Title

INTERRELATIONS OF TRANSFORMATION, VIRUS REPLICATION, AND DIFFERENTIATED FUNCTIONS IN CULTURED CHICK CELLS

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Whai-Jen Soo
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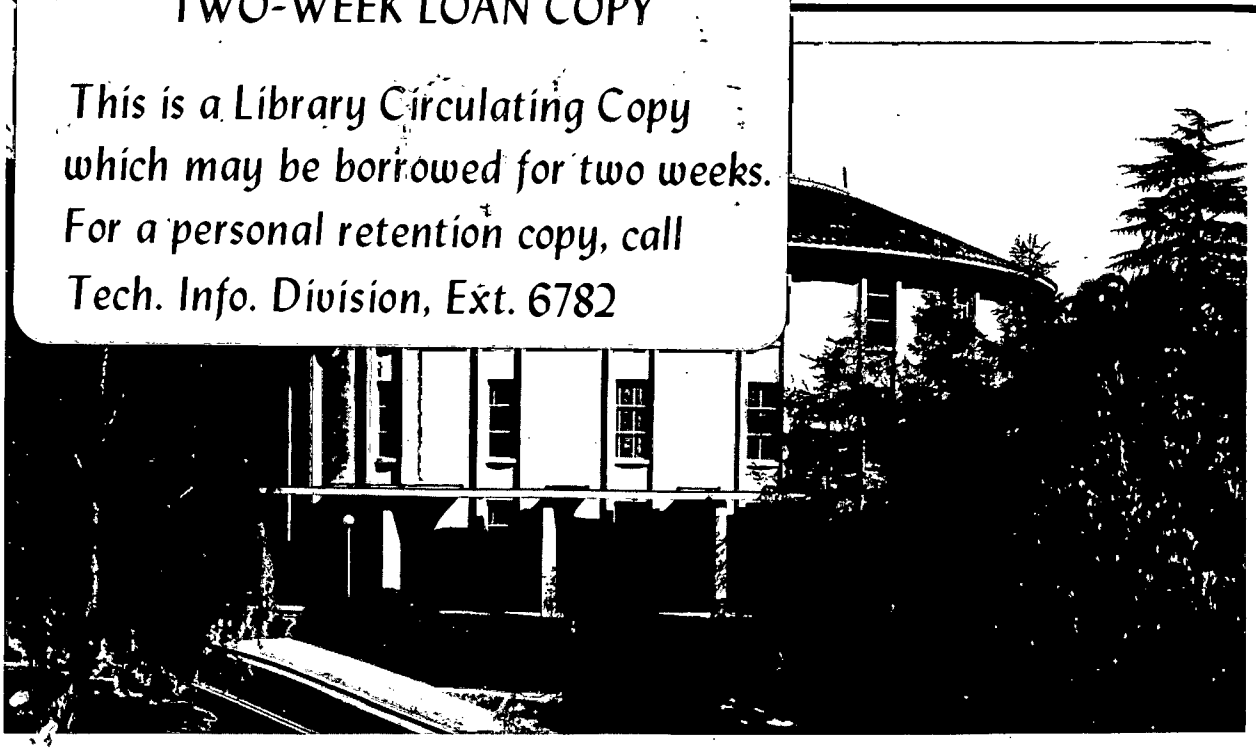

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DISCLAIMER

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ABBREVIATIONS

ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
c-DNA	complimentary DNA
cpm	counts per minute
CMC	carboxymethyl cellulose
DNA	deoxyribonucleic acid
EDTA	disodium ethylenediaminetetra-acetate
M	molar
mCi	millicurie
ml	milliliter
mM	millimolar
RNA	ribonucleic acid
mRNA	messenger RNA
SDS	sodium dodecyl sulfate
ts	temperature-sensitive
td	transformation-defective
TCA cycle	tricarboxylic acid cycle

CHAPTER I INTRODUCTION

"I have always thought that the cure for cancer would be found but heretofore believed it would not be in our generation unless it was fortuitously stumbled upon. I now feel its discovery will come about within our generation."
(Nealon, T. F., 1965).

Cancer and Differentiation

The enigma of cancer has been with clinicians and scientists for more than one hundred and seventy years since the beginning of the first organized oncogenic study (Baillie et al., 1802). Cancer, with its phenotypic schizophrenia including embryonic, fetal and adult characteristics and sometimes even a tumor-specific capacity, has disguised itself under many seemingly unrelated forms and has been more mystifying and elusive than any other disease known to man. Even after numerous intensive investigations, both the clinical and the scientific definitions of cancer are still unclear today (Stewart, 1975; Ponten, 1976).

Although not a single criterion can be used to characterize all types of cancer cells, some cellular characteristics are common to many forms of cancer. These characteristics include uncontrolled growth (Skipper and Schnabel, 1973; Steel, 1973), increased uptake of glucose (Hatanaka, 1974) and amino acids (Foster and Pardee, 1969), Isselbacher, 1972), higher rate of aerobic glycolysis (Wargburg, 1931; Wenner, 1975) and loss of the specialized morphological structures common to their normal counterparts (Auersperg and Worth, 1966; Wilbanks and Shingleton, 1970; Auersperg and Finnegan, 1974). In many cases, cancer cells also suspend or drastically reduce the synthesis of proteins highly characteristic of their parental normal differentiated cell type (Pitot, 1963; Stewart, 1975).

These various morphological and biochemical properties, however, are not only unique to cancer cells but also common to embryonic cells. In addition, the similarity between cancer cells and embryonic cells can be demonstrated by the expression of many fetal antigens (Alexander, 1972; Coggin and Anderson, 1974) and embryonic forms of isozymes (Criss, 1970; Weinhouse, 1971; Schapira, 1973) by neoplastic tissues. Numerous cellular constituents normally present during embryonic or fetal development, but absent in tissues and organs of mature individuals, have been detected in spontaneous or experimentally induced tumors. This parallelism between cancer and embryonic cells had led to a major theory of cancer which postulates that carcinogenesis represents a process of "dedifferentiation" of cells leading to the loss of specialized biochemical and morphological properties unique to a particular differentiated cell type and the reappearance of metabolic and biosynthetic patterns characteristic of undifferentiated embryonic cells (Markert, 1968; Pierce, 1970; Braun, 1974). Cancer cells, according to this theory, are thus "dedifferentiated".

The use of the term "dedifferentiation", however, has its serious limitations. Since the process of differentiation has been shown to involve epigenetic control mechanisms (King, 1966; Gudon, 1968), the use of "dedifferentiation" to define cancer would imply that carcinogenesis is also an epigenetic process in which the basic mechanisms that underlie the neoplastic state are similar to those involved in the normal cellular differentiation and the compositional and behavioral changes observed in tumors due to changes in the programming of normal genes. The strongest evidence for the epigenetic theory was provided by two ingenious transplantation experiments. In the first one by

McKinnell, Deggins and Labat (1969), tumor cells from adult frog with genetic markers were transplanted into activated and enucleated eggs. Normal tadpoles were found to arise from these eggs and bear the same markers. In the second experiment by Mintz and Illmensee (1975), mouse teratocarcinoma cells were injected into blastocysts of a different strain. Mice that were later developed from these blastocysts were found to be cellular genetic mosaics with substantial contriubtions of tumor-derived cells in many developmentally unrelated tissues. Together, these results unequivocally demonstrated the reversibility of neoplastic state and suggested that, in these cases, nuclei of normal and tumor cells were genetically equivalent and that changes in gene structure were not required for the devleopment of malignancy.

The quesiton whether genetic or epigenetic mechanisms are responsible for carcinogenesis in general, however, is still under much debate and it has become more and more clear that the final answer would be a complex one. Enough evidences from studies with some hereditary diseases such as xeroderma pigmentosum (Robbins et al., 1974), retinoblastoma (Knudson, 1973), ataxia-telangiectasia (Wolman and Horland, 1975) and Fanconi's anemia (Poon et al., 1974) suggests that changes in DNA might be indeed the cause of carcinogenesis in these diseases. It is thus likely that either genetic or epigenetic mechanisms might initiate carcinogenesis. Since cancer initiated by genetic mechanisms also regularly manifests those common "dedifferentiated" characteristics such as uncontrolled growth, loss of specialized morphological structures and biochemical properties, etc., it seems that once carcinogenesis is initiated, this process is then accompanied by a stage of "dedifferentiation" which might involve a series of epigenetic changes that are responsible for the progression of carcinogenesis and the retrogenic

phenotypic alterations of cells toward a less differentiated state.

Although the evolution of the cancer phenotype is associated with suppression of normal differentiated phenotype in general, this might not be the case when a specialized differentiated product is considered. Some times, the synthesis of the differentiated product can be suppressed to various degrees as the neoplastic process evolves resulting in a spectrum of cancer phenotypes. For example, insulinomas may produce an excessive amount of insulin per cell, (no insulin per cell) or any variation between these extremes. In cases of malignant melanomas, the neoplasms may be blue-black or black in color reflecting the normal level of melanin synthesis, or they may be colorless as a result of the shutoff of the pigment synthesis. Any variation between these extremes may also occur. The cellular heterogeneity, in the case of leukemia, is not even limited to the expression of a special gene product. Most leukemic populations characteristically contain blast cells and some less differentiated ancestral cells (Metcalf et al., 1965; McCullock et al., 1975). In addition, functional heterogeneity can be demonstrated within apparently uniform leukemic populations and some leukemic cells might actually be able to respond to environmental influences even to the degree of becoming non-dividing end cells with a finite life span (Whang et al., 1963; Jensen and Killman, 1967).

Different mechanisms can be postulated to account for the appearance of this spectrum of cancer phenotypes with regard to a special gene product or functional responsiveness. The various phenotypes can arise as a result of different degrees of "dedifferentiation" during carcinogenesis of cells in a homogeneous population; or they can represent a "maturation arrest" during carcinogenesis in the corresponding stage of differentiation in different populations of cells such as multi-

potent stem cells, progenitor cells or mature differentiated cells related to the same gene product or function; or they can even be the products of progression of neoplastic stem cells to different degrees. Although it is likely that the "dedifferentiation" mechanism might indeed operate during carcinogenesis in many mature, differentiated tissues with a homogeneous cell population, the other mechanisms might take place when the normal populations in a particular tissue are heterogeneous. The term "dedifferentiation" with regard to the change of a special differentiated characteristic during carcinogenesis should be therefore used with caution before detailed histological and biochemical studies are made to identify the initial population of the neoplastic cells.

Transformation of Cultured Cells in General

Besides extensive knowledge about pathology and histochemistry of cancer, the *in vivo* experiments provide relatively little information of the structural and functional differences between normal and cancer cells at the cellular level. Furthermore, it is usually hard to identify the initial neoplastic population during tumor formation, so the study of the early events of carcinogenesis *in vivo* is always a formidable, if not impossible task. To study the mechanisms of carcinogenesis at the cellular and the molecular level, one has thus to turn to the cell culture system, which, after years of development (Shannon, Earle and Walt, 1952), has finally emerged as one of the best systems for biological studies. Since massive culture of cells in homogeneous population can be maintained under fairly-defined conditions, the cell culture system offers decisive advantages over an *in vivo* one for many biochemical studies on the functional and structural changes of cells during carcinogenesis.

Based on the same criteria used for cancer cells *in vivo* such as uncontrolled growth (Steel, 1973), altered metabolic pattern (Bissell, 1976), loss of differentiated functions (Stewart, 1975), etc., "cancer" can now be induced in many types of cultured cells by various oncogenic agents under defined conditions. This process is known as "transformation" in culture, and is often a valid recapitulation of *in vivo* carcinogenesis especially for avian and human cells (Ponten, 1976).

Transformation can be induced either by the introduction of "transforming genes" from the outside such as from an oncogenic DNA or RNA virus (Tooze, 1973) or by perturbations in the preexisting cellular structures brought by chemical carcinogens (Heidelberger, 1975), by irradiation (Borek and Sachs, 1967), or even spontaneously (Earle, 1943). Although the latter mechanism might actually account for a high percentage of human cancer, the rate of spontaneous transformation and the efficiency of transformation by chemical and physical carcinogens are extremely low in culture for non-rodent cells. Moreover, transformation by this mechanism usually leads to a heterogeneous population of transformants (Prehn, 1968; Embleton and Heidelberger, 1972), so that comparison between normal and transformed cells is hampered by the difference among transformants themselves. It thus appears that, despite the theoretical and practical importance for the development of model systems in culture, chemical and physical carcinogenesis still remain underdeveloped.

DNA viruses represent a distinct class of oncogenic events. *In vivo* these viruses only cause tumors in a foreign species (non-permissive host) but not in their natural host (permissive host), so their role as natural carcinogens is still disputed in many instances. In culture, cells of both permissive and non-permissive hosts can be transformed by DNA viruses. Attention has been focused mainly on the non-permissive

host cells since they offer two solid advantages: the frequency of transformation is higher in these cells and they do not allow the virus to replicate in a lytic cycle as do most of the permissive host cells. Consequently, the events of transformation can be better studied in these cells without the complication of a preponderance of lytic events going on concurrently in the culture. A good correlation seems to exist for the DNA viruses with respect to their activity in vivo and in culture, and valuable information about the mechanism of carcinogenesis has been obtained by comparing normal cells to cells transformed by these viruses. The transformation by DNA viruses, however, is a very inefficient process even with non-permissive host cells (Stoker and Abel, 1962; Macpherson and Montagnier, 1964), and the transformed cells revert back to normal phenotype at a high frequency. A homogeneous population of transformed cells can be obtained only through a tedious procedure of infection, selection and replating. This slow process of transformation in culture thus makes DNA viruses a less attractive model system for studies of initial events of carcinogenesis.

Much information about the process of transformation is available from studies with RNA tumor viruses. These viruses have been isolated from many different species of animals (Tooze, 1973) and can be categorized functionally into three major groups: sarcoma viruses, leukosis viruses and mammary tumor viruses. Although they can cause a variety of malignancies in their natural hosts, most sarcoma viruses induce connective tissue tumors, most leukosis viruses cause leukemias and mammary tumor viruses cause mammary carcinomas. When grown in culture, sarcoma viruses can induce transformation in fibroblasts with a high freuqency and leukosis viruses in hemopoietic cells (Graf, 1973, Rosenberg, Baltimore and Scher, 1975). Thus in both cases, the same histiotypic target cells

are used for transformation by the respective viruses *in vivo* and in culture. In addition, various cytological and biochemical studies have shown a perfect correspondence between the behavior of tumors in animals and that of transformed cells in culture. Most likely, transformation by sarcoma and leukosis viruses in culture can therefore serve as a valid model system and reflect faithfully sarcomagenesis and leukemogenesis *in vivo*. None of the currently available cultured cells, however, can be transformed by mammary tumor viruses, and it is not yet clear if the complicated process of mammary carcinoma formation *in vivo* could one day be recapitulated in culture (Ownes and Hackett, 1972; Parks *et al.*, 1974).

Rous Sarcoma Virus: A Useful Tool for Study of Transformation in Culture

Among RNA tumor viruses, the most widely used and best characterized is avian sarcoma virus. This virus was first noted by Rous (1911) who found that a filterable agent (smaller than a bacterium) from a spontaneous chicken sarcoma could induce a tumor when inoculated into chickens. This agent was subsequently isolated, purified, characterized and given the name avian or Rous sarcoma virus.

Rous sarcoma virus is an enveloped, roughly spherical particle with a diameter of about 100 n.m. The particle is organized with a nucelocapsid core with helical symmetry surrounded by a probably icosahedral core shell which, in turn, is surrounded by a membraneous outer envelope. The nucleocapid contains a structural protein with the molecular weight around 12,000 and a single-stranded 60S- 70S RNA complex consisting of two identical 35S subunits (Duesberg *et al.*, 1975; Baluda *et al.*, 1975; Cooper and Wyke, 1975). The core shell is composed mainly of a different structural protein with the molecular weight of 27,000. The viral

envelope contains lipids and two glycoproteins. The glycoproteins protrude from the viral envelope as spikes and knobs, and are responsible for the initial absorption and infection of host cells (De Giuli et al., 1975). Three more structural proteins have also been identified with the virus, but the location and function of these virion proteins are still unknown (Bolognesi, 1974).

Minor structural alterations have been introduced to the glucoproteins and other structural proteins of Rous sarcoma virus during passage through cells, so a heterogeneity of host range and antigenecity can be observed among individual viruses. Based on this host-specificity and distinct antigenicity, Rous sarcoma virus can be classified into different strains and subgroups (Tooze, 1973). The life cycle and mechanisms of infection, replication and transformation, however, are the same for viruses of different strains and subgroups.

Entry of Rous sarcoma virus into a host cell requires a specific recognition of cell surface receptors by viral envelope glycoproteins, followed by fusion or dissolution of viral and cellular membranes (Crittenden, 1968; Dales and Hanafusa, 1972). After entry of the virus into the cell, the virion RNA-dependent DNA polymerase becomes activated (Baltimore, 1970, Temin and Mizutani, 1970) and virion RNA is reverse-transcribed first into single and then into double stranded DNA (Bishop and Varmus, 1975; Coffin, 1976; Haseltine and Baltimore, 1976; Stoll et al., 1977) which is later integrated into cellular DNA (Varmus et al., 1975; Gianni and Weinberg, 1975). Although it seems that integration of the viral genome into cellular DNA is a prerequisite for transformation and for virus production (Guntaka et al., 1975), the possibility that some DNA provirus can remain unintegrated and function

in a free state has not been completely ruled out.

Three virus-specific RNA species with sedimentation coefficients of 35S, 24S and 21S are synthesized from integrated DNA provirus. The 35S RNA contains four known genes: (from 5' end to 3' end) gag, encoding structural proteins of the viral core; pol, encoding the viral RNA-dependent DNA polymerase; env, encoding the viral RNA-dependent DNA polymerase; env, encoding the glycoproteins of the viral envelope; and src, which is responsible for the neoplastic transformation of the host cell (Duesberg et al., 1976). This 35S RNA probably serves both as viral genome and as messengers for the synthesis of viral core proteins and polymerase. The polycistronic translation of gag or gag-pol into precursor proteins is followed by selective proteolytic cleavages (Vogt, Eisenman and Diggelman, 1975; Pawson, Martin and Smith, 1976). The 28S RNA contains env and src and the 21S RNA contains src. These RNAs probably serve as messengers for, respectively, viral glycoproteins and the src gene product or transforming protein (Weiss, Varmus and Bishop, 1977; Brugge and Erikson, 1977).

The extract mechanism by which the viral particle is assembled inside the cell is still not celar. The release of the virus takes place by budding from the plasma membrane. The newly released virus usually undergoes a process of extracellular maturation in which a morphological change is accompanied by the formation of the stable 70S RNA inside the virion (Dalton, 1962; Canaani et al., 1973).

One unique advantage of Rous sarcoma virus for the study of trnasformation is the availability of a variety of mutants, among which two classes deserve special attention. The first class of mutants are no longer able to transform fibroblasts in culture or to induce sarcomas in animals and hence are termed transformation-defective (td) viruses,

but still synthesize infectious progeny virus in a single infection. Genetic and biochemical analysis show that the only difference between td viruses and non-defective or wild-type viruses is that the former have deletions in src gene (Wang et al., 1975; Bernstein, MacCormick and Martin, 1976). Comparison between cells infected by td viruses and by wild-type viruses would thus reveal events that are truly transformation-specific. The second class of mutants are temperature-sensitive (ts) viruses which can replicate inside the infected cells at both permissive (35°) and non-permissive (41°) temperatures but only transform cells at permissive temperature (Martin, 1970; Wyke and Linial, 1973). A synchronized process of transformation can thus be induced simply by shifting the infected cells from non-permissive temperature to permissive temperature, and any change in cellular properties observed during the temperature shift would be strictly due to transformation. By following the courses of events after the temperature shift, the association in time between different transformation-specific events can be studied.

Transformation of Chick Embryo Fibroblasts in Culture by Rous Sarcoma Virus

Much information about transformation has been obtained from studies with chick embryo fibroblasts infected by Rous sarcoma virus. Chick embryo fibroblasts are stable in behavior, and, unlike rodent cells, maintain their normal karyotype and growth regulatory properties in culture (Ponten, 1976). The stability of these cells thus minimizes the "background noise" when normal and transformed phenotypes are compared. Furthermore, transformation frequency of these cells by Rous sarcoma virus is usually high and can be 100% under optimal conditions

(Rubin and Colby, 1968), so a quantitative comparison between normal and transformed phenotypes is possible.

Two pieces of evidence suggest that the transforming protein or src gene product is responsible for the initiation of transformation. This protein can be translated in vitro using the 3' third of Rous sarcoma virus genome (Purchio, Erikson and Erikson, 1977) and is present only in cells transformed by wild-type virus but not in cells infected by td virus. The molecular weight of this protein is estimated to be around 60,000 (Brugge and Erikson, 1977). The function of this protein has been tentatively determined to be either to phosphorylate or to induce phosphorylation of some target proteins the alteration of which might eventually lead to the manifestation of the transformed state (Collett and Erikson, 1978).

Although many differences have been observed beween transformed and normal cells, few of them have been monitored in the ts system. Among those monitored by a temperature shift-down, the increase in glucose uptake represents one of the earlier events of transformation (Martin et al., 1971). Transformed cells usually have a rate of glucose transport three to five times higher than normal cells. When cells infected with ts virus were shifted from non-permissive to permissive temperature, change in glucose uptake can be detected as early as two hours and is complete within twelve hours after shift (Kawai and Hanafusa, 1971; Hynes and Wyke, 1975). This change in glucose uptake is probably a result of accelerated synthesis and hence an increase number of functional transport sites (Kletzien and Perdue, 1975). The increase in glucose uptake has been suggested by Bissell (1976) to be the cause of increased aerobic glycolysis in transformed cells.

Transformation-specific events can be categorized into three major types: surface changes, changes in growth properties, and metabolic changes. Morphologically, transformed cells are distinct from normal cells when attached to a culture dish. The former are round and spindle-shaped, more refractile, and may have surface blebs or ruffles (Ambros, Chen and Buchanan, 1975; Weber, Hale and Losasso, 1977), the latter are flat and better-attached. The morphological change after transformation might be related to both the disruption of internal cytoskeletal structures responsible for cellular locomotion and many transmembrane control mechanisms (Nicholson, 1976; Edleman and Yahara, 1976; Wang and Goldberg, 1976) and the loss of a specific surface protein which might be involved in the formation of an extra-cellular meshwork for cell attachment (Hynes and Wyke, 1975; Ali et al., 1977; Olden and Uamada, 1977; Bornstein and Ash, 1977). The disruption of the organized cytoskeletal structures containing microfilaments and microtubules might also be responsible for the observation that transformed cells are more readily agglutinated than normal cells by bivalent plant lectins such as concanavalin A or wheat germ agglutinin (Burger and Martin, 1972, Ash and Singer, 1976). Transformation is also found to induce alterations in the general pattern of membrane proteins (Warren, Fuhrer and Buck, 1973; Hynes, 1973; Isaka, et al., 1975) and cause the appearance of tumor specific transplantation antigens (Rohrschneider, Kurth and Bauer, 1975). In addition, changes in the composition of carbohydrates and the complexity of glycolipids have been reported to occur in the membrane of transformed cells (Perdue, Kletzien and Wray, 1972; Hakomori, Sasito and Vogt, 1971). Among these changes, increased appearance of sialic acid and hyaluronic acid in the membrane components (Moscatelli and Rubin, 1974; Critchley, Wyke and Hynes, 1976) deserve

special attention since these acids can lead to a change in the surface charge and hence the make-up and the social behavior of the membrane.

Under optimal conditions, the growth rates of normal and transformed chick cells are generally the same. Under a variety of limited conditions, however, normal cells either stop growing or grow at a much reduced rate, whereas transformed cells continue to grow. These conditions which discriminate between normal and transformed cells include: growth at high density; growth in media containing low concentration of serum or serum depleted of growth factors; and growth in agar suspension culture (Benjamin, 1974). The reason why the growth of transformed cells is insensitive to high density is not clear. This insensitivity, however, might be related to the fact that transformed cells can proliferate without anchorage or while being held in any cell shape and therefore can surmount the diffusion problems of crowding (Gurney, 1969; Folkman and Moscona, 1978). Transformation probally alters the efficiency with which cells can utilize the growth-promoting substances, so the growth of transformed cells is also less serum-dependent. In agar suspension, substratum is not provided and one or more growth-promoting substance could be bound by sulfated polysaccharides in agar. Under this double limitation, normal cells do not grow whereas transformed cells will grow into colonies. It has been suggested that the altered growth properties in transformed cells are the consequences of proteolytic activation since proteases are known to stimulate growth of chick embryo fibroblasts (Sefton and Rubin, 1970; Carney and Cunningham, 1977) and transformed cells are found to produce proteolytic enzymes (Unkeless et al., 1974). However, clones of transformed cells that produce low levels of proteolytic activity have been found (Wolff and Goldberg, 1976). The relationship between production of proteolytic enzyme and changes in growth properties therefore needs further clarification.

In addition to increased aerobic glycolysis, the metabolic changes in transformed cells involve a decrease in the level of cyclic adenosine 3', 5'-monophosphate (cAMP) (Otten et al., 1972). The level of cAMP might be lowered as a result of a reduction in adenylate cyclase activity (Anderson, Johnson and Pastan, 1973; Yoshida, Owada and Toyoshima, 1975). The lowered level of cAMP has been shown to induce changes in morphology (Johnson, Friedman and Pastan, 1971), growth rate (Otten, Johnson and Pastan, 1971) and agglutin ability by plant lectins (Sheppard and Lehman, 1972). However, due to its universal involvement in so many normal cellular responses (Robinson, Butcher and Sutherland, 1968), it is not clear if this nucleotide can play a defined role as a specific messenger for transformation.

Transformation-specific phenotypic changes in membrane, growth properties and metabolism might not be all under a single coordinate control since some of these changes but not others can be selectively reversed by treating the transformed cells with certain agents (Ali et al., 1977; Johnson, Friend and Pastan, 1971). If the phosphorylation caused by src gene product does indeed initiate the process of transformation, there might be more than one target protein which could be phosphorylated. These phosphorylated proteins might then serve as initiators for mutually independent transformation-specific events. Although the relationship between various transformation-specific events is still unknown, a map of these events in a ts shift together with information about early protein phosphorylation after transformation might eventually help define the mechanism of carcinogenesis.

