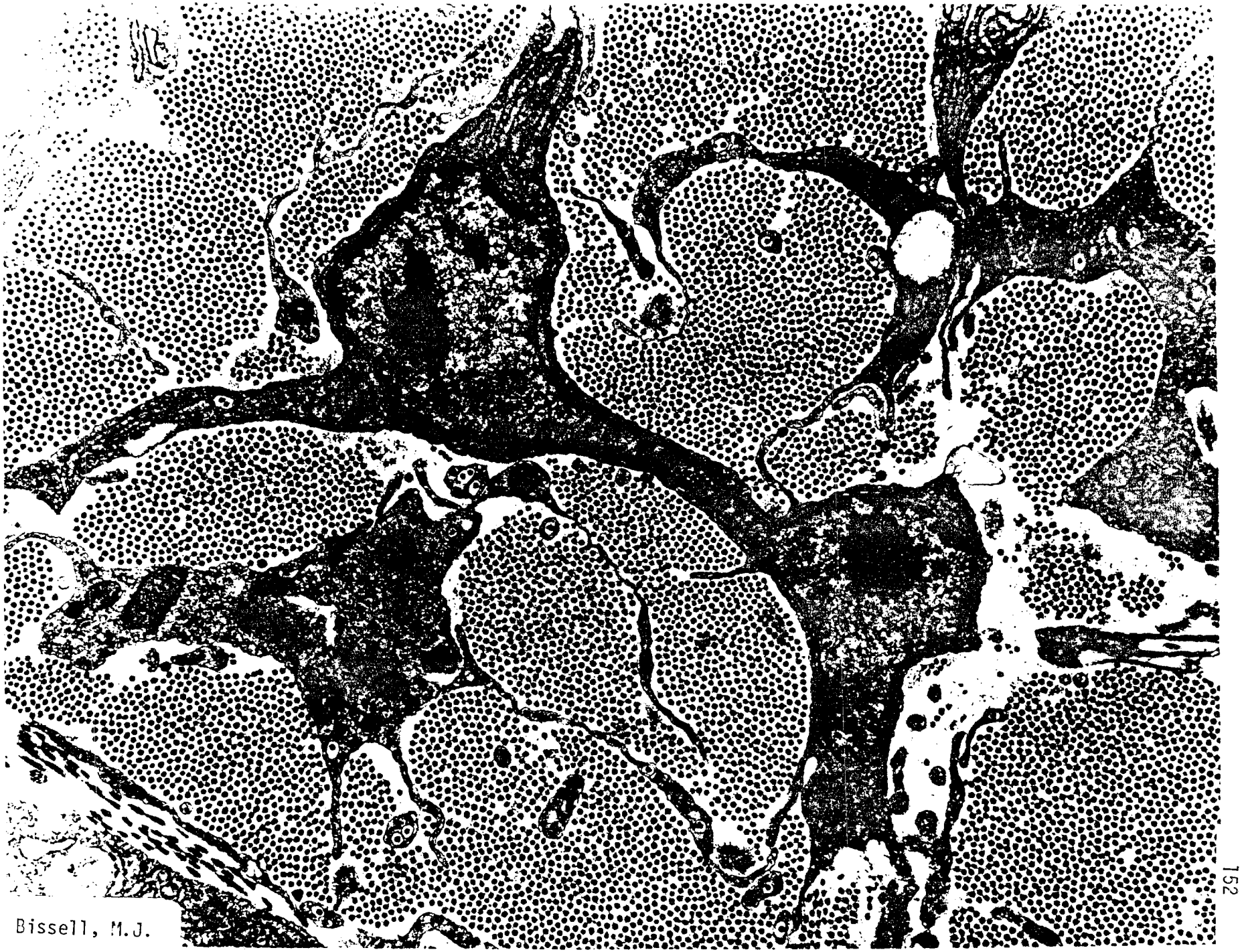
Bissell, M.J.
Figure 19



Bissell, M.J.

Figure 20

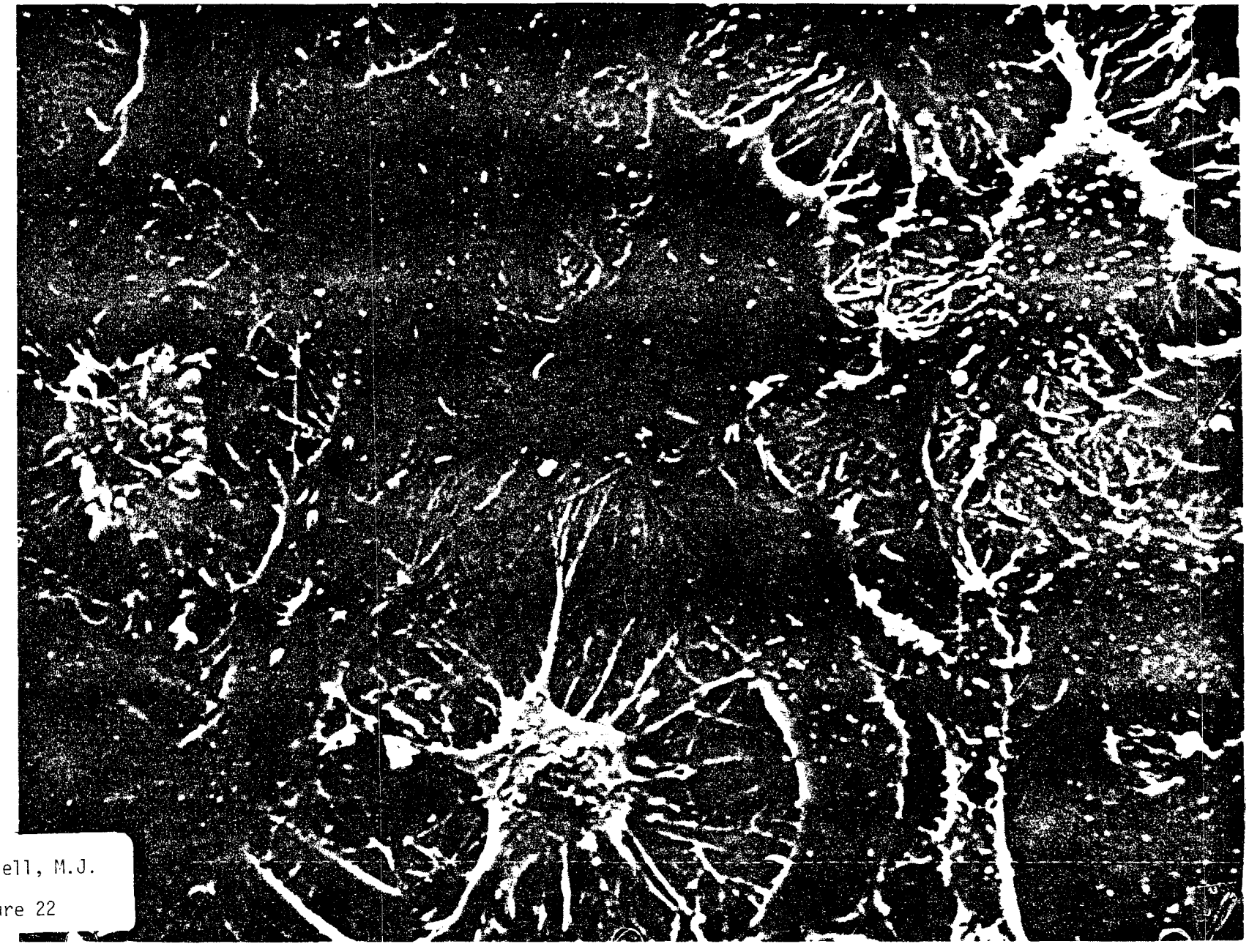


Bissell, M.J.

Figure 21

XBB 771-284

PRIMARY AVIAN TENDON CELLS IN CULTURE



Bissell, M.J.

Figure 22

X 2700