Transformation of Differentiated Cells in Culture

Despite the fact that chick embryo fibroblast culture is one of

the most defined systems and indeed serves as a useful model for the study of transformation in general, it is clearly not the best system for the study of the relationship between transformation and differentiation. To study differentiation requires a cell system where cells are differentiated initally and express one or more function which could be easily defined. The chick embryo fibroblasts, however, are obtained from the whole body wall of 10-day old chick embryos. The cells are still at a rather early stage of differentiation, are relatively uncommitted and heterogeneous. The differentiated characteristics of these cells therefore are hard to define functionally. Recently, a number of cell systems have been developed in which the differentiated function can be much better-defined. These cells are usually able to synthesize a unique molecule which serves as a biochemical marker to help define their differentiated state. Any change in the state of differentiation of these cells can therefore be monitored by following the synthesis of the marker molecules.

Among the currently available differentiated cell systems, some are "terminally" differentiated cells such as melanoblasts characterized by the synthesis of melanin and formation of melanosomes and chondroblasts characterized by the synthesis of sulfated proteoglycan; others are functionally committed cells such as myoblasts characterized by their ability to synthesize muscle-specific myosin and to fuse under defined conditions. Transformation of the terminally differentiated cells seems to cause a "dedifferentiation" of these cells since the synthesis of the marker molecule is either stopped or drastically reduced after oncogenic viral infection of melanoblasts (Boettiger et al., 1977; Robyet et al., 1976) and Holtzer, 1976). On the other hand, transformation of functionally committed cells seems to at least arrest these cells

at their corresponding stage of differentiation since transformed myoblasts neither initiate myosin synthesis nor fuse to form myotubes under favorable conditions (Holtzer et al., 1977). When myoblasts transformed by a ts virus are shifted from permissive to non-permissive temperature, they begin synthesis and fuse into myotubes (Moss et al., 1977). Transformation by oncogenic virus therefore does not cancel the program of differentiation in these cells.

Although studies on transformation of differentiated cells in culture have only just begun, preliminary results suggest that some discrepancies do exist between malignancy in vivo and transformation in culture by viruses. Melanomas in vivo range from fully-differentiated to fully-dedifferentiated, yet all transformed melanoblasts in culture seem to dedifferentiate to the same extent and form a homogeneous population. This homogeneity of transformed cells in culture, however, could be looked at as an asset rather than a liability if one uses the transformation of differentiated cells in culture as a model system to study "dedifferentiation" in vivo during carcinogenesis. The homogeneity of the transformed population, in this case, could provide a uniform system for biochemical analysis so that the mechanism of "dedifferentiation" after transformation can be investigated at the molecular level. Moreover, since the state of differentiation is usually characterized by the synthesis of the marker molecule in the differentiated cell system, the study on the process of "dedifferentiation" during transformation can be practically narrowed down to following the fate of the marker molecule.

The Use of Collagen as Marker for Differentiated State

To define the mechanism of dedifferentiation at the molecular level requires a clear knowledge of the biosynthetic pathway of the marker

molecules. This would allow one to unveil the regulatory step(s) at which the synthesis of these molecules is blocked or decreased after transformation. Unfortunately, the steps involved in the synthesis and processing of the marker molecules used to characterize various differentiated cell systems are still either unclear as in the case of melanin formation (Zimmerman et al., 1974; Hearing and Ekel, 1976) or incomplete as in the case of proteoglycans (Gowtinck, Pennypacker and Royal, 1974; Haskall, 1977) and myosin (Hyne, et al., 1976; Emerson and Becker, 1975).

The disadvantage of using these marker molecules can be overcome by a better understanding of their biosynthetic pathway inside the cell. Alternatively, another differentiated cell system can be developed in which the marker molecule is well-studied. This latter approach was used by Todaro, Green and Goldberg (1964), Temin (1965), Peterkofsky and Prather (1974), and Schwarz (1974). These investigators have studied the possibility of using collagen as the marker for the differentiated state of various cell systems.

The structure, function and synthesis of collagen has been under intensive investigation for the past ten years (Prockop et al., 1976; Bornstein, 1974).

The collagen monomer is made of 3 polypeptide chains, each about 1000 amino acids long, in a triple helical configuration. At least 4 types of collagen have been identified (Miller and Matukas, 1974) each containing different types of collagen polypeptide chains. The synthesis of a particular type of collagen is tissue-specific in vivo.

The biosynthesis of collagen is a multi-step process. Collagen m-RNA is first translated on membrane bound polysomes (Diegelmann et al., 1973) to give a precursor of collagen called "protocollagen" with a molecular weight around 150,000. About half of the prolines and

one-fifth of the lysines in protocollagen are then hydroxylated (Margolis and Lukens, 1971; Uitto and Prockop, 1974) and some of the hydroxylysyl residues are later galactocosylated and in some cases further glucosylated (Myllyla, Risteli and Kivirikko, 1975). The glycosylated protocollagen, now called procollagen, forms a triple helix and is secreted. After secretion, both the amino-terminal (molecular weight 20,000) and the carboxy-terminal (molecular weight 34,000) of procollagen are cleaved from the molecule (Fessler, Morris and Fessler, 1975; Uitto, 1977) and the resulting collagen molecules aggregate in a specific manner to form collagen fibers (Bailey, Robins and Balian, 1974).

This detailed information about many steps involved in the synthesis and processing of collagen makes it possible to test if collagen synthesis is regulated at the level of translation, hydroxylation, glycosylation, conversion of procollagen to collagen or collagen fiber formation. Furthermore, the technology of c-DNA production (Howard et al., 1977; Sobel et al., 1978) and in vitro translation (Benveniast et al., 1976; Adams et al., 1977) have been applied to collagen m-RNA, making it possible to test whether or not collagen synthesis is regulated at the transcriptional level. The existence of this extensive knowledge and tehcnology about collagen thus makes it an especially attractive marker in studying the effect of transformation on the expression of the differentiated function.

Transformation and Collagen Synthesis in Cultured Cells

The relationship between transformation and collagen synthesis has been previously studied in 3T3 cells and in chick embryo fibroblasts. In the case of 3T3 cells, Todaro, Green and Goldberg (1964) first reported an increase in collagen synthesis after these cells were transformed

by oncogenic DNA viruses. The same investigators, however, observed a decrease in their later study when ascorbate was present (Green, Goldberg and Todaro, 1966). Peterkofsky and Prather (1974) reported a reduction of collagen synthesis in Kirsten sarcoma virus-transformed 3T3 cells. These contradictory results are not unexpected since collagen synthesis in "normal" cell lines is already unstable and decreases with passages of the cells (Green and Goldberg, 1963; Peterkofsky and Kiegelmann, 1971). In addition, collagen synthesis in these cells does not respond to the stimulation by ascorbate in the same way as it does in vivo. Consequently, 3T3 cells are definitely not a good system for studying the regulation of collagen synthesis in culture.

Transformation of chick embryo fibroblasts by Rous sarcoma virus was first reported by Temin (1965) to cause, if at all, only a slight reduction of collagen synthesis. Later study by Levinson, Bhatoagar and Liu (1975) suggested a 2-fold reduction of collagen synthesis based on the analysis of the incorporation of ^{3}H-proline into collagen in these cells. Further study by Kamine and Rubin (1977) indicate a 10-fold reduction of collagen synthesis after transformation. Although the reason why these contradictory results could arise is still not clear, it might well be the low level of collagen produced by these cells that makes the assay for collagen synthesis very difficult. Furthermore, the low level of collagen raises the doubt if collagen synthesis does represent a major differentiated function of these cells in vivo. It thus appears that chick embryo fibroblast is also a less desirable system for the study of the regulation of collagen sythesis after transformation.

Chick Tendon Cells

Schwarz (1974) has recently established a cell culture which con-

Due to the homogeneity and the stability of these cells as well as their ability to synthesize collagen at a high and in vivo level and to respond to environmental stimulus in the same way as do their counterpart in vivo, chick tendon cells are superior to both 3T3 cells and chick embryo fibroblasts in terms of expressing a major differentiated function and therefore provide a better system for the study of the relationship between transformation and differentiation.

Previous study in this laboratory (Schwarz, Farson and Soo, see Appendix II) demonstrated that chick tendon cells can be transformed by Rous sarcoma virus as judged by standard criteria of altered morphology, loss of density-dependent growth control and increase in 2-deoxy-D-glucose uptake The study also showed that collagen synthesis by these cells drops drastically upon transformation. To study the time course of this event, I have followed the change in collagen synthesis after transformation by a temperature-sensitive mutant of the virus. This change is then compared to changes in other cellular properties. The mechanism by which collagen synthesis is regulated after transformation has also been investigated in this thesis.

CHAPTER II MATERIALS AND METHODS

Viruses and Cells

The wild-type, the temperature-sensitive mutant (LA 24) and the transformation-defective mutant of Prague strain Rous sarcoma virus were kindly provided by Dr. G. S. Martin, and the ts mutant was recloned in our Laboratory. Tendon cells were prepared according to techniques described previously by Schwarz, Colarusso and Doty (1976). Briefly, tendons from 16-day old chick embryos were dissociated into single cells and 0.8×10^6 cells were allowed to attach in each 25 cm^2 flask or 60 mm plate in 2 ml of F12 medium for one hour. The medium was then replaced and changed daily. Cells were grown in F12 with 0.2% fetal calf serum (Gibco, Grand Island, NY; deactivated for 30 min at 56° C) supplemented with freshly prepared ascorbic acid (50 ug/ml, J. T. Baker Chem. Co., Phillipsberg, NJ) unless otherwise indicated.

Infection and Transformation

After tendon cells had atached, the medium was changed to 1.5 ml of F12 with 0.2% fetal calf serum containing the virus at a multiplicity of 10. The cells were incubated for another hour before an additional portion of medium was added. The final volume of medium was 10 ml for each flask and 5 ml for each plate. Normal cells and cells infected with either wild-type or td virus were kept at 39° for the next 7 days and cells infected with LA 24 were kept at 39° for the first 2 days after infection and switched to either 35° or 41° on the third day after infection. By the seventh day post-infection, more than 90% of the wild-type virus-infected cells maintained at 39° and LA 24-infected cells at 35° were transformed as judged by morphology. The temperature-shifts were usually made on the seventh day post-infection.

Measurement of Radioactive Collagen and Non-collagen Proteins

For regular collagen assay, cells were with 1.5 ml of F12 containing 50 µCi of ^{3}H-proline (New England Nuclear, Boston MA) for 3 hours. Shorter labeling periods were used for the time-course studies. If a labeling was chased, the labeling medium was replaced by a fresh medium containing the same ingredients except radioactive proline. The labeling or chase was stopped by the addition of 5 M NaOH to a final concentration of 0.25 M. In turn-over studies, cells and medium were separated by removing medium from the plate and adding 1.5 ml of fresh medium into the plate. NaOH was then added to both fractions.

Collagen was assayed by a collagenase method described first by Peterkofsky (1972a) and modified by Schwarz, Colarusso and Doty (1976). Samples (0.5 ml) were neutralized by 0.1 ml 1N HCl and then added to the incubation mixture (0.8 ml) containing 0.1 M NaCl, 0.17 M Tes (N-Tris(hydroxymethyl)-methyl-amino-ethanesulfonic acid, Cal Biochem.), 1 mM $CaCl_2$, 87 mM proline. The mixture was divided into six portions. Duplicate portions were incubated with either collagenase or pronase at 37° for one hour. The remaining two portions served as controls and were incubated with buffer only. The difference between the trichloro-acetic acid precipitable counts in control and collagenase-treated portions represented collagen counts and the difference between those in control and pronase-treated portions represented total protein counts. Since proline is found 5.2 times more frequently in collagen than in the average non-collagen proteins, collagen is preferentially labeled by proline. To correct for this biased labeling, the following formula was used as described by Schwarz, Colarusso and Doty (1976). Corrected % of collagen in total protein = 0.19 x uncorrected % / 1 - 0.81 x uncorrected %.

Focus Assay

Assays were performed essentially as described by Rubin (1960). In brief, four hours after secondary normal chick embryo cells were seeded at 2.0 x 10^6 cells per 60 mm dish, medium was removed and cells were exposed to the appropriate dilution of virus in 1 ml of medium for one hour at 39° C. The liquid medium was then removed and agar medium added. Rous sarcoma foci were counted 7 days later.

Measurement of RNA-dependent DNA Polymerase Activity

The enzyme activity was measured as previously described by Duesberg, Von Del Helm and Canaani (1971) and modified by Szabo, Bissell and Calvin (1976). The culture media were centrifuged at 8,000 x g for 20 min to remove cell debris. Virus was pelleted by centrifugation at 25,000 rpm in the Spinco no. 30 rotor for 1.5 hr. The pellets were resuspended in standard buffer (0.01 M Tris, pH 7.4, 0.1 M NaCl, and 0.0001 M EDTA). A portion of the resuspended virus preparation was added directly to the standard assay mixture containing 0.05 M Tris (pH 8.0), 0.05 M KCl, 0.006 M magnesium acetate, 5 mM dithiothreitol, 0.02 mg of template-primer per ml "poly(rA):oligo(dT)", and the appropriate substrate (^{3}H-TTP) at a concentration of 0.02 mM and a specific activity of 1 Ci/mmole. The enzymatic activity was solubilized by the inclusion of 0.2% Triton X-100 in the assay. The reaction mixtures were incubated at 37° C for 1 hr and the reaction was then terminated by the addition of 5% trichloroacetic acid containing 0.01 M sodium pyrophosphate. Direct measurement of DNA polymerase activity in the growth medium of infected cells yielded results that were comparable to those obtained from the resuspended pellets. Both methods of measurement were used.

2-deoxy-D-glucose Uptake

The uptake of 2-deoxy-D-glucose was measured using standard techniques described by Bissell, Farson and Tung (1977). Cells in 60 mm plates were washed with warm, glucose-free Hank's buffer and then incubated for 5 min at 39° for normal cells and 35° or 41° for LA 24-infected cells with the same solution containing 2 µCi/ml of ^{3}H-2-deoxy-D-glucose (New England Nuclear, 10.0 Ci/mmole). The cells were then washed three times with ice-cold Hank's solution containing 10 mg/ml cold glucose, drained, and taken up directly into 2 mls of 0.1% SDS-0.01 N NaOH solution. 0.2 ml of the sample was used for scintillation counting and another 0.2 ml was used for protein determination by the method described by Lowry et al. (1951).

Cell Counts

Cells were removed from the flask with trypsin (0.05%) and gently pipetted to eliminate clumps. They were then counted in a Coulter counter.

Purification of Radioactive Collagen

Collagen synthesis by cultured cells was purified from the medium of these cells using the procedures described by Hata and Peterkofsky (1977). Briefly, normal or infected cells were labeled on their 7th day in culture in 3 ml of F12 containing 50 µg/ml ascorbic acid and 0.5 mM β-aminoproprionitrile (Sigma, St. Louis, MO). After a 15 min preincubation at the respective temperature, 75 µCi of ^{3}H-proline (New England Nuclear, 20 µCi/mmole) was added to each dish, which was then incubated for another 6 hr. Medium was collected, dialyzed, and lyophilized. Lyphilized medium was then dissolved in Tris/NaCl buffer and collagenous material was precipitated by the addition of ammonium sulfate (176 mg/ml). This collagenous material was further treated with pepsin for 6 hr at 15°

to give collagen. Type I collagen standard for reference was purified from chick calvaria according to the procedures described by Siegel (1974).

SDS-polyacrylamide Gel Electrophoresis of Purified Collagen

Acrylamide gel electrophoresis of collagen was carried our using Bio-phore prepared 4% polyacrylamide gels (Bio Rad Labs, Richmond, CA) as described by Little et al. (1977). Briefly, the samples were dissolved in SDS sample buffer (1% SDS, 0.04 M Tris-acetate, pH 8.0, 1 mM EDTA) and heated to 65° for 30 min with or without 40 mM dithiothreitol. The samples were than layered over 4% polyacrylamide gels equilibrated in Bio-Rad SDS electrophoresis buffer (0.2 M Tris-acetate, 0.1% SDS, pH 6.4). Electrophoresis was carried out at 4 mA per gel for 1 hr and then at 7 mA per gel for another 3 hrs. Following electrophoresis, the gels were stained, destained according to Fairbanks, Steck, and Wallach (1971), and sliced into 1 mm slices. Each slice was then solubilized in toluene-permalfour-protosol mixture (New England Nuclear) and counted.

Carboxymethyl-cellulose (CMC) Chromatography

CMC chromatography under denaturing conditions was used as described by Oohira et al. (1974). 2 mg of calf skin acid-soluble collagen (Cal Biochem) was used as carrier with each sample.

SDS-polyacrylamide Gel Electrophoresis of Extracts from Labeled Tendon Cells

The electrophoresis was carried out as described by Laemmli (1970). Cells were labeled with either ^{14}C-proline (New England Nuclear, 255 mCi/mmole) or ^{14}C-leucine (New England Nuclear, 40.2 Ci/mmole) for the designated period of time and solubilized directly on the plate at the end of labeling by the addition of 100 µl of a buffer containing

2% SDS. 50 mM Tris-HCl, pH 6.7, 2 mM phenyl-methylsulphonyl fluoride (PMSF) (Parry and Hawkes, 1978). Samples were solubilized by heating to 100° for 15 min. For samples to be digested with collagenase, cells were scraped off the plate in collagenase buffer (0.1 M Tris, 0.5 M NaCl, 37.5 mM $CaCl_2$, pH 8.0) and heated to 100° for 2 min. The cell homogenates were treated with purified bacterial collagenase for 1 hr at 37° and the mixture was made 2% SDS by the addition of appropriate amount of SDS. Autoradiograms were prepared by drying the gels onto filter paper and exposing them to Kodak X-ray film.

Collagenase Assays

Collagenase activity was assayed as described previously by Werb and Burleigh (1974). Type I collagen labeled with ^{14}C-proline was produced by chick calvaria in culture as described by Siegel (1974) and acid-soluble calf skin carrier collagen was obtained from Cal Biochem. Labeled collagen was used directly for soluble collagen assay in which medium was incubated with collagen substrate for 3, 6 or 24 hrs. At the termination of the incubation, 20% trichloroacetic acid was added to the reaction mixture and acid precipitable counts were determined by filtering the mixture through a nitrocellulose filter (Millipore Corp.) and counting the dried filter. For insoluble collagen assay, labeled collagen and carrier collagen were mixed at a concentration of 2 mg.ml. The mixture was dialyzed for 24 hrs against two changes of collagenase buffer (0.1 M Tris, pH 8.0, 0.5 M NaCl, 37.5 $CaCl_2$). The collagen solution was dispensed in 200 ul aliquots and gelled for 30 min at 37°. The medium being tested was then added to the gelled collagen substrate and the mixture incubated for another 3, 6, or 24 hrs. The remaining collagen after incubation was determined by trichloroacetic acid precipitation.

CHAPTER III EFFECTS OF TRANSFORMATION ON COLLAGEN SYNTHESIS BY CHICK TENDON CELLS

Collagen synthesis can be expressed either as a percentage of total protein synthesis or by the absolute rate of collagen synthesis. The former expression was adopted by Schwarz (1974) and Schwarz and Bissell (1977), was used in our paper (in Appendix II), and will be used in this chapter and chapter IV since this expression can correct for fluctuations in the uptake of proline and overall metabolism as the cells change their density or growth rates. In addition, the use of percentage emphasizes the preferential synthesis of collagen as opposed to other cellular proteins and therefore gives a better indication of the differentiated state of the cell under study. The absolute rate of collagen synthesis, however, is also important since this value gives information about the regulation of collagen synthesis itself. The absolute rate of collagen synthesis in normal and transformed tendon cells will be studied in chapter VI.

Previous work in this laboratory (Schwarz, Farson, Soo and Bissell, see Appendix II) showed that collagen synthesis by chick tendon cells drops drastically upon transformation by Rous sarcoma virus. By using a transformation-defective (td) mutant of the virus, it was also demonstrated that the drop in collagen synthesis is transformation-specific. In this work, however, no data were provided to show that cells in those plates that had been treated with td virus were indeed infected. This question is answered in the present chapter by measuring the virus-specific RNA-dependent DNA polymerase activity in the medium of td virus-infected cultures.

Collagen Synthesis by Normal and Transformed Tendon Cells

Table III-1 shows the relative level of collagen synthesis in normal and transformed tendon cells as tabulated from Fig. 4 of the paper by Schwarz et al. (see Appendix II). In this particular experiment, unfortunately, the process of transformation was not complete so that not all the cells in the "transformed" cultures were transformed as judged by their morphology. There was, nevertheless, a 2 to 3-fold difference in collagen synthesis between normal and "transformed" cells. (Most of the experiments in which transformation of the culture was achieved showed a 5 to 10-fold difference between normal and transformed cells; for example, see Fig. 5 of the same paper).

Change in Collagen Synthesis is Transformation-Specific

The reduction in collagen synthesis could be due to either malignant transformation or the replication of the virus since transformed tendon cells allow Rous sarcoma virus to replicate (Table III-2). To test if the change in collagen synthesis was transformation-specific, a td virus of the same strain was used for comparison. As shown in Table III-2, cells infected by td virus were as active as those transformed by the wild-type virus in producing viral particles as judged by the virus-specific RNA-dependent DNA polymerase activity in the medium. The collagen synthesis in td virus-infected cells, however, did not show any significant drop when compared to that in normal cells (Table III-1). These results suggested that the reduction in collagen synthesis is the result of transformation rather than the virus infection per se.

Discussion

Previous studies on the effect of non-transforming viruses on collagen synthesis in chick embryo fibroblasts have led to different

TABLE III-1 Collagen synthesis by normal and transformed tendon cells[a]

Infecting virus	Collagen synthesis (as percentage of total protein synthesis	
	6 days after infection	7 days after infection
None	21.9	24.9
td virus	18.1	21.9
Wild-type virus	10.9	7.2

[a]Tendon cells were plated at a density of 8×10^5 cells per flask. After cells had attached, the medium was changed to 1.5 ml of F12 with 0.2% serum plus either the wild-type or a transformation-defective (td) mutant of Prague C strain Rous sarcoma virus. The cells were incubated for another hour before an additional 9 ml of medium was added. The medium was changed daily. By the 6th day postinfection, more than 80% of the cells infected with wild-type virus was transformed as judged by morphology. Collagen assay was done in the 6th and 7th days postinfection as described in Materials and Methods.

Table III-2 Virus production by infected avian tendon cells[a]

Infecting virus	Day after Infection	Focus-forming units	RNA-dependent DNA polymerase activity[b]
Wild-type virus	6	8×10^5	4350
	7	1.2×10^6	500
td virus	6	0	4050
	7	0	5250

[a]Chick tendon cells were plated in a 25 cm^2 flask and infected as described in Table III-1. Th e medium was changed every day and collected in a 3-hr period on the 6th and the 7th days after infection. The titer of the transforming virus in the collected medium was then determined by focus assays and that of physical particles of the virus by RNA-dependence DNA polymerase assay.

[b]Activity measured by incorporation of ^{3}H-thymidine-5'-triphosphate as described in Materials and Methods. The units were counts incorporated per minute per 25 µl of medium. In these cultures there were 10^6 cell per 25 µl of medium.

conclusions. Levinson, Bhatnagar and Liu first reported that the infection of chick embryo cells by an avian leukemia virus (RAV-1) did not affect the level of collagen synthesis by these cells. Later study by Banes, Smith and Mechanic (1978) using a non-transforming avian myeloblastosis virus (MAV-2(0)), however, observed a 3-fold increase in the rate of collagen synthesis by these cells after viral infection. It is possible that the different results obtained by these two groups reflect a difference in the intrinsic properties of the viruses used. However, due to the low level of collagen synthesis by chick embryo fibroblasts and the lack of accuracy of collagen assay in dealing with a small amount of collagen, these different results could also merely reflect experimental errors. Since collagen synthesis by tendon cells is ten times higher percentage wise than that by chick embroy fibroblasts, this ambiguity should not exist when tendon cells are used.

Collagen synthesis by tendon cells is drastically reduced after transformation of these cells by a wild-type Rous sarcoma virus. The results in this chapter have shown that cells infected by a transformation-defective virus can still synthesize collagen at the normal level while producing a similar amount of virus as transformed cells. This observation indicates that the viral infection per se does not alter collagen synthesis and virus-specific translation does not affect the highly biased translation of collagen m-RNA. The reduction of collagen synthesis is therefore transformation-specific.

CHAPTER IV CHANGE IN COLLAGEN SYNTHESIS AS AN EARLY EVENT DURING TRANSFORMATION OF CHICK TENDON CELLS

Introduction

Tendon cells are dedifferentiated after transformation by Rous sarcoma virus in terms of the phenotypic expression of their major differentiated function --- collagen synthesis. The mechansim by which transformation causes dedifferentiation is unknown. One way to study the relationship between the process of transformation and dedifferentiation is to analyze the kinetics of the dedifferentiation process during transformation. This approach requires a continuous monitoring of the state of differentiation of cells undergoing transformation. Although eventually all tendon cells can be transformed in a week in culture by Rous sarcoma virus, only a fraction of cells are initially infected so that a complete viral transformation usually requires secondary infections through spreading of viurses by these initially infected cells to other uninfected cells. The process of transformation is therefore not synchronized for all cells in culture and the presence of the uninfected cells could hamper the study of the events that occur during transformation.

This problem can be overcome by the use of temperature-sensitive (ts) mutants of the virus. Since collagen synthesis is not affected by the viral infection per se and only viral infection and replication but not transformation by ts viruses can occur at non-permissive temperature, a completely infected culture that behaves normally with regard to collagen synthesis can be obtained by keeping the ts-infected cells at this temperature for a week. A synchronized process of transformation in these cells can then be induced simply by shifting the cells from

non-permissive temperature to permissive temperature, and quantitative analysis on the kinetics of different transformation-specific events can be made.

Using tendon cells infected by a ts mutant of Prague A strain of Rous sarcoma virus, the kinetics of reduction in collagen synthesis during transformation is examined in this chapter. In addition, by following the courses of events after the temperature shift, the association between the change in collagen synthesis and other transformation-specific events is also studied.

Characterization of Tendon Cells Infected by a Temperature-sensitive Mutant of Rous Sarcoma Virus

The temperature-sensitive system was first characterized. Tendon cells isolated from 16-day old chick embryos were infected with a temperature-sensitive mutant of Prague A strain Rous sarcoma virus, LA 24. Infected cells were maintained first at 39° for two days and then switched to either non-permissive (41°) or permissive (35°) temperatures for another five days, and their morphology, sugar uptake and sensitivity to density-dependent growth control were characterized.

Change in morphology is the most common criterion for transformation. Fig. IV-1 shows the morphology of these cells kept at either 41° or 35° at the end of the week. Cells maintained at 41° were polygonal and flat. Cells maintained at 35° were either spindle-shaped or round, were more refractile, and usually piled up into areas resembling foci. The uptake of 2-deoxy-D-glucose is another commonly used criterion for transformation. Table IV-1 shows that the rate of uptake of 2-deoxy-D-glucose by these cells at 35° is approximately 3.5 times higher at the end of thw week. This magnitude of change is comparable to what has been reported for chick embryo fibroblasts and other cell

TABLE IV-1 Characterization of LA 24-infected primary tendon cells[a]

Parameter	41°	35°
^{3}H-2-deoxy-glucose Uptake (cpm/ug proteins/5 min)	44.8	88.2
Collagen Synthesis (as percentage of total protein synthesis	21%	4.1%
Production of Infectious Virus (focus-forming units/10^6 cells)	0.6×10^6	0.5×10^6

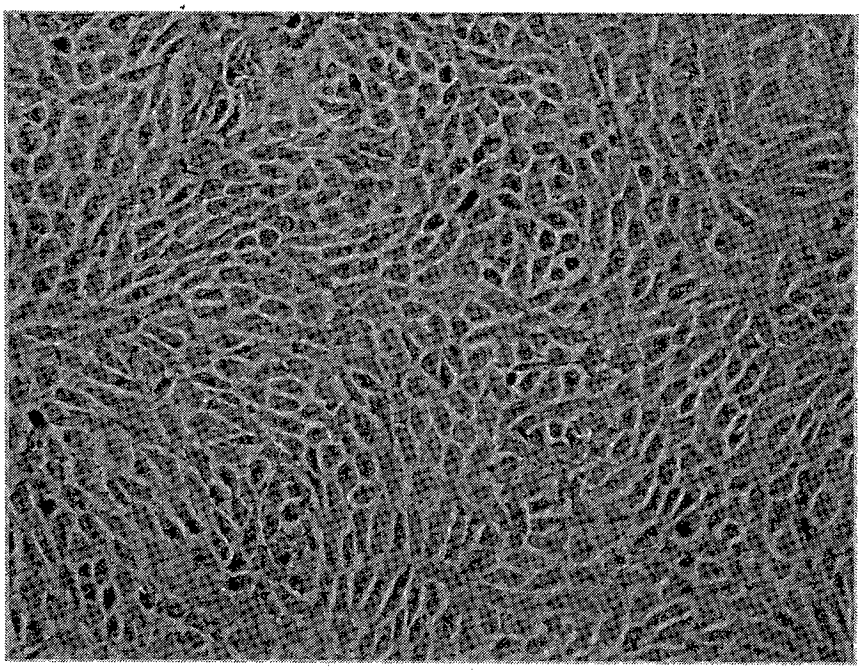
[a] 8×10^5 cells were seeded on each 60 mm plate. Cells were infected with LA 24 and switched to either 41° or 35° as described in Figure IV-1. On the 7th day postinfection, 2-deoxy-D-glucose uptake and collagen synthesis were measured and the titer of the virus was determined by focus assay as described in Materials and Methods. Numbers for each characteristic represent average of 3 determinations.

FIGURE IV-1 Morphology of LA 24-infected tendon cells. Avian tendon cells were plated at a density of 8 x 10^5 cells per flask. One hr after plating, cells were infected with LA 24 at a multiplicity of 10. Infected cells were kept at 39° for the first two days and switched to either 41° or 35° on the third day after infection. Cells were grown in F12 supplemented with 0.1% tetal calf serum and 50 ug/ml ascorbate and the medium was changed daily. The pictures were taken on the 7th day postinfection.

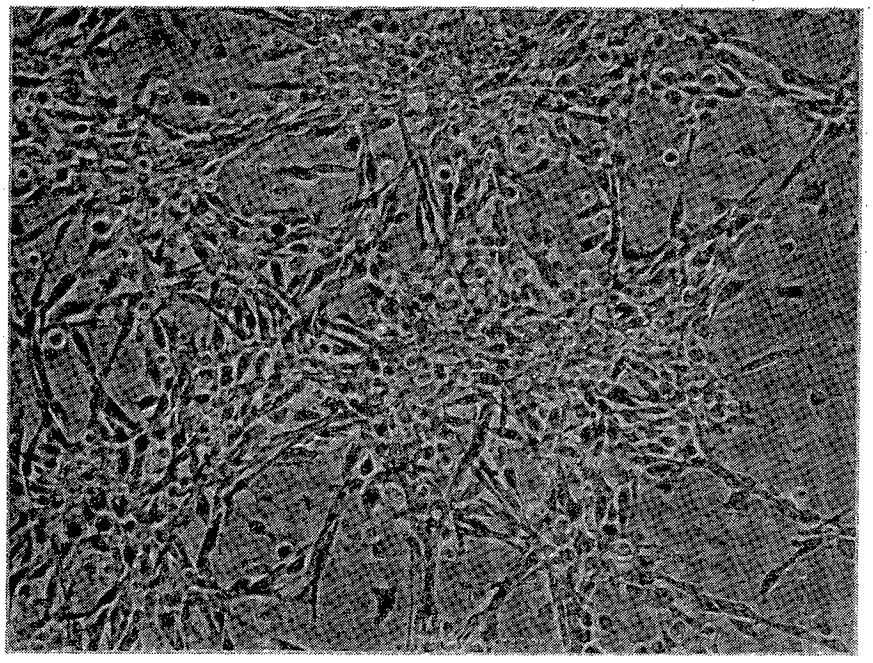
A, cells maintained at 41°

B, cells maintained at 35°

A



B



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systems (Martin et al., 1971; Okayama et al., 1977). The third common criterion for transformation is the sensitivity to density-dependent growth control. The growth curves for these cells at the two temperatures are shown in Fig. IV-2. The growth of cells maintained at 41° was sensitive to density, and cell division became negligible toward the end of the week when confluency was reached. The growth of cells maintained at 35°, however, was not sensitive to high density.

The differences observed were not due to viral replication since these cells released roughly the same amount of infectious virus at both temperatures (Table IV-1). Thus, by the criteria of morphology, sugar uptake and sensitivity to density-dependent growth control, cells maintained at 41° behave similar to normal, uninfected cells whereas cells kept at 35° behave just like cells transformed by a wild-type strain of Rous sarcoma virus.

Collagen Synthesis by Tendon Cells Infected with LA 24

Table IV-1 also shows the level of collagen synthesis at the end of the week in LA 24-infected cells kept at either 41° or 35°. The level of collagen synthesis by cells kept at 41° is at least five times higher than that by cells kept at 35°. Furthermore, the level of collagen synthesis in infected cells kept at 41° is the same as that in normal, uninfected cells, suggesting again that the viral infection per se does not affect the level of collagen synthesized.

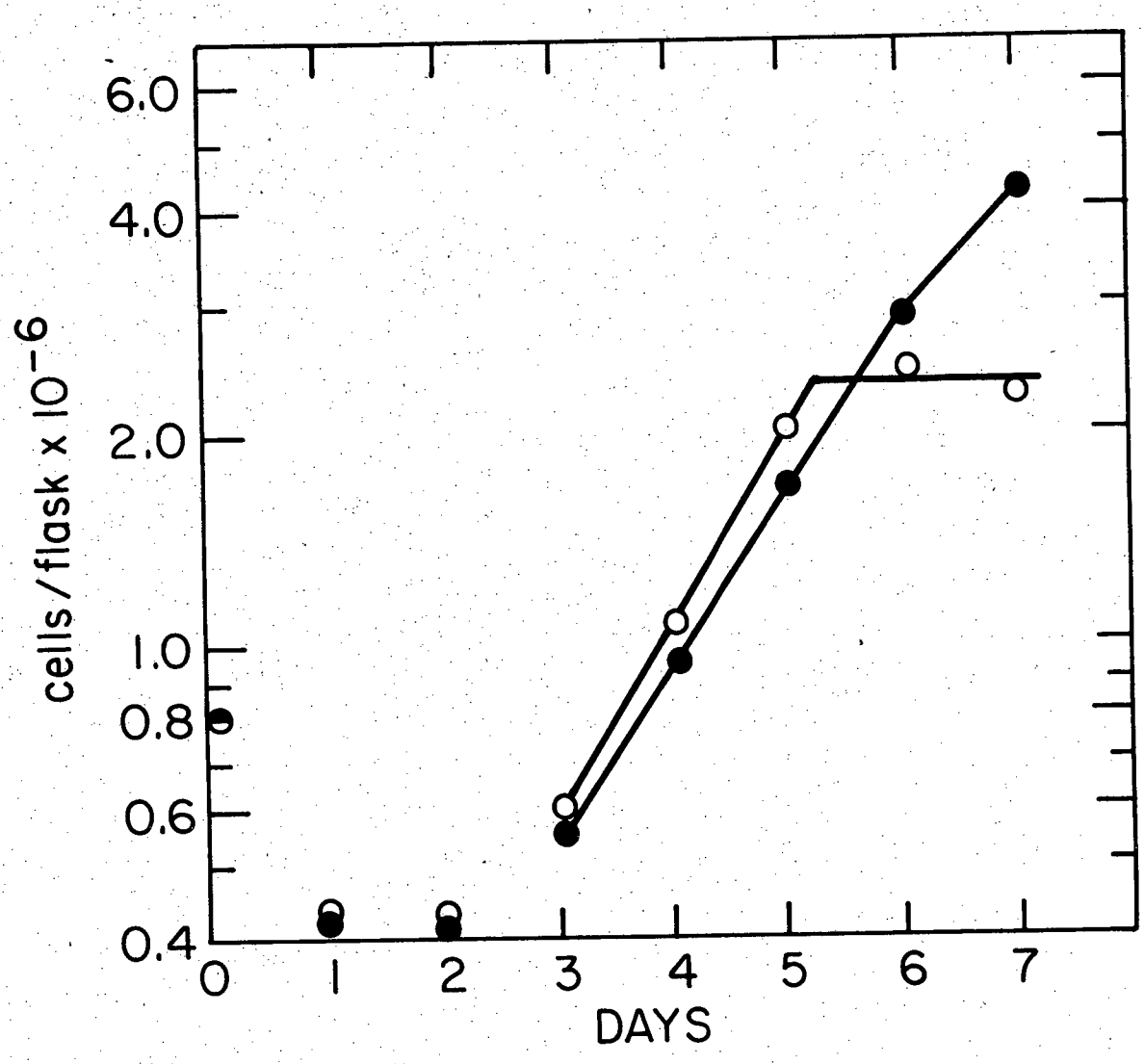
Changes in Morphology and Sugar Uptake with Temperature-Shifts

Since synchronized processes of "transformation" or "reversion to normal phenotype" can be achieved by merely shifting the temperature from 41° to 35° or vice versa, the kinetics of the cellular events which occur during these processes can be studied using temperature shifts.

FIGURE IV-2 Growth curves of LA 24-infected cells maintained at 41° and 35°. Tendon cells were plated and infected as described in Figure IV-1. Infected cells were grown at 35° for the first two days and switched to either 41° or 35° on the third day after infection. Cells were counted as described in Materials and Methods.

o, cells maintained at 41°

●, cells maintained at 35°



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Cells infected with LA 24were cultured at 39° for two days and switched to either 41° or 35° for another four days. Half of the plates were then shifted to the other temperature on the seventh day. Changes in morphology and 2-deoxy-D-glucose uptake were monitored at intervals after the temperature shifts. Morphological changes were visible in the shift-down experiment at about 4 hr, in the shift-up at about 8 hr. Changes in morphology in either direction were more pronounced at 12 hr and were complete at 24 hr. The change in the sugar uptake occurred much faster. In the shift-down it was 75% complete by about 6 hr after shift. In the shift-up it was 75% complete at 4 hr after shift (Fig. IV-3).

As a control, 2-deoxy-D-glucose uptake by normal cells during temperature shifts was also measured. As shown in Fig. IV-4, no change in sugar uptake by normal cells was observed with either shift. The changes observed in this biochemical characteristic of LA 24-infected cells during temperature shifts were therefore strictly related to transformation.

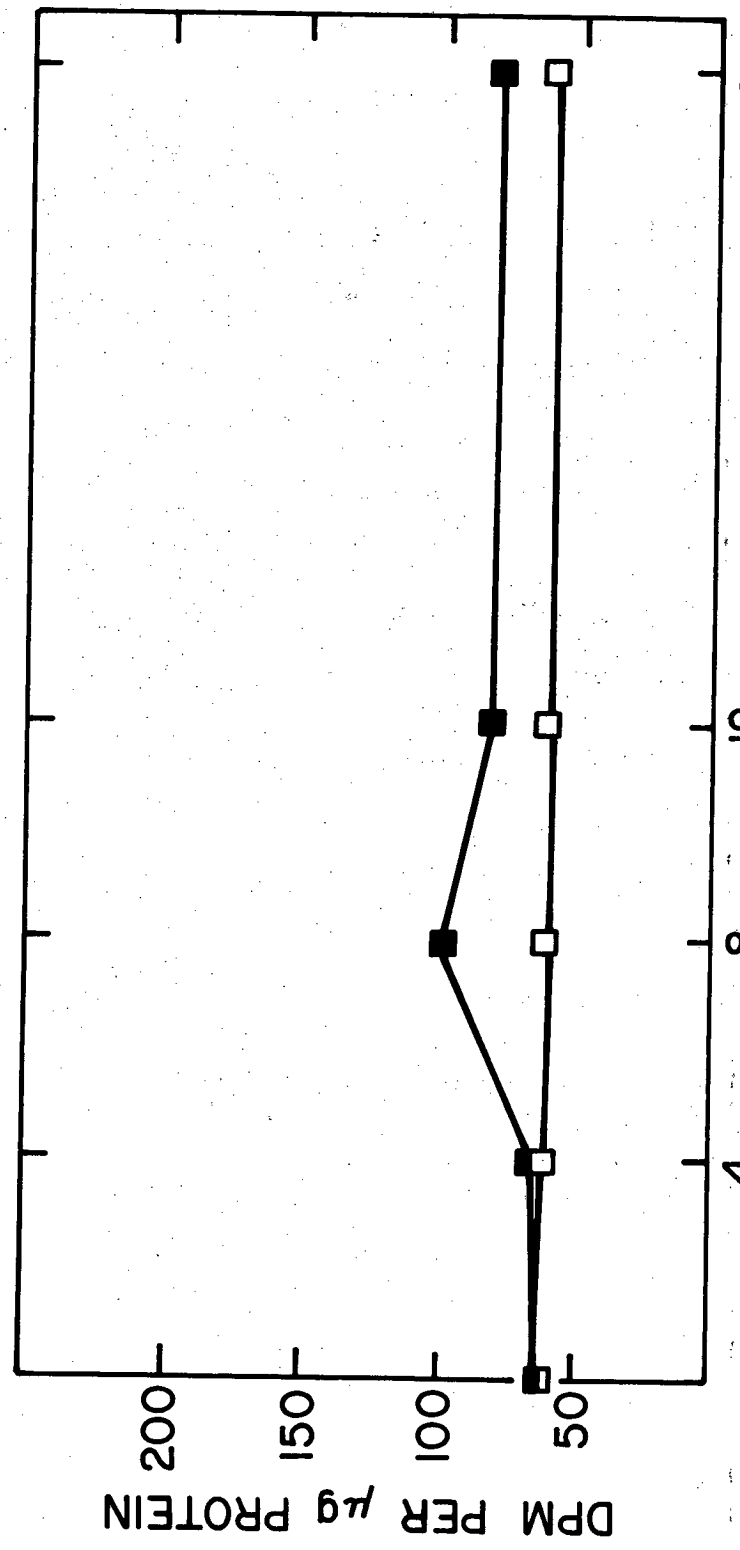
Change in Collagen Synthesis with Temperature-shifts

The time scale required for a change in collagen synthesis after transformation has not been studied in detail previously. Since the change in collagen synthesis is transformation-specific, its kinetics during the process of transformation can also be studied by temperature shifts. As shown in Fig. IV-5, temperature has no effect on the level of collagen synthesis in normal or wild-type virus-transformed cells. A quick drop in the level of collagen synthesis, however, was observed in LA 24-infected cells when they were shifted from 41° to 35° (Fig. IV-6). The decrease in collagen synthesis in these cells was almost 50% complete as early as 4 hr after shift-down. This observation indicates that

FIGURE IV-3 Effect of temperature shift on 2-deoxy-D-glucose by normal tendon cells. 8×10^5 cells were seeded on each 60 mm plate. Cells were grown at 41° in F12 supplemented with 0.1% fetal calf serum and 50 ug/ml ascorbate for 6 days and the medium was changed every day. On the 7th day after seeding, half of the plates were shifted to 35° and the other half remained at 41° as control. 2-deoxy-D-glucose uptake was measured at 4, 8, 12 and 24 hr after shift as described in Materials and Methods.

■, cells remained at 41°

□, cells shifted from 41° to 35°



XBL 7711-4743

FIGURE IV-4 Effect of temperature shifts on 2-deoxy-D-glucose uptake by LA 24-infected cells. 8×10^5 cells were seeded on each 60 mm plate. Cells were infected with LA 24 and switched o either 41° or 35° as described in Fig. IV-1. On the 7th day postinfection, half of the plates at either temperature were shifted to the other temperature and the rest of the plates remained at the original temperature. 2-deoxy-D-glucose uptake was measured at 4, 8, 12 and 24 hr after the temperature shifts as described in Matherals and Methods.

o, cells remained at 35°

●, cells shifted from 35° to 41°

▲, cells remained at 41°

△, cells shifted from 41° to 35°

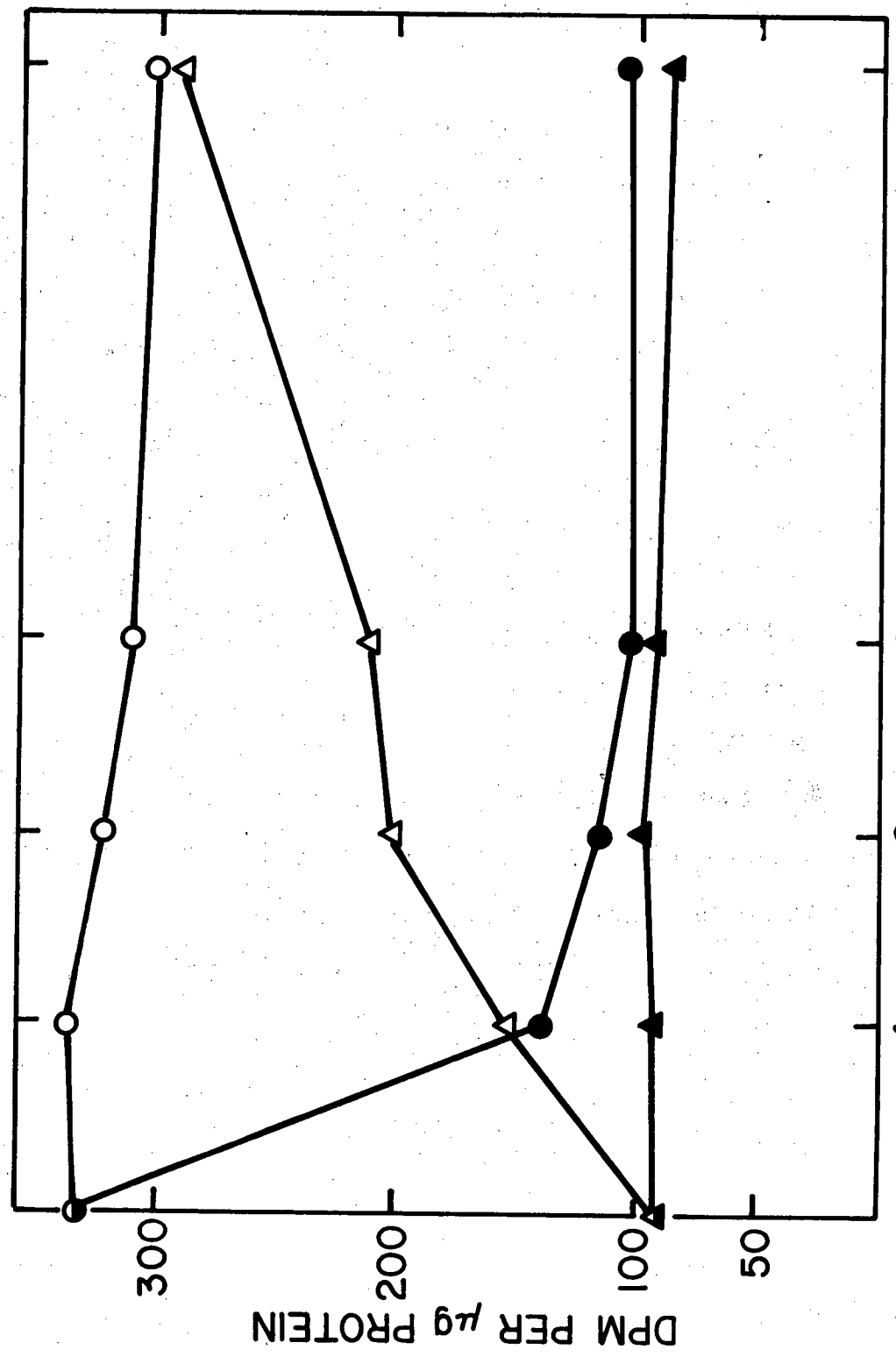


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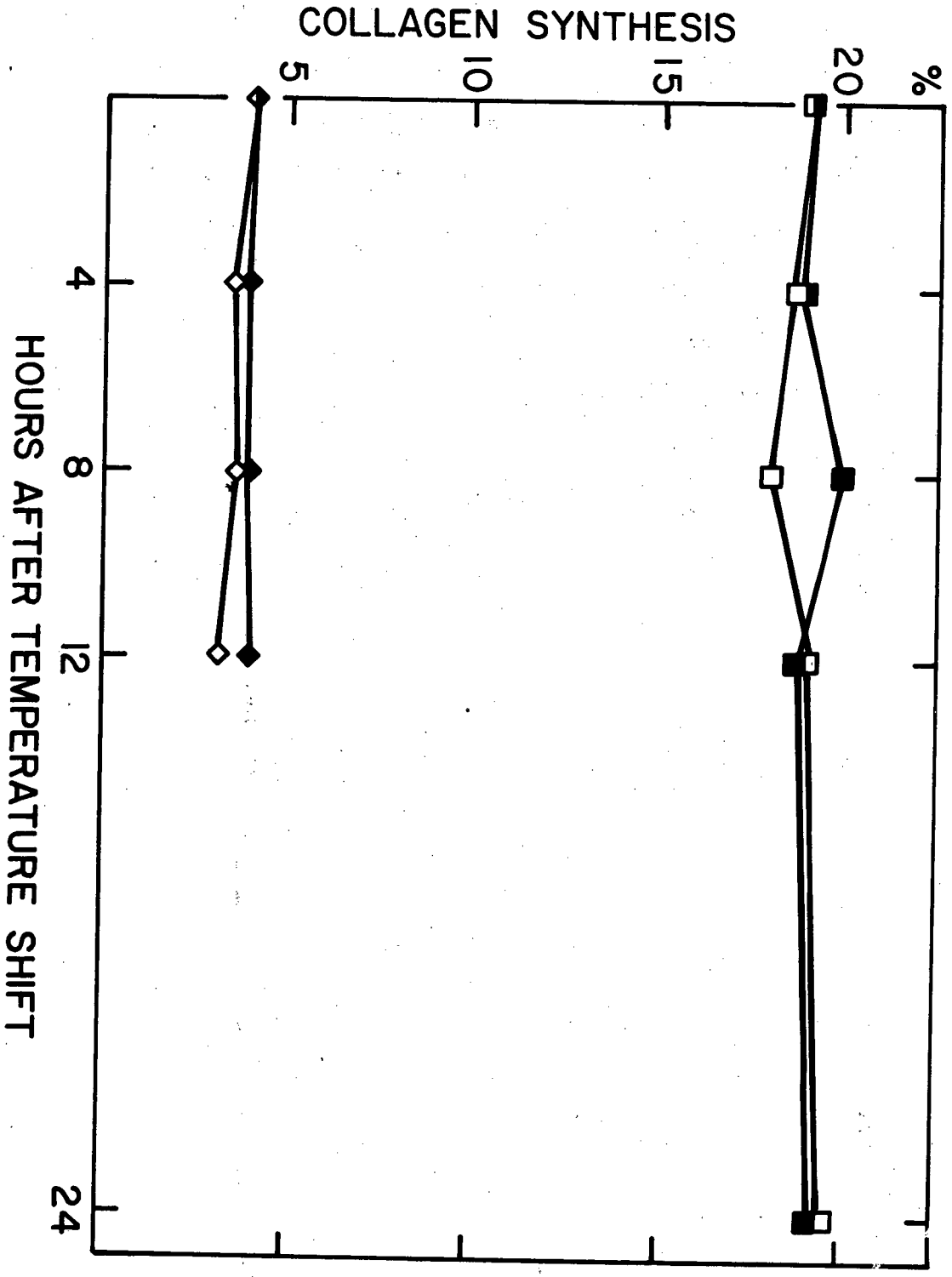
FIGURE IV-5 Effects of temperature shifts on collagen synthesis in normal and Prague A wild-type virus-infected tendon cells. Tendon cells were plated at a density of 8×10^5 cells per flask. After cells had attached, half of the flasks were infected with a wild-type Prague A strain of Rous sarcoma virus in 1.5 mls of F12 with 0.1% fetal calf serum and the other half were mock-infected. the cells were incubated for another hour before an additional 9 mls of medium were added. The medium contained F12 supplemented with 0.1% fetal calf serum and 50 ug/ml ascorbate and was changed daily. Cells were kept at 41° for 6 days. On the 7th day postinfection, half of the flasks were shifted to 35° and the other half remained at 41° as control. Collagen synthesis was measured at 4, 8, 12 and 24 hr after shift for mock-infected cells and at 4, 8 and at 4, 8 and 12 hr after shift for transformed cells ad described in Materials and Methods.

■, mock-infected cells remained at 41°

□, mock-infected cells shifted from 41° to 35°

◆, Prague A-infected cells remained at 41°

◇, Prague A-infected cells shifted from 41° to 35°



XBL7711-4744

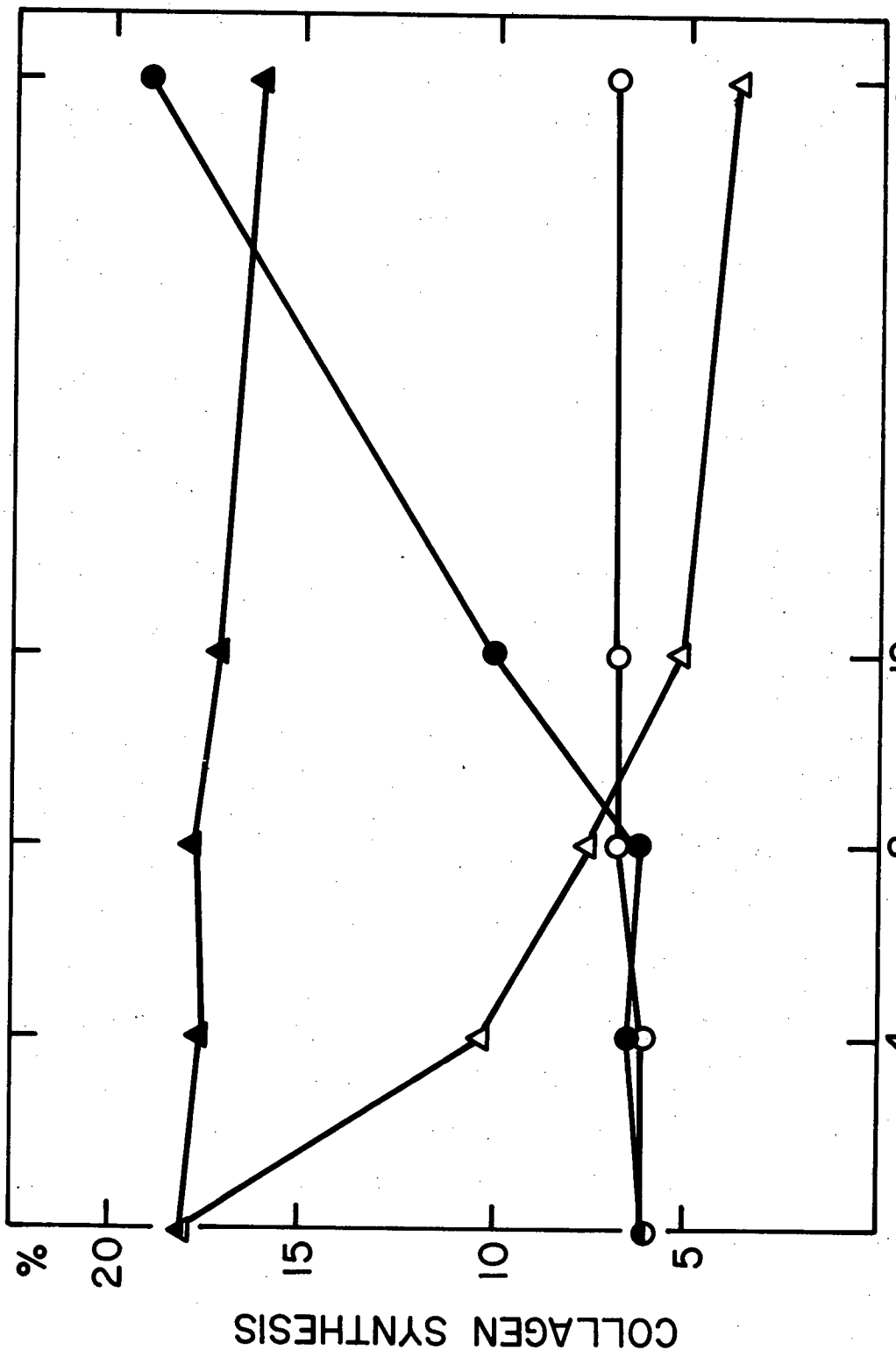
FIGURE IV-6 Effect of temperature shifts on collagen synthesis in LA 24-infected cells. Tendon cells were plated, infected, with LA 24, and switched to either 41° or 35° as described in Fig. IV-1. On the 7th day postinfection, half of the flasks at either temperature were shifted to the other temperature and the rest of the flasks remained at the original temperature. Collagen synthesis was measured at 4, 8, 12 and 24 hr after the temperature shifts as described in Materials and Methods.

o, cells remained at 35°

●, cells shifted from 35° to 41°

▲, cells remained at 41°

△, cells shifted from 41° to 35°



XBL 7711-4745

the alteration in collagen synthesis is an early event during the process of transformation and is closely associated in time with other early events such as sugar uptake.

When LA 24-infected cells were shifted from 35° to 41°, an increase in collagen synthesis was detectable only by about 12 hr after the shift. It appears that a relatively longer period of time is therefore needed to increase collagen synthesis back to its "differentiated" level during the process of reversion to normal phenotype.

Discussion

In this chapter, I have examined the time scale required for the reduction in collagen synthesis and hence the process of dedifferentiation upon temperature shift of tendon cells infected with a temperature-sensitive mutant of Rous sarcoma virus. The change in collagen synthesis was found to be an early event after transformation. The reduction in collagen synthesis was 50% complete as early as 4 hr after shift-down. The early manifestation of this decrease strongly suggests that the dedifferentiation process is a primary event after transformation and occurs as soon as *src* is activated (Collett and Erikson, 1978).

Changes in sugar uptake were almost complete at 12 hr after either shift when those in morphology were still not evident in many cells. It is possible that these two changes actually take place at the same time but that the early changes in morphology were not detected under the phase microscope. The difference in the uptake of 2-deoxy-D-glucose between normal and transformed cells, however, has been shown to occur irrespective of the shape of the cell (Bissell, Farson and Tung, 1977). It is thus more likely that the changes in sugar uptake take place at

local areas in membrane and occur earlier than do gross changes in morphology.

Although a direct relationship between the drop in collagen synthesis and the increase in sugar uptake is hard to imagine, it is possible that the former is operationally associated with another early event of transformation, the stimulation of cell proliferation. In this respect, it is interesting to compare the effect of transformation on collagen synthesis to that of serum which also stimulates cells to proliferate. High concentrations of serum also render normal tendon cells unresponsive to ascorbate modulation and reduce collagen synthesis to a level comparable to that in transformed cells (Schwarz and Bissell, 1977). In addition, the drop in collagen synthesis after serum addition to monolayer cultures occur at least as fast as after transformation and is complete within 6 hr after a high concentration of serum (5%) is added to normal tendon cells (Bissell, unpublished results). The common effect of both serum and transformation on collagen synthesis therefore suggests that the reduction in collagen synthesis by transformation could be a result of cell proliferation.

The molecular mechanism by which collagen synthesis is reduced after transformation has been previously studied by Adams et al. (1977) and Rowe et al. (1978) using chick embryo cell culture system. These investigators reported that the reduction was due to lowered level of translatable collagen m-RNA in the transformed cells. If one assumes that the reduction in collagen synthesis in tendon cells after transformation is under transcriptional control, and that the mean lifetine of collagen m-RNA is about 15 hr (Perry and Kelley, 1973), one could expect a 63% reduction

in collagen synthesis about 15 hr after shift, a kinetic much slower than the observed here. This means that even an instant change in collagen gene transcription upon the onset of transformation process could not account for the rapid decrease in collagen synthesis. Therefore, the initial reduction could well be regulated at the degradation of m-RNA or other post-transcriptional steps in the biosynthetic pathway of collagen, which in turn could lead to the reduction of collagen gene transcription by feedback mechanism. To study these possibilities, the temperature-sensitive system again provides an excellent tool. By following the time course of change at each step during a temperature shift, the relationships among the regulatory steps could be unveiled.

The increase in collagen synthesis with shift-up was not observed until 12 hr after the shift and lagged behind the change in 2-deoxy-D-glucose uptake. Collagen synthesis and sugar uptake, therefore, may be modulated by different events during the process of reversion. It is possible that the increase in collagen synthesis during the reversion to normal phenotype requires de novo transcription whereas the regulation of 2-deoxy-D-glucose uptake is at post-transcriptional level (Kletzien and perdue, 1975). At any event, it seems that extra time is needed for the synthesis and processing of collagen in a shift-up experiment It might be hypothesized that the additional time is required by the transformed cells to revert back first from a transformed to an "untransformed" phenotype characterized by the lower level of sugar uptake, etc., and then to a normal "differentiated" state characterized by the high level of collagen synthesis. To test this hypothesis, the kinetics of other markers for both differentiated and

"untransformed" states have to be examined. Nevertheless, this may be a generalized phenomenon since in another system in which chick myoblasts were infected with LA 29, a temperature-sensitive mutant of Pargue A Rous sarcoma virus similar to LA 24, the reversion of myosin synthesis during the shift-up also lagged behind changes in sugar uptake (Moss and Martin, personal communications).

CHAPTER V COLLAGEN COMPONENTS SYNTHESIZED BY NORMAL AND VIRUS-TRANSFORMED TENDON CELLS IN CULTURE

Introduction

Collagen synthesis *in vivo* is tissue-specific under normal conditions. There are at least five genetically distinct collagen subunits (α-chains) which combine to form four different collagen molecules (Miller, 1976). These collagen subunits can be identified by their amino acid composition, CNBr peptide map and distinct antigenicity. Type 1 collagen is probably the only collagen found in rigid connective tissues such as bone and tendon. In addition, it is also found in most pliable tissues such as skin, blood vessels, intestine, lung, etc. Two of the three polypeptide chains in type I collagen are called $\alpha 1(I)$ and have a different amino acid sequence from the third chain, called $\alpha 2$. Each of the three remaining collagen types contains three identical polypeptide chains which are specific for each type. Thus type II collagen (specific to cartilage) is designated $[\alpha 1(II)]_3$, type III collagen (found also in pliable tissues) is designated $[\alpha(III)]_3$, and finally type IV collagen (specific to basement membrane) is designated $[\alpha 1(IV)]_3$.

Although the tissue-specific collagen synthesis is normally under rigid control, quantitative and qualitative changes could occur in pathological states. For example, a ratio of 2-3 to 1 for type I and type III collagen is usually observed for normal soft tissues, but this ratio shifts toward more type I collagen in arteriosclerosis (McCullagh and Balian, 1975) and pulmonary fibrosis (Seyer, Hutchenson and Kang,

1976). In the case of Ehlers-Danlos syndrome with which patients have fragile but not extensible skin, the synthesis of type III collagen in soft tissue can be completely shut off (Gay et al., 1976). The abnormal collagen synthesis is also observed in osteogenesis imperfecta in which bone cells synthesize both type I and type III collagen; and in one embryonal carcinoma in which the malignant cells synthesize yet a new type of collagen not found in any normal tissue (Little et al., 1977).

The disturbance in collagen synthesis has also been reported to occur in culture. Although most cultured cells under normal conditions seem capable of expressing the type of collagen specific to their tissue origin (Hance and Crystal, 1977; Pacifici et al., 1977), qualitative changes in collagen synthesis can be induced by specific agents such as 5-bromodeoxyuridine (Mayne, Vail and Miller, 1975; Daniel, 1976) and serum (Narayanan and Page, 1977) which affect the state of differentiation of these cells in culture (Daniel et al., 1973; Gospodarowicz and Moran, 1976). Since transformation also affects the differentiated state of cultured cells, it is of interest to know if transforming agents could cause any change in the collagen phenotype in culture.

Hata and Peterkofsky (1977) recently reported that transformation by either 4-nitroquinoline-1-oxide, a chemical carcinogen, or murine sarcoma virus could induce the synthesis of new types of collagen in 3T3 cells. However, the new type of collagen induced by either transforming agent was distinct and seemed to be specifically related to that agent. Furthermore, transformation of 3T3 cells by SV 40, a DNA virus, did not induce any change in the type of collagen synthesized. It is therefore unclear if the change in collagen phenotype is directly related to the

process of transformation per se or some other actions by the transforming agents.

This ambiguity can be avoided if a system is used in which only transformation-specific events are observed. To determine whether transformation affects the type of collagen synthesized by tendon cells in culture, the collagen components synthesized by normal cells, cells transformed by a wild-type Rous sarcoma virus, as well as cells infected by LA 24 are examined in this chapter.

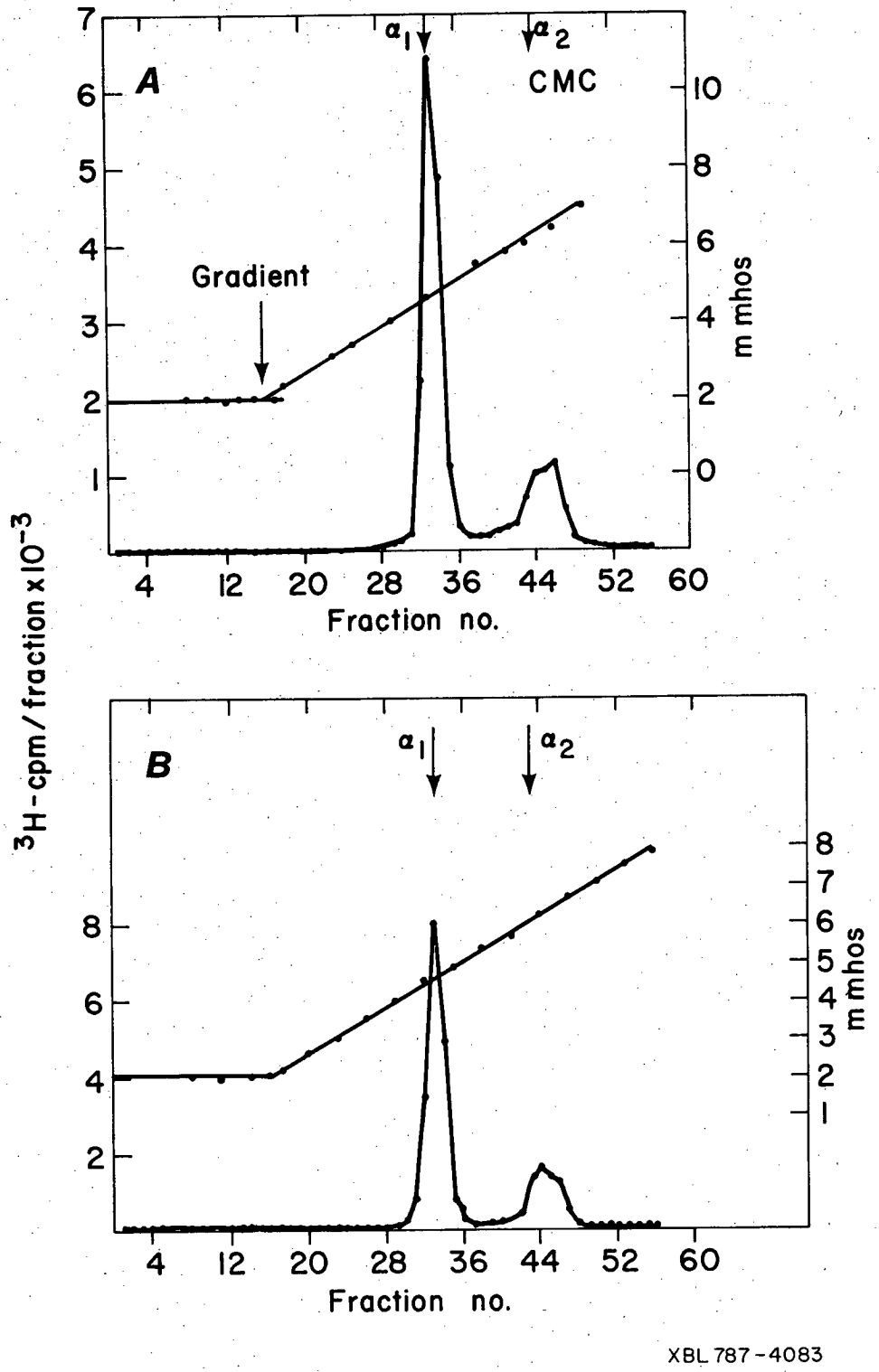
Collagen Components Synthesized by Normal Tendon Cells and Wild-type Rous Sarcoma Virus-transformed Cells

The collageneous material secreted into medium of normal and Rous sarcoma virus-transformed tendon cells was collected and purified. To make sure that the purified material was present only in the form of collagen but not a mixture of both procollagen and collagen (Hata and Peterkofsky, 1977), the material was further treated with pepsin at 15° C for 6 hrs. Under such conditions, all procollagen would be converted to collagen (Bornstein, 1974). The purity of collagen was always tested by a purified collagenase (Schwarz, Colarusso and Doty, 1976). The type of collagen was then identified by carboxymethyl-cellulose (CMC) chromatography and SDS-polyacrylamide gel electrophoresis (PAGE). Fig. V-1 shows the CMC chromatograms of collagen from ^{3}H-proline-labeled normal and transformed tendon cell media. The major collagen components from both normal and transformed cell culture media corresponded to the α_1(I) and α_2 chains of chick calvaria type I collagen. Since only 70 to 90% collagen could usually be recovered from a CMC column, a quantitative analysis of the components was not possible.

FIGURE V-1. Carboxymethyl-cellulose chromatograms of collagen from normal and wild-type Prague A virus-transformed cells. Cells were plated, infected, and grown in culture as described in Table III-1. Radioactive collagen from the medium of normal (3×10^4 cpm) and transformed (35×10^4 cpm) cultures was prepared as described in Materials and Methods, and applied to a CMC column (1.5 cm x 7.5 cm). The column was washed with 50 ml starting buffer (0.03 M sodium acetate/4 M urea at pH 4.8), then developed with a NaCl concentration gradient from 0 to 100 mM with 100 ml each of starting and limiting buffer at 43°.

A, normal cells

B, transformed cells


A
α_1
α_2
CMC
Gradient
m mhos
^{3}H-cpm/fraction x10^{-3}
Fraction no.
B
α_1
α_2
m mhos
Fraction no.

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A better recovery (more than 95%) could be obtained from gel electrophoresis. Fig. V-2 shows the SDS-PAGE profiles of collagen from ^{3}H-proline-labeled normal and tanssformed tendon cell media which were run together with type I collagen standard purified from chick calvaria. In the absence of dithiothreitol, the major collagen components from both normal and transformed cell culture media showed mobilities in SDS-PAGE corresponding to the α_1 : α_2 being 2.33 for normal and 2.28 for the transformed media. These ratios were close to the calculated ratio of type I collagen (2.3) (Miller, 1976). In both profiles, there were some β and γ components which represents dimers $\alpha_1\alpha_2$ ($=\beta_{12}$) and $[\alpha_1(I)]_2$ ($=\beta_{11}$) and trimer $[\alpha_1(I)]_3$ ($=\gamma$) as aggregates. The transformed cells, however, seemed in addition to secrete a different type of collagen which had a higher molecular weight than γ. This type of collagen was not simply an aggregate of α-chains since in different experiments the amount of β and γ chnaged but this component remained 10 to 15% of total collagen. Furthermore, this new type of collagen probably contains subunits of α_1 chains since upon reduction this component disappeared and some radioactivity appeared in the region of α_1 chains. This was evidenced in the SDS-PAGE profile in the presence of dithiothreitol. The α_1 : α_2 ratio increased from 2.28 to 2.49 and the high-molecular-weight component in the non-reduced SDS-PAGE profile of transformed medium disappeared; whereas the α_1 : α_2 ratio of collagen from normal medium was 2.29, showing no change from 2.33 upon reduction.

Collagen Components Synthesized by LA 24-infected Cells

To further determine if the synthesis of the new collagen component was transformation-specific, collagen components synthesized by

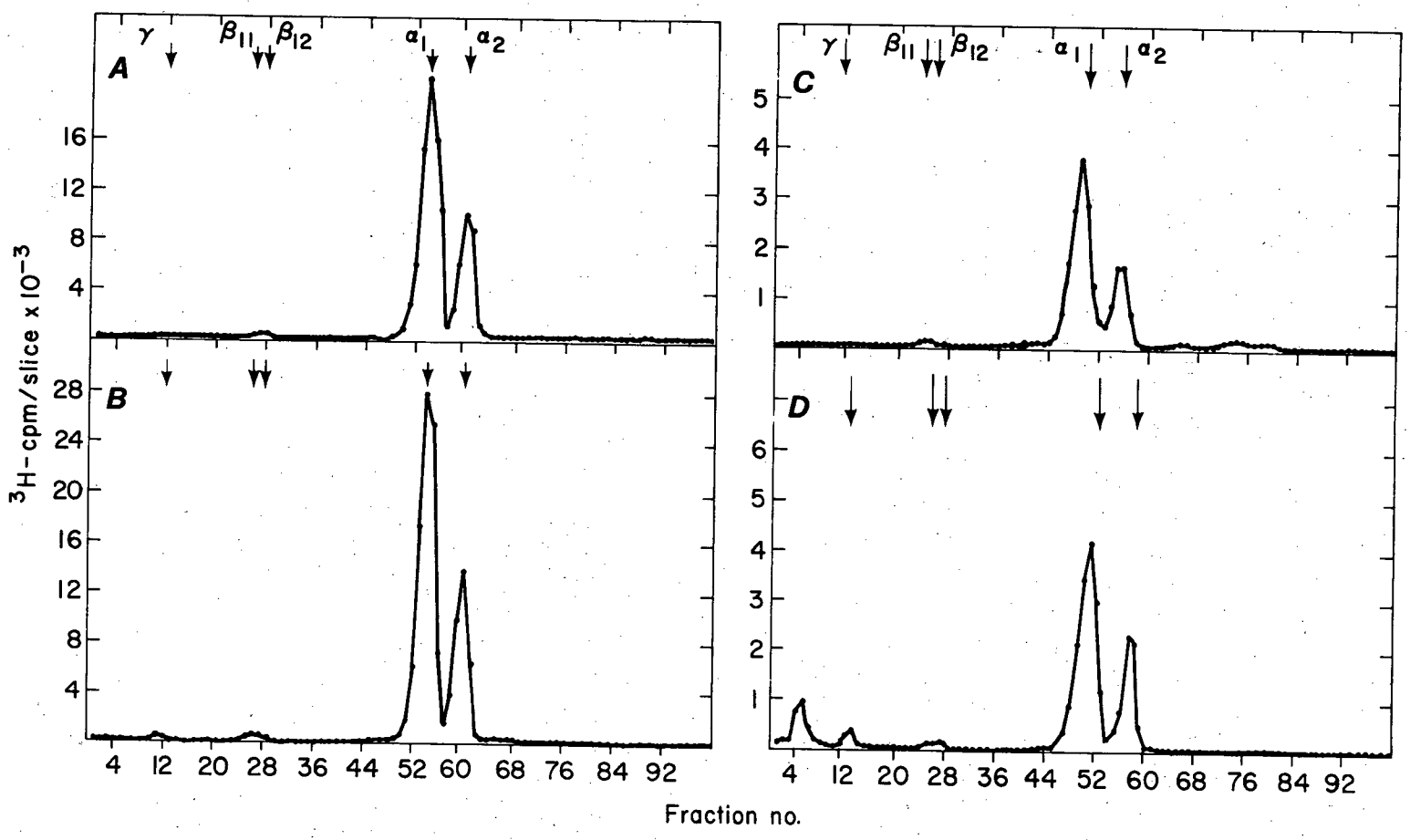
FIGURE V-2 SDS-polyacrylamide gel electrophoresis of collagen from the media of normal and Prague A wild-type virus-transformed cells. Radioactive collagen (13×10^4 and 2.3×10^4 cpm for non-reduced PAGE and 10×10^4 and 2.1×10^4 cpm for reduced PAGE) was prepared as described in Fig. V-1 and analyzed by SDS-PAGE as described in Materials and Methods.

A, normal cells, reduced

B, normal cells, non-reduced

C, transformed cells, reduced

D, transformed cells, non-reduced


γ
β11
β12
α1
α2
A
B
C
D
^{3}H-cpm/slice x 10^{-3}
Fraction no.
4 12 20 28 36 44 52 60 68 76 84 92

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LA 24-infected tendon cells kept at either 41° or 35° were analyzed. Fig. V-3 shows the CMC chromatograms of collagen from ^{3}H-proline-labeled media of LA 24-infected cells maintained at either temperature. Again only α_1(I) and α_2 coule be identified in either culture medium by this technique.

Fig. V-4 shows the SDS-PAGE profiles of collagen from either medium. The additional component was found only from medium of cells maintained at 35° but not at 41°. Moreover, the α_1 : α_2 ratio increased from 2.29 to 2.44 and the new component again disappeared upon reduction in the case of collagen from medium of cells kept at 35°, but the ratio of α_1 : α_2 did not change significantly (from 2.30 to 2.33) upon reduction in the case of collagen from medium of cells kept at 41°. These results suggested that the synthesis of the new collagen was indeed transformation-specific.

Discussion

Tissue specificity of collagen synthesis is another useful criterion for differentiation. This polymorphic expression is probably triggered when cells are first differentiated (Epstein, 1974), so that the particular type of collagen synthesized would help define the structure and function of the tissue (Hance and Crystal, 1977). Thus not only quantity but also quality are important for the differentiated state of cells. Tendon tissue in vivo synthesizes type I collagen which forms densely packed fibrils responsible for the toughness of the tissue. It was therefore necessary to show that tendon cells in culture synthesize the same type of collagen under conditions these cells were maintained. This was especially true regarding the fact that cultured tendon cells were grown in the presence of serum which was known to modulate the type of

FIGURE V-3. Carboxymethyl-cellulose chromatograms of collagen from LA 24-infected tendon cells. Radioactive collagen from the medium of LA 24-infected tendon cell cultures kept at either 35° (3.5×10^4 cpm) or 41° (4.5×10^4 cpm) was analyzed on CMC as described in Fig. V-1.

A, cells maintained at 41°

B, cells maintained at 35°

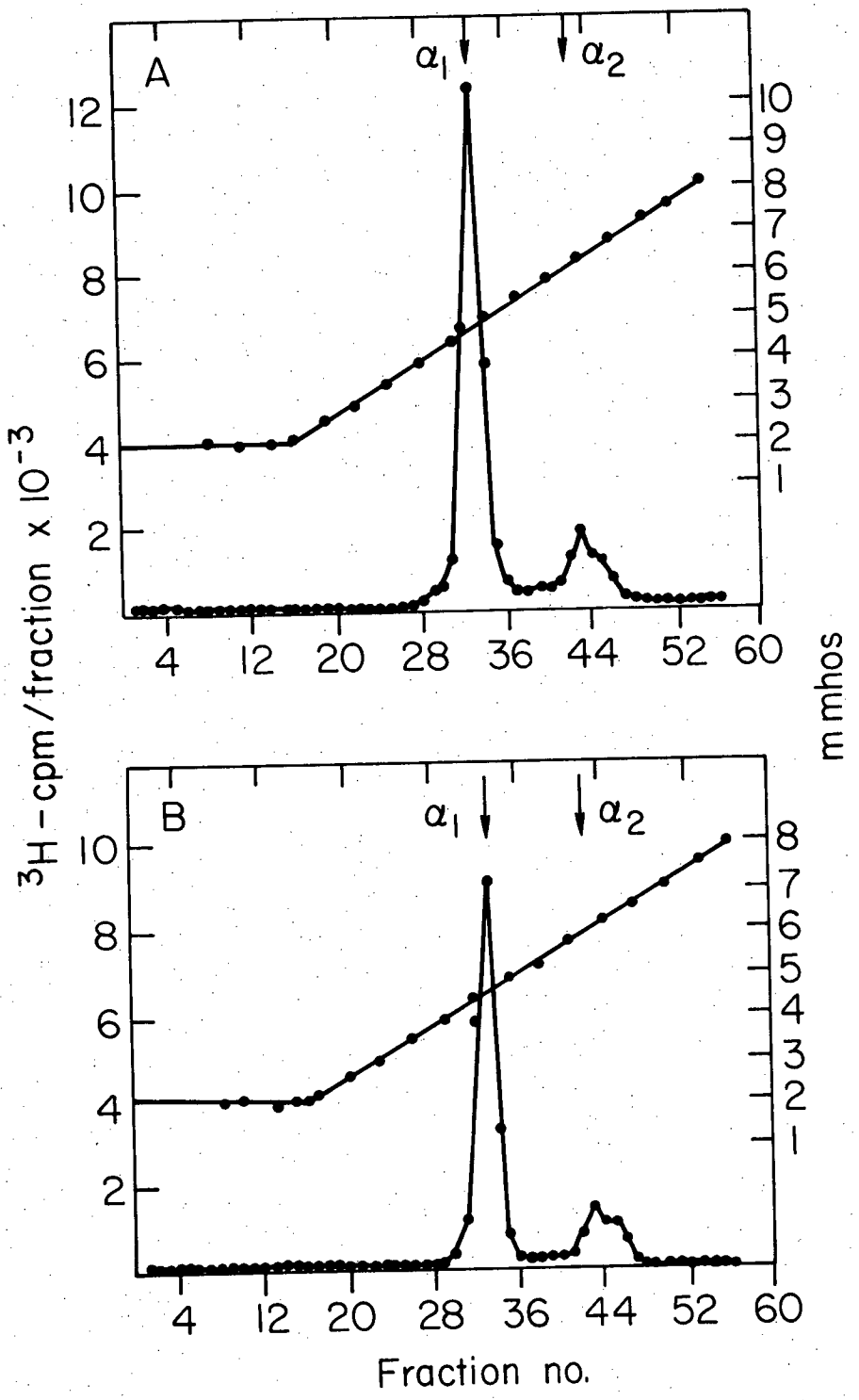
A
B
α_1
α_2
^{3}H - cpm / fraction x 10^{-3}
m mhos
Fraction no.
12
10
8
6
4
2
10
9
8
7
6
5
4
3
2
1
4
12
20
28
36
44
52
60

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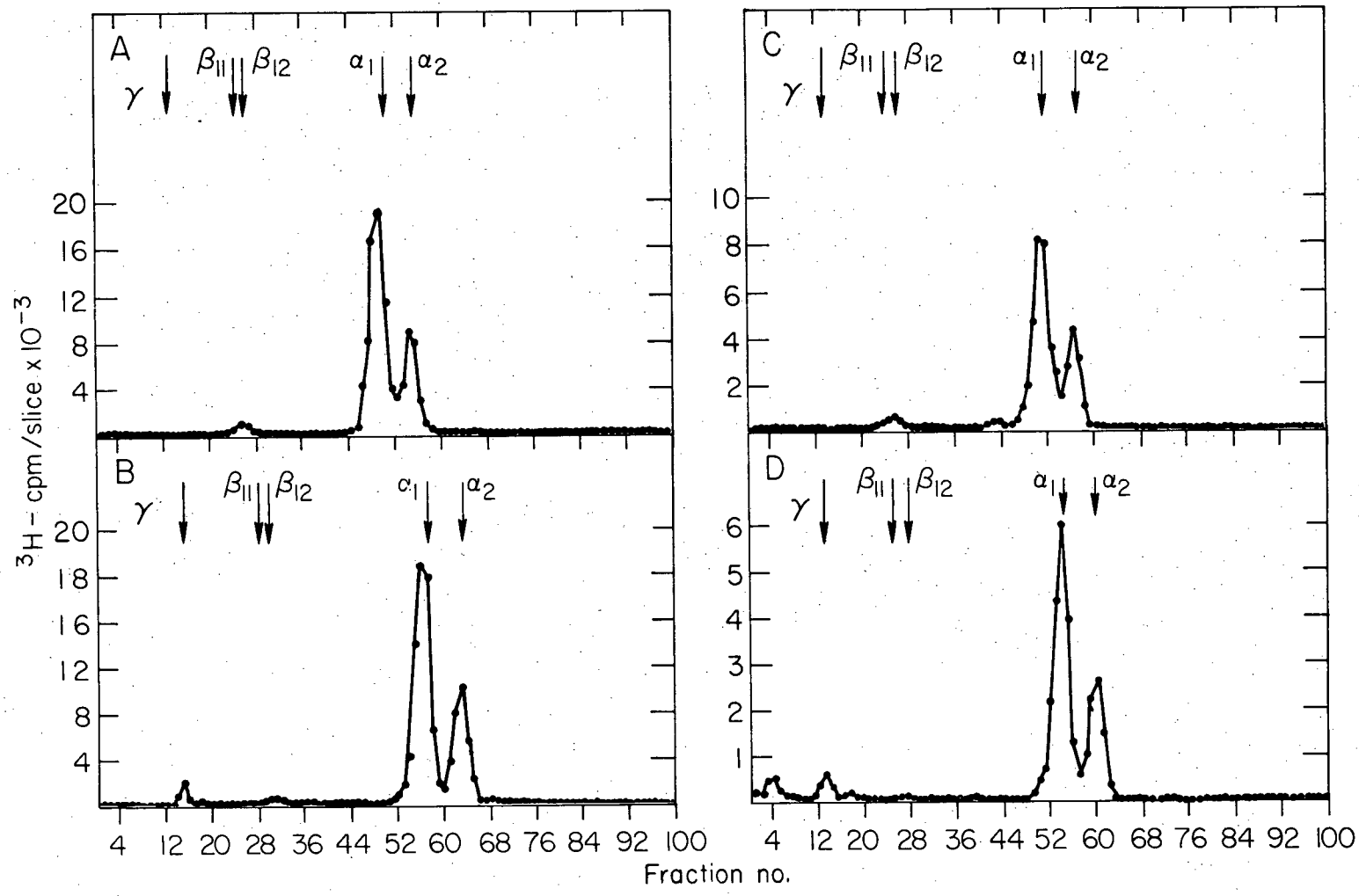
FIGURE V-4. SDS-polyacrylamide gel electrophoresis of collagen from the media of LA 24-infected cells. Radioactive collagen (11×10^4 cpm and 2.8×10^4 cpm for non-reduced PAGE and 9.5×10^4 cpm and 4.3×10^4 cpm for reduced PAGE) from media of cells kept at either 41° or 35° was prepared and analyzed as described in Fig. V-2.

A, cells maintained at 41°, reduced

B, cells maintained at 41°, non-reduced

C, cells maintained at 35°, reduced

D, cells maintained at 35°, non-reduced


A
B
C
D
γ
β11
β12
α1
α2
^{3}H – cpm/slice x 10^{-3}
Fraction no.
4 12 20 28 36 44 52 60 68 76 84 92 100

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collagen synthesized by human fibroblasts in culture (Narayanan and Page, 1977). Studies in this chapter have shown that, after one week in culture, the tendon cells not only synthesized the same level of collagen as their in vivo counterparts but also maintain the same type of collagen. These results indicate that cultured tendon cell system quantitatively and qualitatively a valid model for the study of differentiation in culture.

Transformation by Rous sarcoma virus does not seem to cause any major change in collagen synthesis in tendon cells. Although the transformed cells might synthesize an additional type of collagen at a very low level, most of the collagen synthesized by these cells is still type I. This observation disagrees with that reported by Hata and Peterkofsky (1977) in which over 30% of the collagen synthesized by virally transformed BALB 3T3 cells belong to a new type, but agrees with that made by Arbogast et al. (1977) in which chick embryo fibroblasts transformed by Rous sarcoma virus might synthesize the same type of collagen as normal cells. These different observations with chick and 3T3 cells may reflect either different regulatory mechanisms in collagen synthesis between species or again the differentiatial stability of rodent cell lines and avian cells in primary culture. It is possible, however, that the additional type of collagen synthesized by transformed tendon cells is similar to that synthesized by transformed BALB 3T3 cells since both contain intrahelical disulfide bonds and are made of subunits of α_1 chains. Whether they actually represent the same type of collagen would require further chemical analysis such as CNBr peptide map and amino acid analysis.

To show that the synthesis of the additional type of collagen is indeed transformation-specific, one has to rule out two other possibili-

ties. The first possibility is that the synthesis of this collagen might only be a result of viral infection but not the direct consequence of transformation. This possibility is ruled out by the fact that LA 24-infected cells maintained at 41° allow virus to replicate but appear to synthesize only type I collagen. The second possibility is that the synthesis of this additional type of collagen also occurs in normal cells, but the low level of this collagne in comparison to type I collagen makes its detection very difficult. The transformation selectively reduces the synthesis of type I collagen, so the additional type of collagen can now be detected. This second possibility can be ruled out by the following calculation. Since the additional type of collagen represents 10 to 15% of total collagen synthesized by transformed cells, and a 10-fold decrease in collagen synthesis is usually observed after transformation, the assumption that transformation only reduces the synthesis of type I collagen would dictate 1.0 to 1.5% of total collagen synthesized by normal cells to be the new type of collagen. If this were the case, at least 1300 cpm of this collagen should have been detected with 13×10^4 cpm of total collagen from normal culture medium was applied to gel electrophoresis in Fig. V-2B. However, no significant increase in counts above background was observed beyond regions associated with type I collagen.

Recent work by Little and Church (1976) suggested the existence of a new type of collagen specific for embryonic cells. Since transformation is known to induce the expression of fetal antigens and the embryonic form of isozymes, it will be interesting to compare the additional type of collagen synthesized by transformed tendon cells to this embryonic type of collagen. Preliminary characterization of the embryonic collagen, however, showed that this type of collagen did not contain any

intrahelical disulfide bond (Little et al., 1977). These two types of collagen might therefore represent distinct gene products.

CHAPTER VI MECHANISM OF THE DECREASE IN THE LEVEL OF COLLAGEN IN CHICK TENDON CELLS AFTER TRANSFORMATION. I. SYNTHESIS AND SECRETION

Introduction

So far in this thesis, it has been established that collagen synthesis in avian tendon cells, as measured by percentage of total protein synthesis, is considerably reduced after transformation of these cells by Rous Sarcoma virus. The percentage has been used for the expression of collagen synthesis since it emphasizes the state of differentiation of these cells. While this method of expression clearly indicates a quantitative "dedifferentiation" of tendon cells after transformation in terms of collagen synthesis, it indeed does not provide any information about the molecular mechanism by which collagen synthesis is changed. Since the rate of protein synthesis in general is higher in transformed cells, the reduction of collagen synthesis as percentage of total protein synthesis could be due to either the decrease in collagen synthesis or the increase in non-collagen protein synthesis at the absolute level or both. To distinguish these possible mechanisms would thus require the measurement of the absolute amount of collagen synthesized by normal and transformed cells.

The problem of determining the mechanism by which collagen synthesis is decreased after transformation is further complicated by the fact that collagen is a secreted protein and the process of secretion has been implied to play a feedback regulatory role on synthesis (Peterkofsky, 1972). Thus even a reduction in the absolute level of collagen synthesis after transformation could be the result of a decrease in the rate of secretion of tropo-procollagen molecule and/or the decrease in the

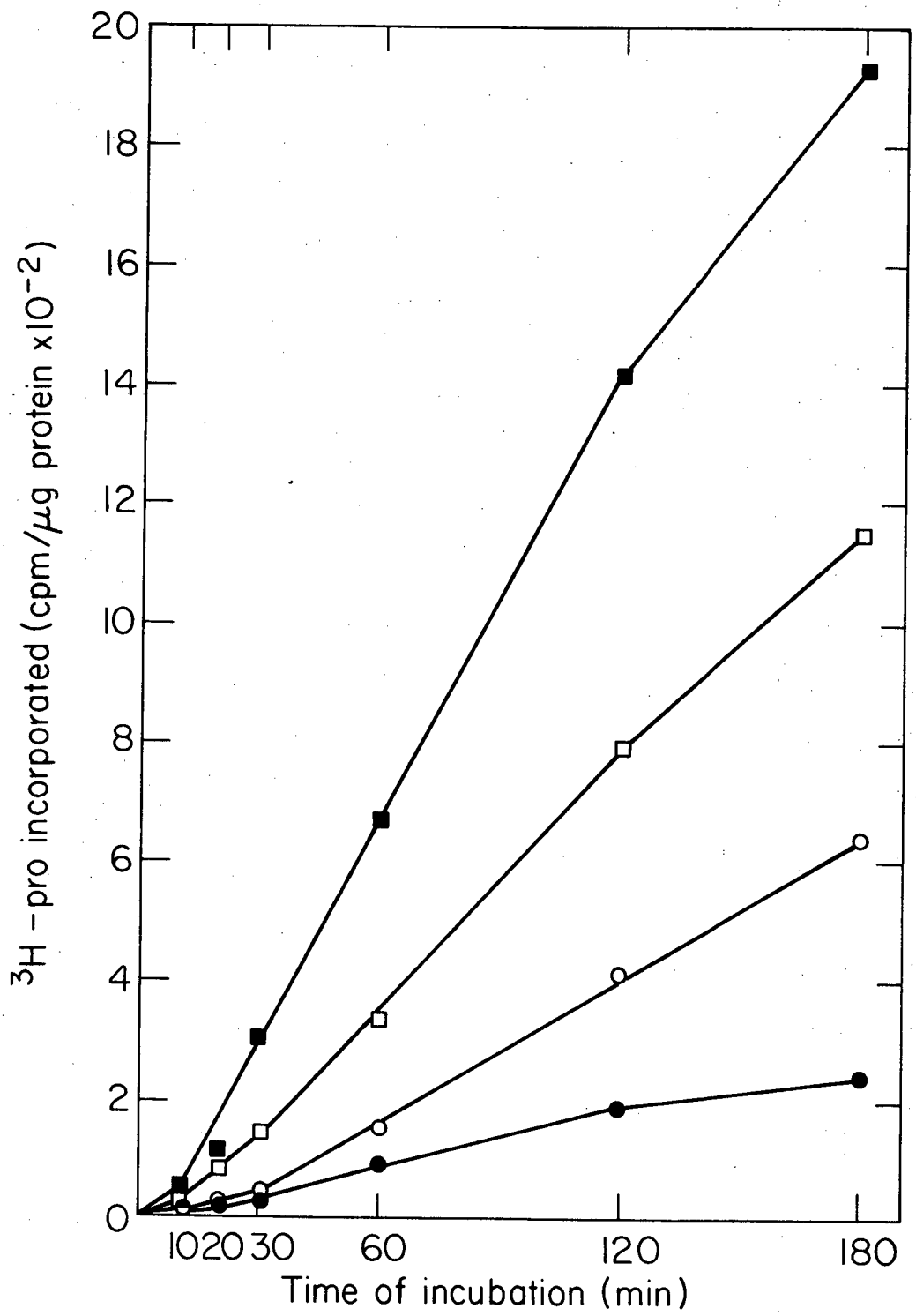
biosynthesis of procollagen polypeptide itself.

In order to determine the precise regulatory mechanisms which are responsible for the reduction in collagen synthesis after transformation, the absolute level of collagen synthesis as well as the rate of secretion of collagen by chick tendon cells before and after transformation need to be examined. This chapter deals with the above considerations.

The Rate of Collagen Synthesis in Normal and Transformed Tendon Cells

To determine the rate of collagen synthesis in normal cells and cells transformed by a wild-type Prague A Rous sarcoma virus, radioactively labeled proline was added to both cultures, and incorporation into collagen and total protein of cells and medium after fixed intervals of incubation was measured as described in Materials and Methods. The kinetics of this incorporation is shown in Fig. VI-1. The incorporation was not linear for both cultures for the first 30 min possibly because of changes in the specific activity of precursor pools of free proline. Non-linearity was also observed for transformed culture with incorporation for more than 2 hr. This non-linearity, however, did not seem to significantly affect the calculation for collagen synthesis expressed as percentage of total protein synthesis so that the results from experiments in Chapters III and IV using 3-hr incorporation were still comparable. The collagen synthesis with 1, 2 and 3 hr incorporation was, respectively, 2.7%, 2.7% amd 2.5% of total protein synthesis. The normal cells synthesized collagen as 19.0% of total protein so a 7-fold reduction was observed after transformation if collagen synthesis was expressed as percentage of total protein synthesis.

FIGURE VI-1. The time course of incorporation of ^{3}H-proline into collagen and total protein of normal and transformed tendon cells. Cells were plated at a density of 8 x 10^{5} cells per 60 mm plate. After cells had attached, half of the plates were infected with a wild-type Prague A strain of Rous sarcoma virus in 1.5 mls of F12 with 0.2% fetal calf serum and the other half were mock-infected. The cells were incubated for another hour before an additional 9 mls of medium were added. Cells were grown in F12 with 0.2% fetal calf serum and 50 ug/ml ascorbate and the medium was changed daily. On the 7th day postinfection, ^{3}H-proline incorporation was carried out for 10, 20, 30, 60, 120 and 180 min and analysis of collagen and total proteins of cells and medium for each time point was carried out as described in Materials and Methods. The results presented in this figure represent the sum of values for the cell and the medium fractions. Symbols: open, normal cells; closed, transformed cells; ○, collagen; □, total protein.


20
18
16
14
12
10
8
6
4
2
0
^3H -pro incorporated (cpm/μg protein x10^{-2})
10 20 30 60 120 180
Time of incubation (min)

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The absolute rate of collagen synthesis in normal and transformed cells could be compared using the linear region of the kinetic curves in Fig. VI-1. When the slopes of incorporation of proline into collagen between 30 min and 2 hr were compared, a 2.4-fold decrease in collagen synthesis was found after transformation of tendon cells. The 7-fold decrease in collagen synthesis as percentage of total protein synthesis was therefore a result of not only the decrease in collagen synthesis at the absolute level but also a 3.3-fold increase in non-collagen protein synthesis after transformation.

Synthesis of Collagen Subunits by Normal and Transformed Cells

The fact that the type I collagen synthesized by tendon cells contains two distinct subunits (α_1 and α_2 chains) made it interesting to test if the synthesis of these subunits were reduced to the same extent by transformation. To analyze the separate synthesis of these two α-chain subunits would require an examination of the intracellular synthesis of the precursors of these polypeptides before they are secreted in the form of tropo-molecules since the latter process would dictate a rigid 2:1 ratio of α_1 to α_2 chains (see Chapter V). As shown in Fig. VI-2, collagen was secreted as early as 30 min after the addition of ^{3}H-proline, so the kinetic analysis of cellular pro-collagen was limited to this period.

Fig. VI-3 and VI-4 show the incorporation of ^{3}H-proline into pro-α_1 and pro-α_2 collagen subunits in normal tendon cells during the first 30 min incubation after the addition of the radioactive precursor. Since collagenase will not distinguish between different collagen α-chains, the SDS-polyacrylamide gel electrophoresis was used to separate pro-α_1 and

FIGURE VI-2. The time course of incorporation of ^{3}H-proline into collagen in the medium and cell layer of normal and transformed tendon cultures. This figure is a magnification of Fig. VI-1 for collagen synthesis. The results for the cell layer and the medium fractions have also been plotted separately to indicate the amount of collagen secreted at each time point. Symbols: open, normal cells; closed, transformed cells; o, total collagen; △, collagen in the medium fraction; □, collagen in the cell fraction.

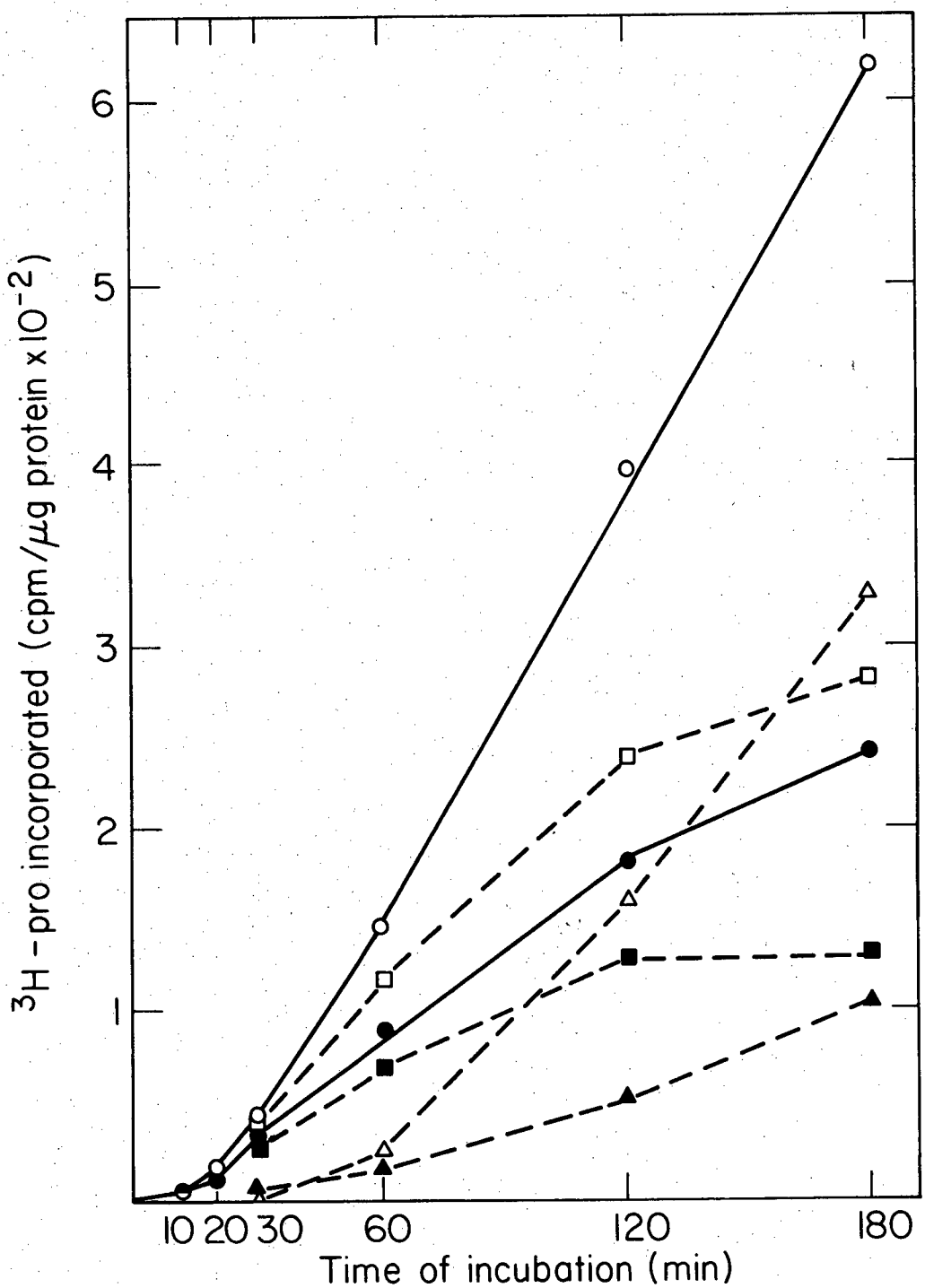
^{3}H - pro incorporated (cpm/μg protein x 10^{-2})
1
2
3
4
5
6
10 20 30
60
120
180
Time of incubation (min)

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FIGURE VI-3. The time course of incorporation of ^{14}C-proline into pro-α_1 and pro-α_2 collagen subunits of normal tendon cells. Cells were seeded in 60 mm plates and maintained in F12 with 0.2% fetal calf serum and 50 ug/ml ascorbate as described in Materials and Methods. On day 7, cells were pulsed with 10 uCi ^{14}C-proline per plate for 10, 20, and 30 min. The labeling medium was then withdrawn and the cells were either homogenized in 2% SDS buffer (A-E) or in collagenase buffer (F,G) as described in Materials and Methods. Sample for F was further treated with purified bacterial collagenase at 37° for 1 hr. Equal amounts of protein were applied to lane A through E while same volume of samples were applied to F and G. SDS-polyacrylamide gel electrophoresis and autoradiography were carried out as described in Materials and Methods. Pro-α_1 and pro-α_2 bands were indicated by arrows. By comparing lane F to G, these two bands could be identified since they were selectively digested by purified collagenase.
Lane A and E, 30 min incorporation.
Lane B, D. F and G, 20 min incorporation.
Lane C, 10 min incorporation.

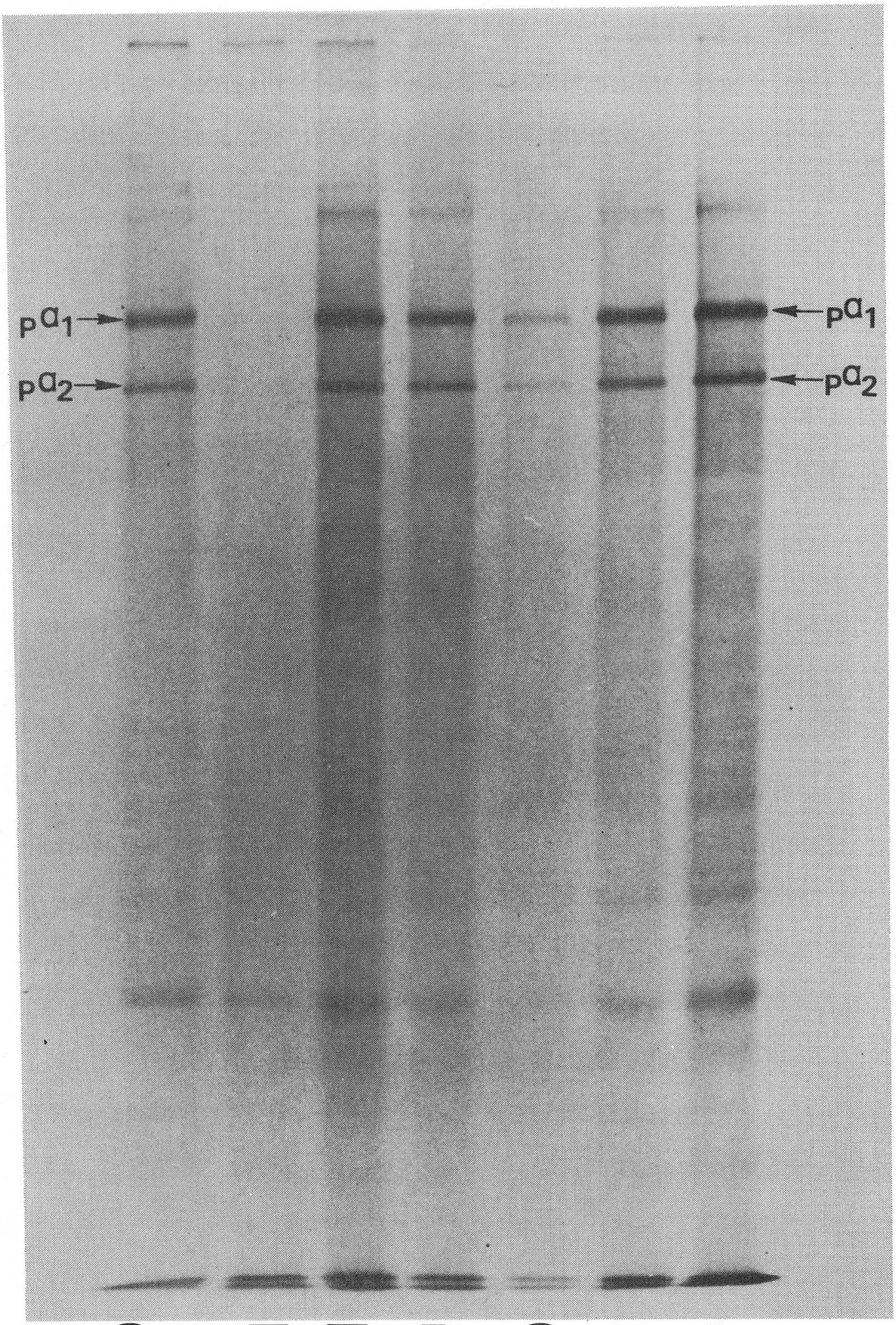

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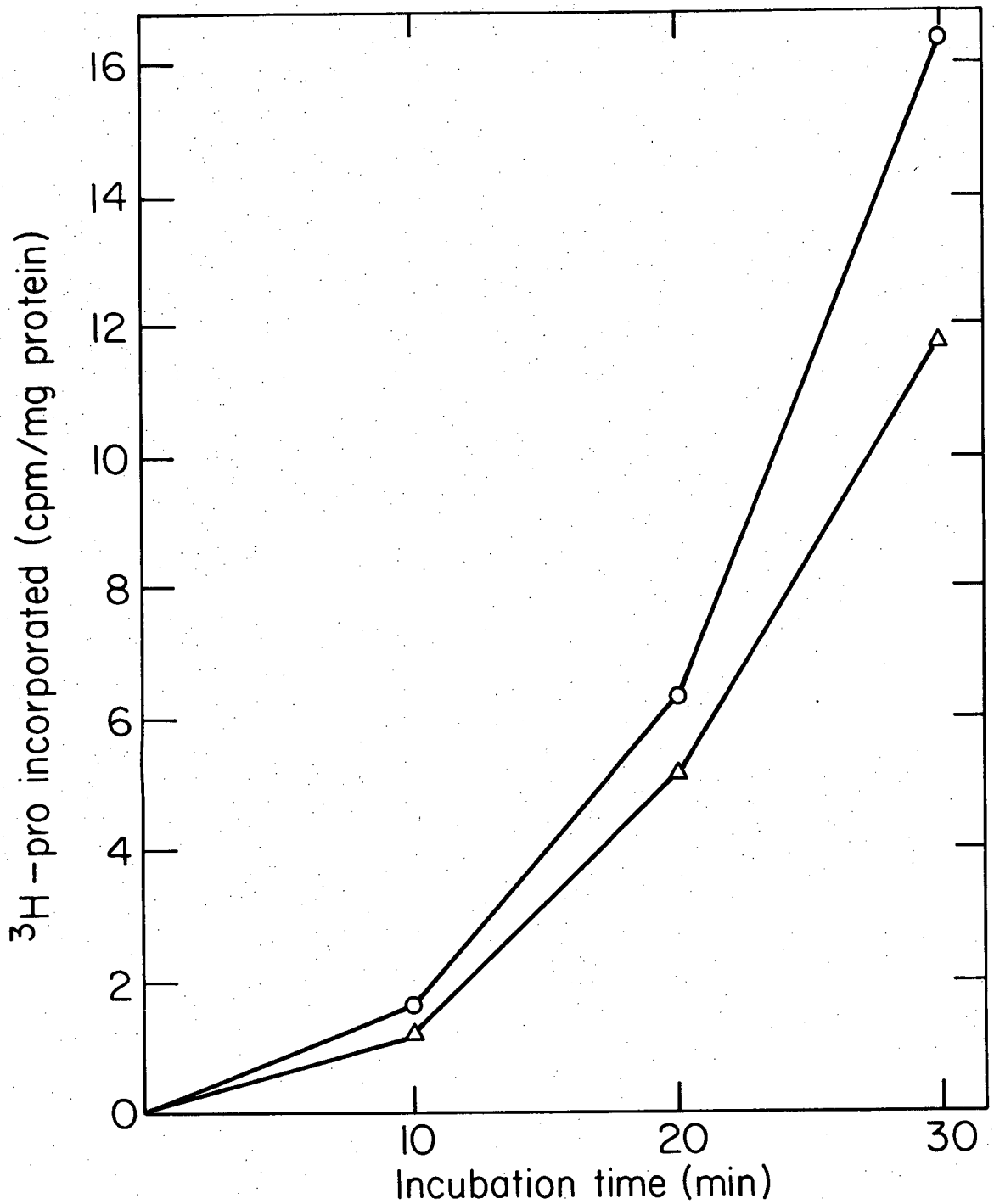
FIGURE VI-4. The time course of incorporation of ^{14}C-proline into pro-α_1 and pro-α_2 collagen subunits of normal tendon cells. The pro-α_1 and pro-α_2 bands in lane A through E were cut from the gel and burned in a Packard automatic combustion apparatus to give ^{3}H-H_2O which was then counted in a scintillation counter. Results from Lanes A and E as well as from B and D did not differ from each other for more than 10% and were thus averaged to give single values.

○, pro-α

▲, pro-α


16
14
12
10
8
6
4
2
0
10
20
30
^{3}H-pro incorporated (cpm/mg protein)
Incubation time (min)

XBL789-4198

pro-α_2 and the incorporation of radioactivity into these polypeptides was determined by counting the corresponding bands on the gel as described in Materials and Methods. As shown in Fig. VI-4, the synthesis of neither of pro-α chains was linear during the first 30 min incorporation and increased with time when ^{3}H-proline was used as the radioactive precursor. This non-linearity thus prevented an adequate calculation of the rate of synthesis of these collagen subunits.

The same problem was not observed when ^{3}H-leucine was used as the radioactive precursor. As shown in Fig. VI-5 and Fig. VI-6, the incorporation of ^{3}H-leucine into pro-α_1 and pro-α_2 was more or less linear in both normal and transformed tendon cells between 10 and 25 min after the addition of the radioactive precursor. The slopes of incorporation of leucine into pro-α_1 and pro-α_2 could therefore be used for the calculation of the rate of synthesis of these polypeptides in normal and transformed cells. The reduction of the synthesis of pro-α_1 and pro-α_2 was, respectively, 2.3- and 2.4-fold after transformation. The synthesis of these two collagen subunits was thus reduced to the same extent by transformation.

Secretion of Collagen by Normal and Transformed Tendon Cells

To follow the secretion of newly synthesized collagen, normal and transformed cells were labeled with ^{3}H-proline for 3 hr and the label was then chased by transferring the cells to fresh medium containing only "cold" proline. At designated intervals, the medium and cells were separated and assayed for ^{3}H-collagen as described in Materials and Methods. To correct for the difference beween the intracellular collagen levels in normal and transformed cells (as shown in Fig. VI-2),

FIGURE VI-5. The time course of incorporation of ^{14}C-leucine into pro-α_1 and pro-α_2 collagen subunits of normal and transformed tendon cells. Cells were plated, infected, and maintained as described in Fig. VI-1. On the 7th day postinfection, cells were pulsed for 10, 15, 20, 25, and 30 min with 10 uCi ^{14}C-leucine per plate. The labeling medium was withdrawn and the cells were then homogenized in 2% SDS buffer as described in Materials and Methods. SDS-polyacrylamide electrophoresis and autoradiography were carried out as described in Materials and Methods. For each time point, approximately equal amounts of radioactivity from normal and transformed samples were applied.

Lane B, D. F. and H, normal cells.

Lane A, C, E, G, and I, transformed cells.

Lane A and B, 10 min incorporation each with about 50,000 cpm

Lane C and D, 15 min incorporation, 150,000 cpm.

Lane G and H, 25 min incorporation, 450,000 cpm.

Lane I, 30 min incorporation, 500,000 cpm.

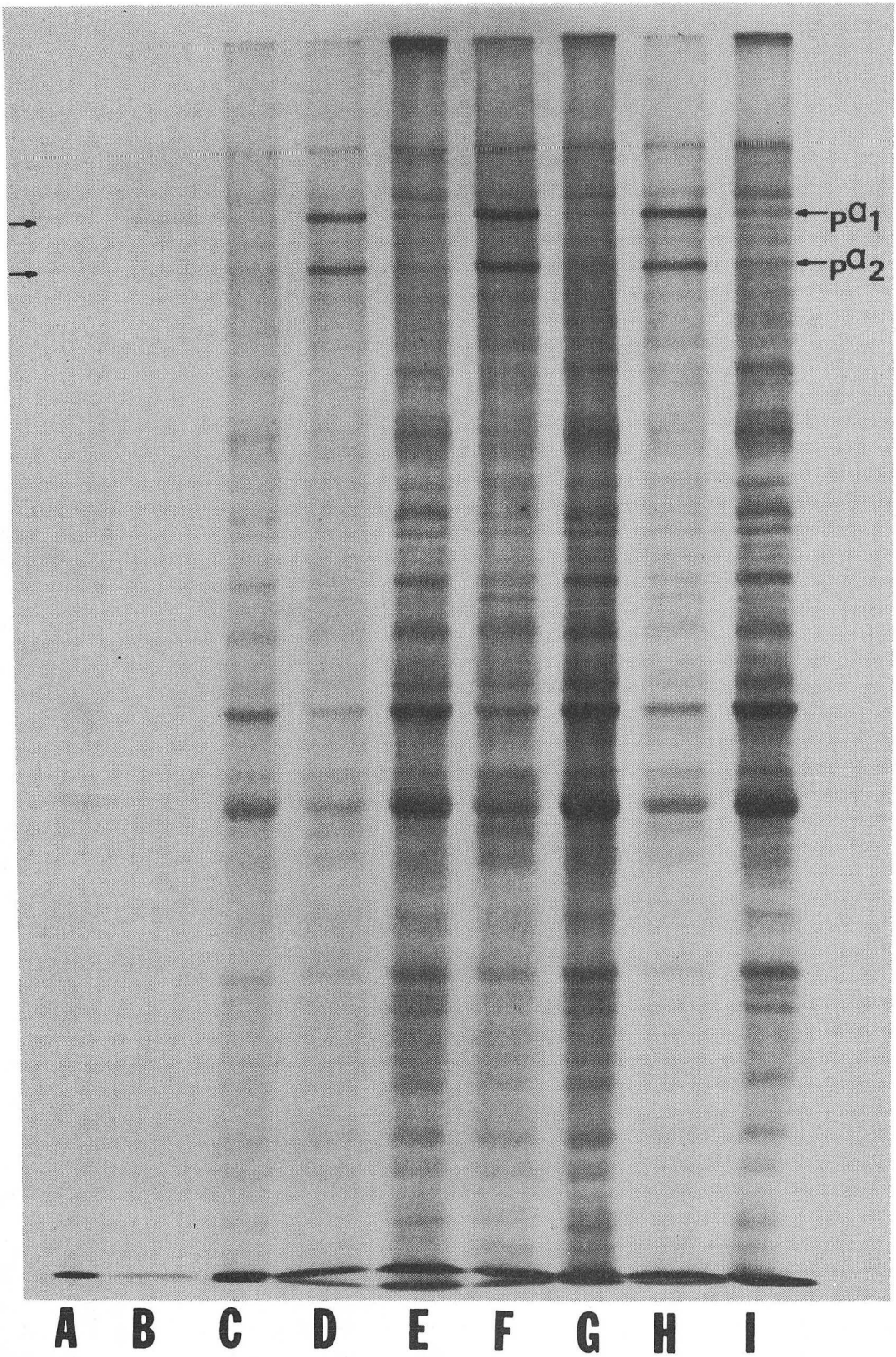


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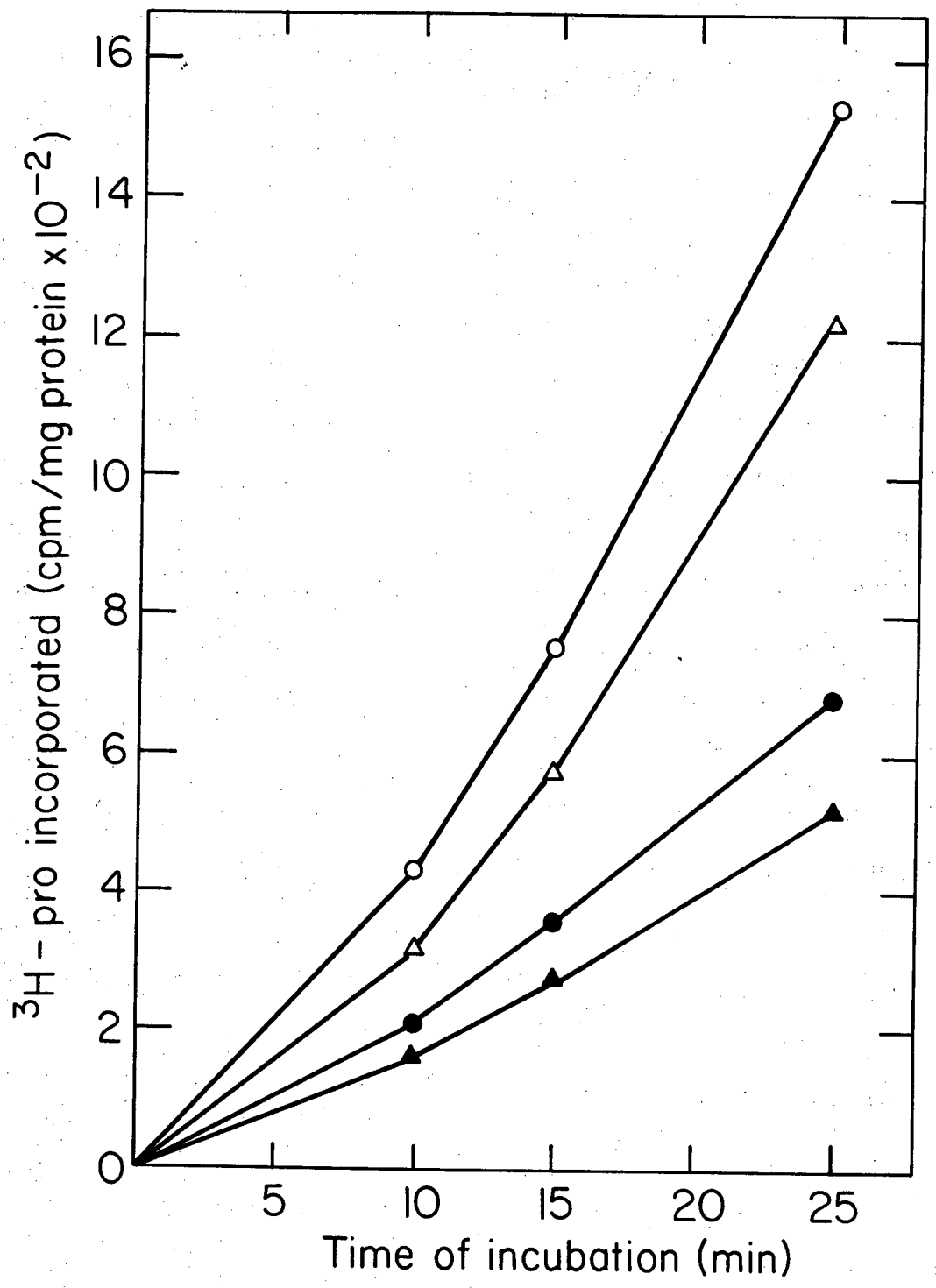
FIGURE VI-6. The time course of incorporation of ^{14}C-leucine into pro-α_1 and pro-α_2 collagen subunits of normal and transformed tendon cells. Since aggregates were found in Lane E, only 10, 15, and 25 min time points were used for calculation. The pro-α_1 and pro-α_2 bands were cut from the gel and burned and counted as described in Fig. VI-4.

Symbols: open, normal cells; closed, transformed cells; o, pro-α_1; Δ, pro-α_2.


^{3}H- pro incorporated (cpm/mg protein x10^{-2})
0
2
4
6
8
10
12
14
16
5
10
15
20
25
Time of incubation (min)

XBL 789-4199

the rate of secretion was expressed as percentage of total collagen secreted into the medium. Fig. VI-7 shows that normal and transformed cells secrete collagen at a similar rate.

This finding is also supported by the observation that the intracellular collagen pools are decreased in size in the same proportion to the decreases in collagen biosynthesis (2-2.5-fold, Figs. VI-2 and VI-6).

Discussion

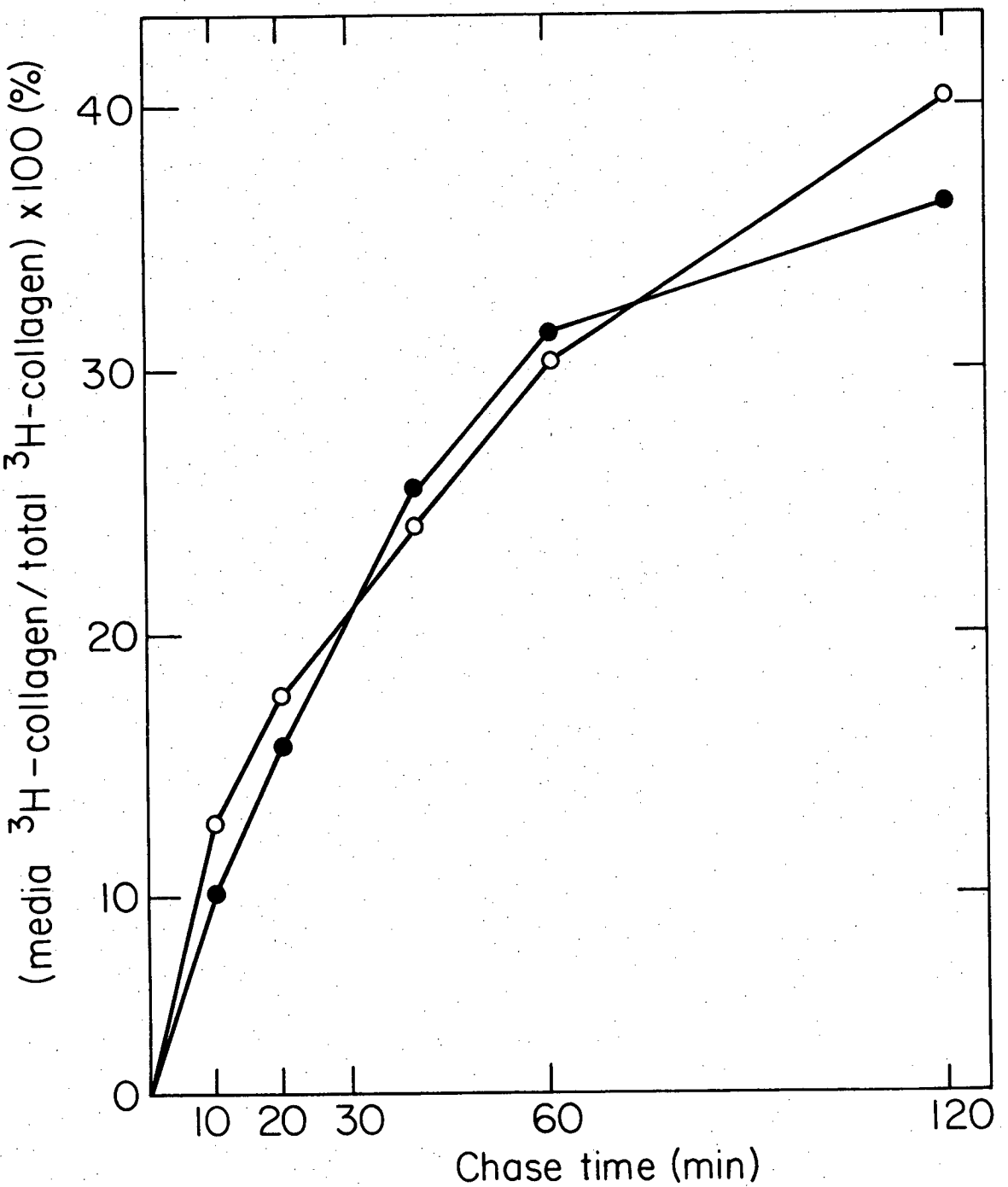
Studies in this chapter indicate that the "dedifferentiation" in transformed tendon cells in terms of collagen synthesis is caused by two mechanisms. First, the rate of collagen synthesis is decreased 2-2.5-fold. Second, the synthesis of other non-collagen protein is increased 3-fold. Although it is not clear how these two mechanisms are related to each other, the results suggest that both are correlated with the switch of tendon cells from a highly differentiated state to a "dedifferentiated" one after transformation. Thus not only the absolute level of collagen synthesis but also the relative expression of collagen to total protein should be used for monitoring the state of differentiation since the latter covers both types of changes and therefore serves as a better indicator for the differentiated state of tendon cells.

It is interesting that the intracellular pool of proline needs a longer time to reach a constant specific activity than that of leucine after the addition of these radioactive amino acids to the medium. This could be due to either a much larger intracellular pool size of proline or the compartmentation of this amino acid inside the cell (Airhart, Vidrich and Khairallah, 1974). In any event, when the linear region of

FIGURE VI-7. Secretion of collagen by normal and transformed tendon cells. Cells were plated, infected, and maintained as described in Fig. VI-1. On the 7th day postinfection, each plate was pulsed with 50 uCi of ^{3}H-proline for 3 hr and the label was chased by replacing the medium with 5 ml of fresh F12 with 0.2% fetal calf serum and 50 ug/ml ascorbate. After chase times indicated, the medium was separated from cells and analysis of collagen of cells and medium was carried out as described in Materials and Methods. Total collagen represents the sum of values for the cell and medium fractions.

o, normal cells.

●, transformed cells.


(media ^{3}H-collagen/ total ^{3}H-collagen) x 100 (%)
0
10
20
30
40
10
20
30
60
120
Chase time (min)

XBL 789 - 4207

the kinetic curve for the incorporation of either amino acid was used for calculation, the same extent of reduction in collagen synthesis was observed in transformed cells. It was noteworthy, however, that when total protein synthesis was compared between normal and transformed cells, the use of leucine gave a 2.5-fold increase after transformation while the use of proline gave only 1.7-fold increase (Fig. VI-1). Although this difference can be explained by the fact that collagen contains 10 times more proline than leucine (21% vs. 2%), and represents 20-25% of total proteins of normal cells but only 2-5% of these of transformed cells, the results suggest that special consideration should be made for the choice of a particular amino acid as the radioactive precursor for the measurement of protein synthesis in a system in which the synthesis of one protein constitutes a high proportion of that of total protein.

The finding that the synthesis of both pro-α_1 and pro-α_2 chains was reduced to the same extent after transformation suggested a coordinate effect on the synthesis of these collagen subunits by transformation. It is surprising, however, that the ratio of intracellular pool of pro-α_1 and pro-α_2 is about 1.3 for both normal and transformed cells using either proline (Fig. VI-4) or leucine (Fig. VI-6) as the radioactive precursor. Since the ratio of α_1 to α_2 in the medium of normal and transfomred cells is, respectively, 2.3 and 2.5, it seems that the synthesis of pro-α_1 and pro-α_2 might be regulated independently. It is possible that this difference in ratios reflects a differential feedback control on either the transcription or the translation of these collagen subunits. A more thorough analysis of this problem therefore requires the measurement

of the levels as well as the translation rates of the mRNA's of these pro-α chains.

When radioactive proline was allowed to incorporate into collagen for more than 2 hr, non-linearity of incorporation was observed for transformed but not normal cells. This non-linearity could be due to either a depletion of proline pool or the degradation of newly synthesized collagen in transformed cells. The latter possibility will be examined in more detail in the following chapter.

CHAPTER VII MECHANISM OF THE DECREASE IN THE LEVEL OF COLLAGEN IN CHICK TENDON CELLS AFTER TRANSFORMATION. II. DEGRADATION

Introduction

Collagenolytic activity has been found to be associated with a certain number of invasive neoplastic tissues (Dresden, Hellman and Schmidt, 1972). These tissues can be of either epithelial or mesenchymal origin (Taylor, Levy and Simpson, 1970). The collagenase activity can be demonstrated in primary cultures (Dabbous, Roberts and Brinkley, 1977) or the homogenates of these tumors (Harris, Faulkner and Wood, 1972) and is postulated to facilitate the tumor invasion by degrading the connective tissue structure of the surrounding normal tissue (Hashimoto et al,m k973). The biochemical properties and the mechanism of action of the collagenase synthesis by tumors in vivo have been well characterized (McCroskery, Richards and Harris, 1975), and the enzyme probably has the same substrate specificity as those synthesized by normal tissues such as skin (Eisen, Jeffrey and Gross, 1968) during tissue remodeling (Eisen, Bauer and Jeffrey, 1970) and wound healing (Donoff, McLennan and Grillo, 1971).

Although the tissue culture or homogenate can provide enough quantity of collagenase for biochemical analysis, neither source affords a model system for the study of the effect of transformation on the synthesis and release of this enzyme at the cellular level. Such a system would require a well-characterized cell culture in which the production of collagenase by cells before and after transformation in culture can be studied. Recently, both bovine and rabbit fibroblasts (Birkedal-Hansen

et al., 1976; Werb and Burleigh, 1974) as well as rat bone cells (Puzas and Brand, 1976) have been reported to produce collagenase in culture. However, no attempt has yet been made to examine the effect of transformation on these cells.

In the previous chapter, there were some evidence suggesting that transformation of tendon cells might increase the rate of collagen degradation in these cells. The increase catabolism of collagen could be due to the induction of some collagenolytic activity in transformed cells. If this were the case, trasnformation of tendon cells in culture would offer a system in which the regulation of the synthesis of collagen could be studied at the cellular or even molecular level. This chapter studies the degradation of collagen in normal and transformed tendon cells and describes the initial characterization of a collagenolytic activity released into the culture medium by the transformed cells.

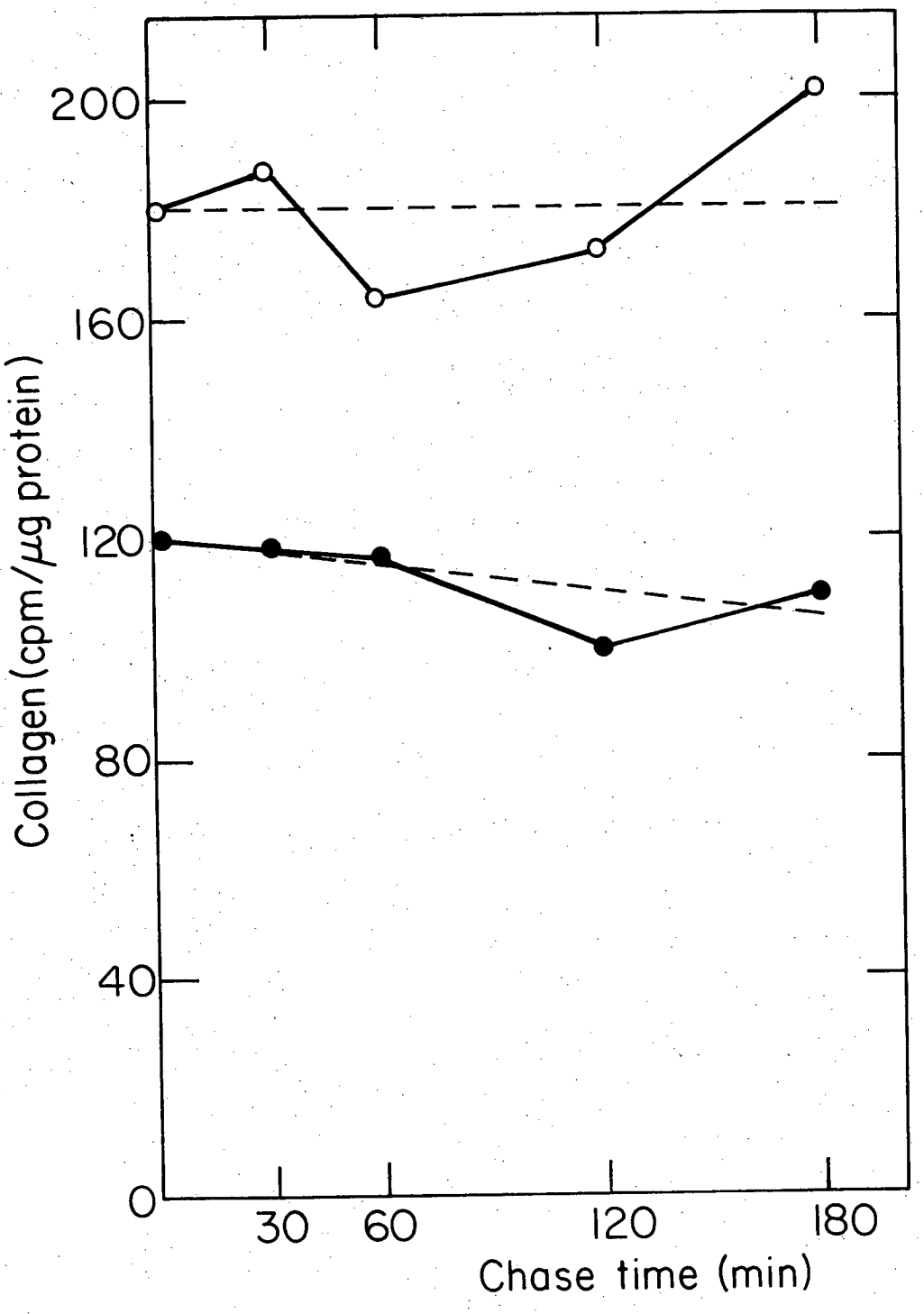
Degradation of Collagen in Normal and Transformed Tendon Cell Cultures

As shown in the last chapter, the incorporation of radioactive proline into collagen of transformed but not normal cells became nonlinear and leveled off between 2 and 3 hrs after the addition of the amino acid. This could be the result of the degradation of the newly synthesized collagen in the transformed culture. To test this possibility, the degradation of collagen in cultures of normal tendon cells as well as cells transformed by a wild-type Prague Rous sarcoma virus was examined. Each plate of normal or transformed cells was prelabeled with 100 uCi of ^{3}H-proline for 24 hrs and then replaced in chase medium containing only unlabeled proline. After the designated periods of time, the amount of collagen in cells and medium combined was analyzed as described in Materials and Methods. Fig. VII-1 shows the results of this

FIGURE VII-1 Degradation of collagen in normal and transformed tendon cell cultures. Cells were plated at a density of 8×10^5 cells per 60 mm plate. After they had attached, half of the plates were infected with a wild-type Prague A strain of Rous sarcoma virus in 1.5 mls of F12 with 0.2% fetal calf serum and the other half were mock-infected. The cells were incubated for another hour before an additional 9 mls of medium were added. Cells were grown in F12 with 0.2% serum and 50 ug/ml ascorbate and the medium was changed daily. On the 7th day postinfection, each plate was pulsed with 100 uCi of ^{3}H-proline for 3 hrs and the label was chased by replacing the medium with 1.5 mls of fresh F12 with 50 ug/ml ascorbate. After chase time indicated, the amount of radioactive collagen in the chase mixture was analyzed as described in Materials and Methods.

o, normal cells.

●, transformed cells.


200
160
120
80
40
0
Collagen (cpm/μg protein)
30
60
120
180
Chase time (min)

XBL789-4200

pulse-chase experiment. Although the large fluctuation of measurements made the conclusion somewhat tentative, it appeared that normal cells did not elaborate any collagenolytic activity during the period of chase, whereas transformed cells degraded up to 10% of the labeled collagen during the same period of time.

At temperatures above 30°, collagen can also be digested by neutral proteases (Evanson, Jeffrey and Krane, 1968). Since transformed cells have been found to produce proteolytic enzymes (Inkeless et al., 1974), the degradation of collagen could be due to the presence of these non-specific proteases. This possibility, however, was ruled out when the degradation of non-collagen protein in normal and transformed cultures was examined. As shown in Fig. VII-2, non-collagen protein was degraded to a much smaller extent than that of collagen in the transformed culture during the same period of chase. The collagenolytic activity in the transformed culture therefore may indicate the presence of a specific collagenase.

Characterization of the Collagenolytic Activity in the Culture Medium from Transformed Cells

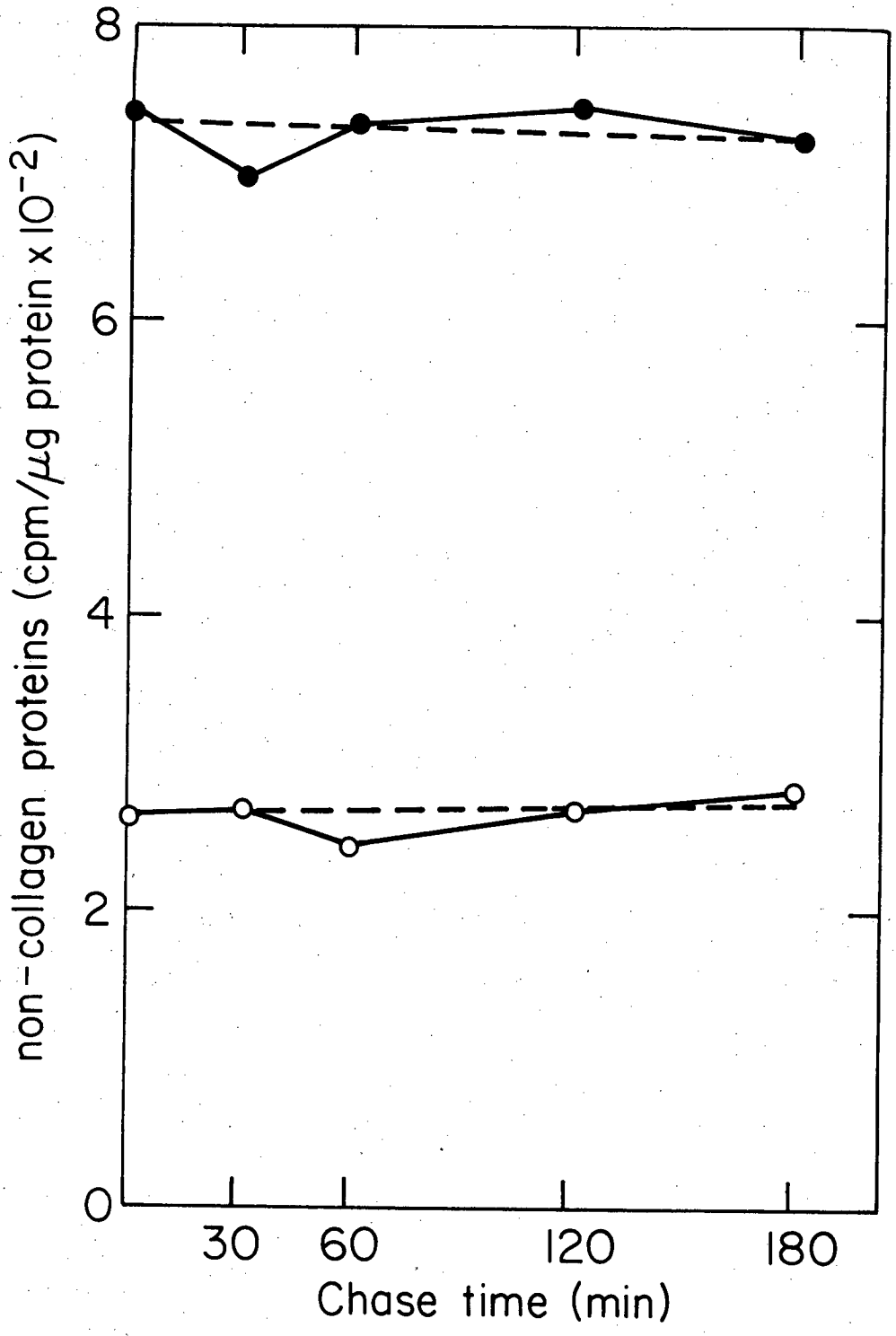
To further determine if collagen degradation took place inside the cell or in the medium, cell layer and medium were separated and tested for collagenolytic activity independently. Since the extracellular collagen molecules could be either soluble as tropomolecules or insoluble as fibrils, both forms of collagen were used as substrates for the tests.

The medium fraction was first tested. Media from normal and transformed cultures were dialyzed against collagenase buffer and tested for collagenolytic activity either in the unconcentrated form or after partial purification by precipitation with ammonium sulfate at a

FIGURE VII-2. Degradation of non-collagen proteins in normal and transformed tendon cell cultures. The results for this figure were obtained from the same experiment as those for Fig. VII-1. After the 3 hr pulse and chase time indicated, the amount of ^{3}H-non-collagen proteins in the chase mixture was determined as described in Materials and Methods.

o, normal cells.

●, transformed cells.


8
6
4
2
0
non-collagen proteins (cpm/μg protein x 10⁻²)
30
60
120
180
Chase time (min)

XBL789-4201

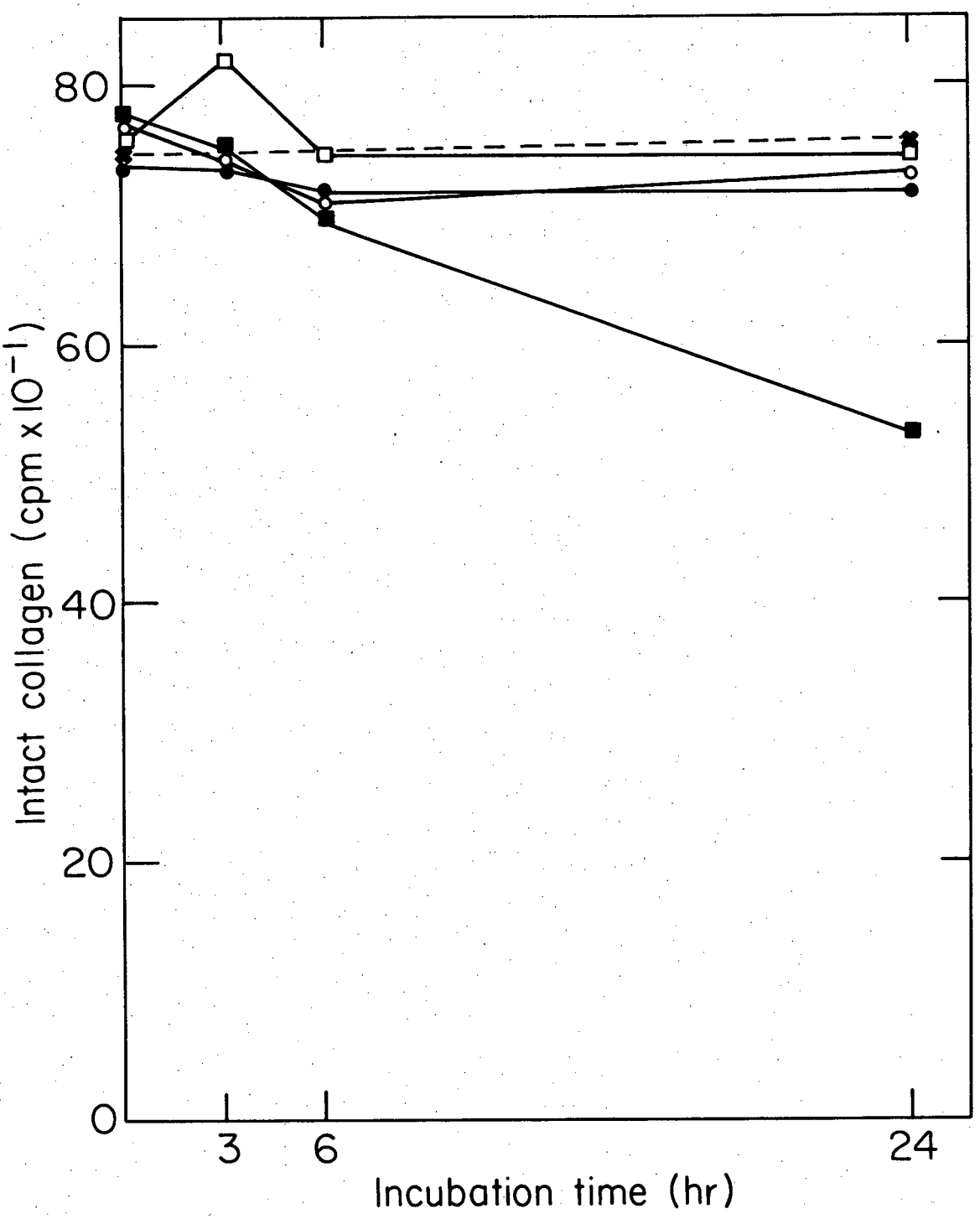
concentration (20%-60%) which normally precipitated all known animal collagenases. Either form of collagen was then added to the testing medium and the mixture incubated for designated periods of time and then analyzed for any degradation of collagen. Figs. VII-3 and VII-4 show that collagenolytic activity could not be detected in the unconcentrated medium for either normal or transformed cultures. The degradation of both forms of collagen by the partially purified medium from transformed culture, however, was significant when the mixture was incubated for more than 3 hrs. This degradation was not observed with partially purified medium from normal culture.

Cells homogenates of normal and transformed cells were also tested for the presence of collagenolytic activity. As shown in Table VII-1, neither the unconcentrated nor the partially purified cell homogenates of normal or transformed cells was found to be associated with significant amount of collagenolytic activity.

Discussion

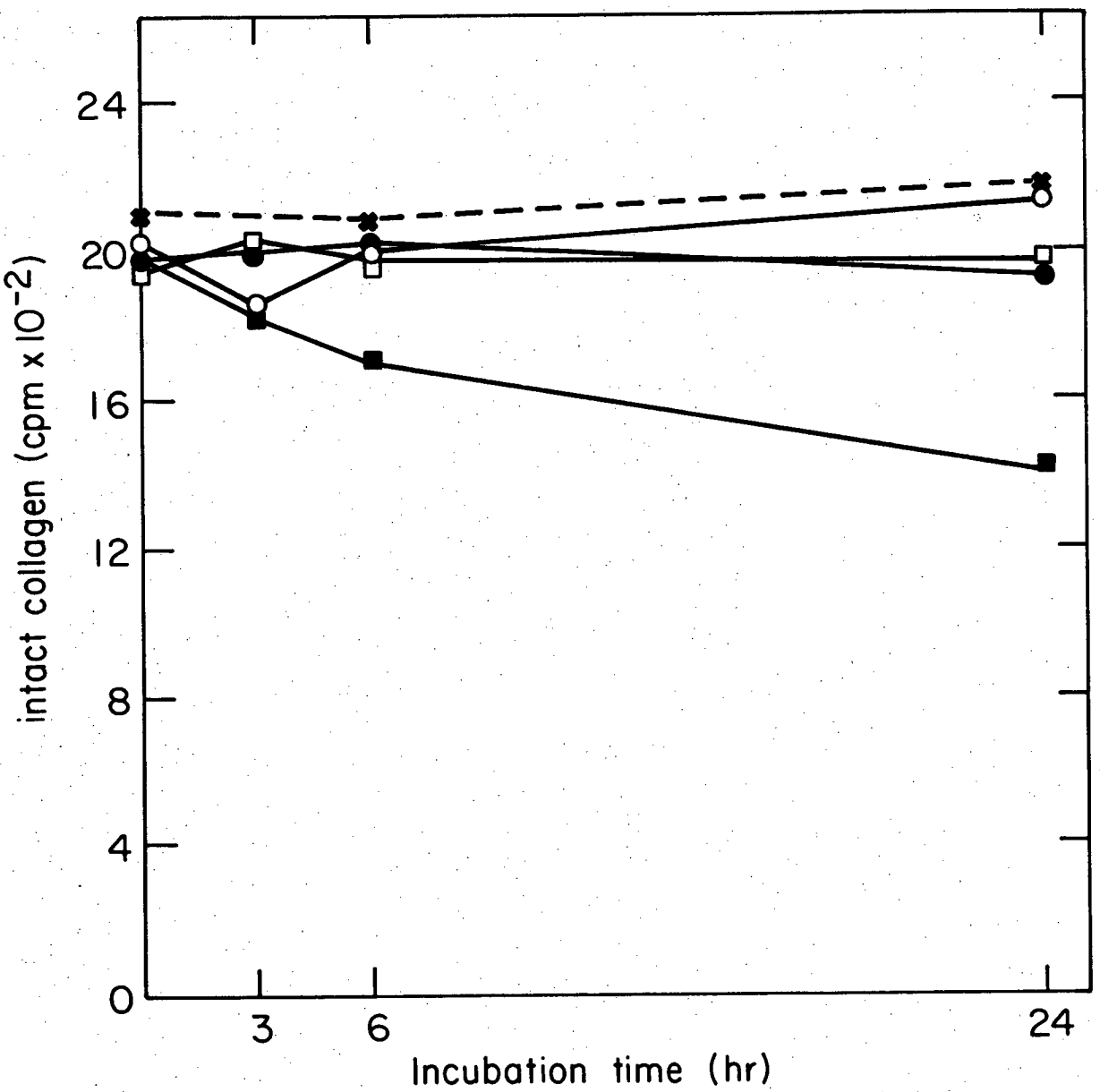
When a specific collagenase was first found in homogenates of tumors in vivo, it was hypothesized that the collagenolytic enzyme was membrane-bound and could confer upon tumor cells a distinct degree of mobility or invasiveness (Harris, Faulkner and Wood, 1972). Later studies with cultures of tumor explants (McCroskery, Richards and Harris, 1975; Dabbous, Roberts and Brinkley, 1977), however, showed that most of the enzymes were actually released into the medium by the tumor tissues in culture. Although it is still not clear how and when the enzyme becomes active during the process of secretion, it is possible that the main function of collagenase is to digest extracellular collagen fibrillar matrix not necessarily in contact with the cells. The results

FIGURE VII-3. Soluble collagen assay for collagenolytic activity in normal and transformed culture media. Cells were plated, infected, and maintained as described in Fig. VII-1, and switched to serum-free medium on the 6th day after plating. 24 hrs later, culture media were collected, partially purified, and dialyzed against collagenase buffer (0.1 M Tris/0.5 M NaCl.37.5 mM $CaCl_2$, pH 8.0). For each assaying tube, the reaction mixture contained 200 ul dialysed medium to which were added ^{3}H-collagen purified from chick calvaria and 20 ug Bovine serum albumin. Control tubes contained collagenase buffer instead of medium. After designated time, the assay was stopped and the remaining collagen in each tube was determined as described in Materials and Methods.
Symbols: open, normal cells; closed, transformed cells; o, unconcentrated media; □, partially purified media; **x**, control tubes.


80
60
40
20
0
Intact collagen (cpm x 10⁻¹)
3
6
24
Incubation time (hr)

XBL789-4210

FIGURE VII-4. Insoluble collagen assay for collagenolytic activity in normal and transformed culture media. Unconcentrated and partially purified media were prepared as described in Fig. VII-3. The insoluble collagen assay contained essentially the same reaction mixture as the soluble collagen assay except that the collagen used was non-cross-linked and was dried to a thin film for the assay. Symbols: open, normal cells; closed, transformed cells; o, unconcentrated media; □, partially purified media; **x**, control tubes.


24
20
16
12
8
4
0
intact collagen (cpm x 10⁻²)
3
6
24
Incubation time (hr)

XBL789-4209

TABLE VII-1 Radiocollagen assays for collagenolytic activity in normal and transformed cell homogenates[a]

Type of assay	Soluble collagen assay[b]		Insoluble collagen assay[b]	
	Amount of collagen (cpm)		Amount of collagen (cpm)	
	at "0" time	after 24 hr incubation	at "0" time	After 24 hr incubation
Normal cell				
homogenate	2038	2113	814	780
ammonium sulfate precipitate	2245	2178	733	935
Transformed cell				
homogenate	2143	2097	780	859
ammonium sulfate precipitate	2089	2190	885	756

[a]Cells were plated, infected, and maintained as described in Figure VII-1, and switched to serum-free medium on the 6th day after plating. 24 hrs later, culture media were separated, and the cell layer scraped off, homogenized, dialyzed, and partially purified as described in Materials and Methods. Soluble and insoluble collagen assays were carried out as described in Figure VII-3 and Figure VII-4 except that dialyzed cell homogenates were used.

[b]Average of duplicates.

in this chapter demonstrated for the first time that transfomred cells in culture could synthesize a specific collagenolytic enzyme. In addition, this enzyme was probably also a secreted one since most of the activities were found in the medium. Preliminary characterization of this collagenase showed that it might have the same substrate specificity as other collagenases purified from cultures of normal and tumor tissues.

It is not known if the process of transformation actually induces the synthesis and release of collagenase or merely activates an inactive form of this enzyme. Normal fibroblast cell lines such as 3T3 cells have been reported to release little collagenolytic activity (Werb and Burleigh, 1974). On the other hand, there is mounting evidence in some culture systems of normal cells for the existence of inactive precursors of collagenase (Harper, Bloch and Gross, 1971; Oronsky, Perper and Schroder, 1973; Werb et al., 1977). Moreover, it has been shown that the zymogen of collagenase could be activated by proteolytic enzymes (Birkedal-Hansen et al., 1976). It is possible that normal tendon cells also synthesize an inactive form of collagenase which is activated after transformation by non-specific proteases synthesized by transformed cells. The most unequivocal way of testing this possibility would be to raise antibodies against purified collagenase from transformed culture medium and look for immuno-cross-reacting protein in the normal culture counterpart.

Bornstein and Ash (1977) recently suggested that extracellular collagen fibrils might be involved in the formation of an external protein meshwork responsible for cell attachment and the flattened morphology of chick embryo fibroblasts. Since tendon cells synthesize collagen at a higher level and are even more "flattened" than those fibroblasts,

it is possible that the same type of collagen meshwork is also present in this cell type. If this is the case, the reduced level of collagen synthesis after transformation could be intimately related to the change in cellular motility and morphology. In addition, the synthesis and release of a specific collagenase by transformed cells could further reduce the level of collagen already associated with cell membrane or extracellular matrix and therefore enable these cells to completely sever themselves from their previous ties with the substratum. Studies with antibodies against collagen as well as collagenase should help elucidate the mechansims of this destructive process.

Since transfomred tendon cells also synthesize a low level of collagen, the simultaneous synthesis and breakdown of collagen by these cells raise an interesting question of how these apparently opposing processes are regulated. It could be speculated that the decrease of collagen synthesis and the appearance of collagenolytic activity might be under a coordinated control to reduce the level of collagen in transformed cells as well as in their milieu. Alternatively, the regulation of the production of collagenase could be totally independent from that of collagen synthesis since cells not capable of synthesizing collagen can still be induced to produce collagenase. Fruther study on the balance between these two processes under different conditions is needed to distinguish between the alternatives.

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APPENDIX I

Effect of 2-deoxy-D-glucose on Cellular Metabolism and Virus Maturation

The word described in this appendix was carried out during the first two years of my graduate study. While this work and the major work of my thesis were not strictly related, they were related in the sense that both dealt with virus-cell interactions. The knowledge that I gained and the techniques that I learned from this project about cell culture, virus, viral infection, and transformation thus provided a useful background for my subsequent study with the transformation of check tendon cells. This project also helped me to go through the transition from a pure precision-seeking chemist who was initially both doubtful and depressed about the messiness of the cell culture studies to an open-minded biochemist who appreciated the beauty as well as the limits of cell biology. I was much more prepared scientifically and relaxed psychologically when I later began to work on tendon cell culture.

Part of this work was published in the form of an abstract in the publication of the 61st annual meeting of the American Society of Biological Chemists (Federation Proceedings 36, 741, 1977).

ABSTRACT

Effect of 2-deoxy-D-glucose on Cellular Metabolism and Virus Maturation

Whai-Jen Soo

The effect of 2-deoxy-D-glucose on the cellular metabolism of chick embryo fibroblasts as well as on the replication of Rous sarcoma virus in infected chick cells was studied in this project. A possible relationship between host cellular metabolism and virus replication was also explored.

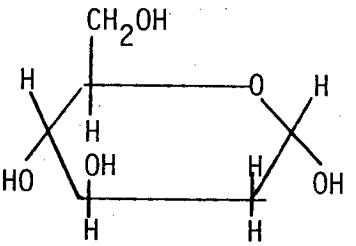
2-deoxy-D-glucose affected cellular glucose metabolism first by inhibiting the uptake and phosphorylation of glucose. Inhibition of these two steps led to an immediate drop in the level of glucose-6-phosphate which in turn limited glycolysis. The phosphorylation of 2-deoxy-D-glucose into 2-deoxy-D-glucose-6-phosphate further interferred with the conversion of glucose-6-phosphate to fructose-6-phosphate and reduced the rate of glycolysis. The phosphorylation step itself also depleted the pool size of some important energy metabolites inside the cell and introduced a serious stess to the cell. These effects together caused most of the cellular machinery to stumble. In response to this stress, however, the cells were able to operate a partial TCA cycle and managed to recover glycolysis to a certain extent.

The replication of Rous sarcoma virus was affected by 2-deoxy-D-glucose by perhaps two different mechanisms. The inhibition of glycolysis and the depletion of energy source led to a reduction in the cellular macromolecular synthesis of the host cell. Consequently, the corresponding viral RNA and protein synthesis were also reduced and the production of virions inhibited. In addition, 2-deoxy-D-glucose may have interfered with the normal chain elongation of the carbohydrate moiety of the

viral glycoprotein by possibly being incorporated into the oligosaccharide side chains as a substitute for mannose. This process appears to further render most of the released viruses non-infectious.

CHAPTER 1 INTRODUCTION

2-deoxy-D-glucose differs from glucose in the substitution of the 2-OH group by a hydrogen atom and may be represented in the pyranose form as



It was first examined by Cramer and Woodward (1952) for its ability to interfere with glycolysis and to inhibit the growth of malignant tumors on the assumption that, as an analogue, 2-deoxy-D-glucose might specifically block the glycolytic pathway and also reduce the respiration of the neoplastic tissue through the Crabtree Effect (Crabtree, 1929). The fact that this sugar analogue did produce some regression of Walker carcinoma had raised the possibility that it might be useful in the chemotherapy of certain neoplasms. Later studies, however, found that 2-deoxy-D-glucose was also toxic to many normal tissues so that its use as a carcinostatic agent was greatly limited. This sugar analogue, nevertheless, was found to be a valuable tool in biochemical investigations and has been extremely useful in helping to elucidate the regulatory mechanisms of glycolysis, respiration, energy metabolism and their relationships inside the cell. Furthermore, recent studies have suggested that this sugar analogue can also be used to examine the role of carbohydrates in the synthesis and processing of glycoproteins and in their biological functions.

Uptake and Metabolism of 2-deoxy-D-glucose

I will discuss the uptake and the fate of 2-deoxy-D-glucose in cells before turning to the metabolic disturbance produced. 2-deoxy-D-glucose enters the cell by the same transport system as that utilized by glucose (Kletzien and Perdue, 1974; Renner, Plagemann and Bernlohr, 1972). At lower concentrations, it is mainly taken up by facilitated diffusion; and at higher concentrations, by simple diffusion (Dolberg, Bassham and Bissell, 1975). The Km for 2-deoxy-D-glucose uptake is only slightly higher than that for glucose, so at equal concentrations, this analogue can inhibit the uptake of glucose effectively.

Early studies by Kipnis and Cori (1959) and by Barban and Schulze (1961) showed that 2-deoxy-D-glucose could be readily phosphorylated to 2-deoxy-D-gluclse-6-phosphate in animal cells. For a while, it was believed that the formation of 2-deoxy-D-glucose was the end of 2-deoxy-D-glucose metabolism (Hatanaka and Hanafusa, 1970; Plagemann, 1973). Recent study by Schmidt, Schwarz and Scholtissek (1974), however, showed that other derivatives of 2-deoxy-D-glucose could also be found in many types of animal cells although in much smaller quantities. These derivatives include 2-deoxy-D-glucose-1-phosphate, 2-deoxy-D-glucose-1,6-diphosphate, UDP-2-deoxy-D-glucose, GDP-2-deoxy-D-glucose and 2-deoxy-D-gluconic acid-6-phosphate. The formation of GDP-2-deoxy-D-glucose indicates that 2-deoxy-D-glucose can also act as an analogue for mannose since mannose but not glucose is activated to GDP derivative. 2-deoxy-D-glucose was also found to be incorporated in the unaltered form into the carbohydrate moiety of glycoproteins and glycolipids of animal cells (Steiner, Courtney and Melnick, 1973; Steiner, Somers and Steiner, 1974).

Many derivatives of 2-deoxy-D-glucose can be found inside the cell, nevertheless, 2-deoxy-D-glucose is considered to be an unmetabolizable substrate in the sense that it cannot be used as an energy source by the cell. In fact, many of its derivatives are inhibitors of many important cellular processes.

Effects of 2-deoxy-D-glucose on Cellular Metabolism and Respiration

2-deoxy-D-glucose has been shown to inhibit glycolysis in many types of cells (Webb, 1966). The free sugar analogue competes with glucose for the same transport and phosphorylation system and the phosphorylated product, 2-deoxy-D-glucose-6-phosphate, has been shown to inhibit the activity of the phosphoglucose isomerase (Wick et al. 1957; Nirenberg and Hogg, 1958). The inhibition of this enzyme in turn leads to an accumulation of glucose-6-phosphate which might further inhibit the hexokinase activity inside the cell (Wick et al. 1957). All these effects together then severely limit the input of glucose for metabolism and greatly reduce the rate of glycolysis.

Ibsen, Coe and McKee (1960) first reported that 2-deoxy-D-glucose inhibited the respiration of Ehrlich ascites carcinoma cells. Based on the observation that this analogue inhibits respiration to about the same degree as glucose, these authors concluded that the two acted by the same mechanism, a phenomenon often called the Crabtree, or reversed Pasteur, effect (Crabtree, 1929). Although it has been stated that an acceleration of glycolysis inhibits the oxidation of pyruvate, there is no evidence to link the entire glycolytic pathway with respiratory control. That 2-deoxy-D-glucose inhibits glycolysis but nevertheless reduces the rate of respiration in fact suggests that glycolysis is not necessary for the Crabtree effect (Ram et al. 1963). Based on measurements

of the capacity for ATP synthesis by respiration and glycolysis, Yushok (1974) concluded that the maintenance of a uniform rate of ATP synthesis might be the basis of Crabtree effect. Since 2-deoxy-D-glucose is an ATP-depleting agent while glucose is an ATP-producing agent, it is hard to explain how they could alter ATP synthesis in the same manner. It seems more likely that the initial reaction necessary for respiratory inhibition is the phosphorylation by hexose kinase since the rate of respiratory inhibition by different sugars is correlated with the rate of phosphorylation (Webb, 1966). It is not known, however, how the phosphorylation step can affect the rate of respiration especially with regard to the report by Detwiler (1971) that only glucose but not 2-deoxy-D-glucose inhibited the respiration of platelets. One has to await the elucidation of this interesting effect.

One unique effect of 2-deoxy-D-glucose on cellular metabolism is the induced degradation of adenylates (McComb and Yushok, 1964). Due to the phosphorylation of this analogue, most of the ATP inside the cell is converted to ADP and AMP. These adenylates are further dephosphorylated and deaminated to form IMP and inosine so the total amount of adenine nucleotides is greatly reduced. It has been suggested that the degradation of these adenylates would allow the cell to utilize the acid-stable as well as the acid-labile phosphorus of the degraded molecules for the synthesis of high-energy compounds, especially ATP. However, there is no strong evidence that phosphate is indeed the pacemaker for ATP synthesis and further study is needed to clarify this point.

Effects of 2-deoxy-D-glucose on Synthesis, Secretion and Glycosylation of Glycoproteins

The effect of 2-deoxy-D-Glucose on the synthesis of carbohydrate-protein complex was first noticed by Farkas, Svoboda and Bauer (1970)

who found that 2-deoxy-D-glucose inhibited the regeneration of yeast protoplasts by blocking the synthesis of the mannan-protein matrix. The action of 2-deoxy-D-glucose was considered to be primarily the interference with the synthesis or secretion of mannan, and the selective blocking of mannan synthesis further prevents the appearance of the protein counterpart. Further study following the synthesis and secretion of glycoproteins by the yeast protoplasts in the presence of 2-deoxy-D-glucose suggested that the inhibition of the carbohydrate synthesis could cause both an inhibition of the secretion of the glycoproteins and a reduced synthesis of the protein backbone of these molecules (Liras and Gascon, 1971). In the animal cells, 2-deoxy-D-glucose was found to inhibit the synthesis and secretion of several types of glycoproteins including immunoglobulin (Melchers, 1973), interferon (Havell et al. 1975), and collagen (Blumenkrantz, Rosenbloom and Prockop, 1969; Kruse and Bornstein, 1975). Studies on the intracellular processing of these glycoproteins showed that 2-deoxy-D-glucose could inhibit not only the glycosylation but also the intracellular movement of the unglycosylated precursor of these molecules from membrane-bound polyribosomes into endoplasmic reticulum. It was suggested that the inhibition of the glycosylation was the cause for the block of the intracellular transport and the process of glycosylation might provide the hydrophilic driving force for the intracellular movement of the glycoproteins (Melchers, 1973). It is not clear, however, how general this mechanism might be, and this view has been challenged by Kruse and Bornstein (1975) who suggested that 2-deoxy-D-glucose inhibited the transcellular movement of collagen simply by its interference with the energy metabolism.

The mechanism by which 2-deoxy-D-glucose inhibits glycosylation was

first suggested by Biely and Bauer (1968) that 2-deoxy-D-glucose could be incorporated into the carbohydrate moiety of a glycoprotein or protein-polysaccharide complex as an analogue for mannose and terminate further chain elongation. This view is strongly supported by the findings that GDP-2-deoxy-D-glucose can be formed inside the cell and that 2-deoxy-D-glucose can be incorporated into the carbohydrate moiety of the glycoproteins in animal cells (Schmidt, Schwarz and Scholtissek, 1974; Steiner, Courtney and Melnick, 1973). 2-deoxy-D-glucose, however, could potentially also inhibit the conversion of glucose to most of the sugars inside the cell. This could reduce the pool size of these sugars and decrease their incorporation into the glycoprotein. This hypothesis has not been tested.

The effect of 2-deoxy-D-glucose on the synthesis of membrane glcoproteins has never been studied. It is likely that the glycosylation of these glycoproteins could also be inhibited by 2-deoxy-D-glucose and the structural integrity of the membrane might be impaired. Amos et al. (1976) reported that drastic morphological changes of L cells were observed when glucose-containing media were supplemented with "secondary" sugars. It would be interesting to see if 2-deoxy-D-glucose could cause any major structural change in membrane.

Effects of 2-deoxy-D-glucose on Virus Replication

A virus is a simple assemblage of nucleic acids, proteins, and sometimes lipids. It is not capable of converting food sources into energy through any metabolic pathway. When a virus replicates inside the cell, it takes advantage of the available cellular energy source and macromolecule-synthetic machinery to produce its own viral components. Based on this hypothesis, it might be reasonable to assume that the replication

of the virus should be affected to the same extent as the cellular metabolism when the latter was perturbed by a metabolic inhibitor. This was not the case, however, when the effect of 2-deoxy-D-glucose on the replication of some viruses was examined. Kaluza, Scholtissek and Rott (1973) found that 2-deoxy-D-glucose inhibited the replication of Semliki Forest virus to a much greater extent than would be expected by its interference with the cellular energy supply alone. Later studies (Courtney, Steiner and Benyesh-Meonick, 1973; Schnitzer, et al. 1975; Scholtissek, et al. 1974) showed that the replication of many other cytopathic enveloped viruses was inhibited by 2-deoxy-D-glucose in a similar way. Although the exact mechanism by which 2-deoxy-D-glucose inhibits the replication of these viruses is still unknown, it has been suggested that, by acting also as an analogue for mannose, this sugar interferes with the glycosylation of the viral glycoproteins thus inhibiting the proper assembly of the viral components and the release of viral particles.

While this mechanism accounted reasonably for the effect of 2-deoxy-D-glucose on the replication of cytopathic viruses, it could not explain how the replication of tumor virus was affected by this sugar. Prochownik, Panem and Kirsten (1975) showed that even at high concentration of 2-deoxy-D-glucose when the RNA and protein synthesis in host cells was strongly inhibited, the replication of murine sarcoma-leukemia virus in rat kidney cells was not affected, but the released virus was no longer infectious. These authors suggested that the interference with glycosylation by 2-deoxy-D-glucose, in this case, might only render the virus non-infectious but did not inhibit the assembly and release of the virus. The authors, however, did not explain how the

production of viral RNA and proteins was not affected even though the cellular RNA and protein synthesis were strongly inhibited.

Since the replication cycle and the host-virus relationship are different for cytopathic and tumor viruses, it is possible that 2-deoxy-D-glucose acts differently on the replication of these viruses. On the other hand, it is not known if the observation made with murine sarcoma-leukemia virus actually represents a general case for tumor virus, given the fact that murine viruses are complex and defective and need more than one component to replicate. In addition, murine system is usually unstable and prone to have secondary alterations. It is therefore important to study the effect of 2-deoxy-D-glucose on the replication of a better-characterized tumor virus in a stable cell system.

The main purpose of this project is to study the different effects of 2-deoxy-D-glucose on cellular metabolism of chick embryo fibroblasts and on the replication of Rous sarcoma virus in these cells.

CHAPTER 2 MATERIALS AND METHODS

Chemicals and Isotopes

2-deoxy-D-glucose (Calbiochem., San Diego CA) and glucosamine (Sigma Chemical Co., St. Louis, Mo.) were prepared fresh immediately before each experiment and were sterilized by passage through a Millipore filter. Uridine-5-^{3}H (specific activity 27Ci/mmole), uridine-2-^{14}C (48Ci/mmole), 2-deoxy-D-glucose-^{3}H (10Ci/mmole), L-fucose-^{3}H (12Ci/mmole), D-galactose-^{3}H (1.643Ci/mmole), D-glucosamine-1-^{14}C (51mCi/mmole), DL-leucine-4,5-^{3}H (60Ci/mmole), DL-leucine-1-^{14}C (51Ci/mmole) and ^{3}H-deoxy-thymidine-5'-triphosphate (18.5Ci/mmole) were purchased from New England Nuclear, Boston, MA. For steady-state experiments, the ^{32}P as $H_3{}^{32}P_4$ carrier free) in H_2O was purchased from New England Nuclear, and the glucose-^{14}C (306Ci/mmole) from Amersham.

Cell Culture and Virus Infection

Primary and secondary cultures of chick embryo cells were prepared as described previously by Rein and Rubin (1968) and modified by Bissell et al. (1973). Ten-day old C/O or C/B type SPF chick embryos were removed from eggs. After decapitation and evisceration, they were minced and washed with tri-saline buffer, then stirred with a magnetic stirrer in 0.25% trypsin (Sigma). At 15-min intervals the suspended single cells were decanted into a "stop bath" containing 2/3 cold medium 199 (Grand Island) and 1.3 calf serum (Microbiological Associates). The procedure was repeated twice. The cells were centrifuged and resuspended in medium 199 containing 2% tryptose phosphate broth, 1% calf serum, and 1% heat inactivated chicken serum (referred to as "2-1-1"). Primary cultures were seeded at 8×10^{6} cells per 100nm Petri dish in 10 ml medium. The cultures were incubated at 38-39° C in an atmosphere of 5% CO_2 in air to maintain pH of

7.3-7.4. The medium was changed to fresh 199 (2-1-1) on the third day. For studies with transformed cultures, half the cells of a single embryo were infected 4 hr after primary seeding with Prague C strain of Rous sarcoma virus at the multiplicity of 0.1. Secondary cultures were prepared 4 days after the primary seeding. Cells were removed from dishes by trypsinization and seeded at either 1 x 10^6 cells per 35mm dish in 2 ml medium or 2 x 10^6 cells per 60mm dish in 5 ml medium. For secondary cultures, an additional 1 mg/ml of glucose was added to the medium bringing the glucose concentration to 11.0 mM. While penicillin and streptomycin were included, Fungizone was eliminated due to harmful side effects (Dolberg and Bissell, 1974).

Focus Assay

Assays were performed essentially as described by Rubin (1960). In brief, four hours after secondary normal cells were seeded at 2.5 x 10^6 cells per 60mm dish, medium was removed and cells were exposed to the appropriate dilution of virus in 1 ml of medium for 1 hr. The liquid medium was then removed and agar medium added. Rous sarcoma foci were counted 7 days later.

Measurement of RNA-dependent DNA Polymerase Activity

The enzyme activity was measured as previously described by Duesberg, Von del Helm and Canaani (1971) and modified by Szabo, Bissell and Calvin (1976). The culture media were centrifuged at 8,000 x g for 20 min to remove cell debris. Virus was pelleted by centrifugation at 25,000 rpm in the Spinco No. 30 rotor for 1.5 hr. The pellets were resuspended in standard buffer (0.01 M Tris, pH 7.4, 0.1 M NaCl, and 0.001 M EDTA). A portion of the resuspended virus preparation was added directly to the standard assay mixture containing 0.05 M Tris (pH 8.0), 0.05 M KCl, 0.006 M

magnesium acetate, 5 mM dithiothreitol, 0.02 mg of template-primer per ml "poly(rA):oligo(dT)", and the appropriate substrate (^{3}H-TTP) at a concentration of 0.02 mM and a specific activity of 1 Ci/mmole. The enzymatic activity was solubilized by the inclusion of 0.2% Triton X-100 in the assay. The assays were incubated at 37°C for 1 hr and then terminated by the addition of 5% trichloroacetic acid containing 0.01 M sodium pyrophosphate. Direct measurement of DNA polymerase activity in the growth medium of infected cells yielded results that were comparable to those obtained from the resuspended pellets. Both methods of measurement were used.

Labeling of Viral Particles

Secondary cultures were incubated for 36 hr after seeding. The medium was aspirated and replaced with 5.0 ml new medium containing 0 or 5 mM 2-deoxy-D-glucose. The cultures were then labeled with either D-galactose-6-^{3}H (5 µCi/ml), L-fucose-^{3}H (5 µCi/ml), or D-glucosamine-1-^{14}C (2 µCi/ml) at 18 hrs after 2-deoxy-D-glcose was added. The medium was collected 18 hrs later and labeled virus particles were analysed. Since it was reported that 2-deoxy-D-glucose interfered with ^{3}H-uridine transport (Prochownick, Panem and Kirsten, 1975), the cultures were labeled with uridine-5-^{3}H (10 µCi/ml) 8 hrs before the sugar was added. The cultures were then continuously labeled with uridine until the medium was collected. Uridine-2-^{14}C (1 µCi/ml) was also used for labeling occasionally. For cultures labeled with ^{3}H-deoxy-D-glucose, the cells were grown in a medium in which glucose was replaced by 5 mM pyruvate, and the isotope was added to a final concentration of 5 µCi/ml.

Virus Purification

Media were clarified by centrifugation at 8.000 x g for 20 min. Virus was pelleted from the supernatant by centrifugation for 90 min at 25,000 rpm in the Spinco No. 30 rotor. Virus was then resuspended in the standard buffer and was centrifuged to equilibrium on a linear 20 to 55% sucrose gradient for 3 hr at 45,000 rpm in a Spinco SW 50.1 rotor. To determine radioactivity incorporated, the gradients were fractionated, and labeled virus was either precipated with trichloroacetic acid, collected on membrane filters (Millipore Corp.) and subsequently counted in toluene-bases fluid or alternatively aliquots of the gradient were counted directly in Aquasol using a Packard TriCarb liquid scintillation counter.

Steady-state Analysis and Tracer Studies

Thirty-two hours after secondary seeding the medium of secondary cell cultures was replaced by one which contained 5.5 mM ^{14}C-glucose (56 µCi/ml, specific activity 10.2 Ci/mole), 1.25 mM inorganic ^{32}P-phosphate (1.25 µCi/ml, specific activity 1 Ci/mole), and the cells were allowed to metaoblize for at least 15 hrs under the usual growth conditions (tissue culture incubators, 5% CO_2 in air, 39°C). The cells were then transferred to a steady-state apparatus as described by Bassham, Bissell and White (1974). One hour after the medium change, 20 mM 2-deoxy-D-glucose was added to half of the cultures to perturb the steady state. Each experiment included a series of kinetic points to follow the levels of metabolite pools after the perturbation.

Immediately before the cells were killed, the medium was removed and the culture was rapidly washed with cold Hank's buffer containing unlabeled glucose. The cells were killed by the addition of cold 80%

methanol (less than 15 sec after removal of medium). The dead cells were then scraped from the dish with a rubber policeman and disrupted by sonic oscillation (Bissell et al., 1973), after which an aliquot portion was applied to filter paper for analysis by two-dimensional paper chromatography as described by Pedersen, Kirk, and Bassham (1965). The samples were first developed with phenol-water acetic acid (84:16:1) for either 24 or 48 hr. After the paper was dried, it was turned 90° and developed with butanol-water-propionic acid (50:28:22) for another 24 or 48 hr. After the paper was again dried, the locations of the labeled metabolites were detected by radioautography and the content of ^{14}C in each was determined.

Protein concentrations were determined on aliquots of each sample by the method of Lowry et al. (1951).

Cellular RNA and Protein Synthesis

Secondary cultures were incubated for 36 hrs after seeding. The medium was replaced by 5.0 ml fresh medium containing either 0 mM, 5 mM or 20 mM 2-deoxy-D-glucose. 3 hr, 6 hr, 12 hr, and 24 hr later, the medium was removed and the cells pulsed with medium 199 containing the same concentration of 2-deoxy-D-glucose plus either 10 μCi/ml uridine-^{3}H for the measurement of RNA synthesis or 10 μCi/ml leucine-^{3}H for the measurement of protein synthesis. After a one hour pulse, the medium was removed and cells quickly washed with ice-cold Hank's buffer. 1.5 ml of 5% trichloroacetic acid was then used to extract TCA-soluble materials for scintillation counting. The TCA-precipitable materials were solubilized into 2 ml of 0.1% SDS- 0.01 N NaOH solution. 0.2 ml of this sample was used for scintillation counting and another 0.2 ml for protein determination.

CHAPTER 3 EFFECT OF 2-DEOXY-D-GLUCOSE ON CELLULAR METABOLISM IN CHICK EMBRYO FIBROBLASTS IN CULTURE

Introduction

Previous studies with different types of cells showed that although the glycolysis was inhibited and energy metabolism changed by 2-deoxy-D-glucose, the respiratory rate could either increase (Hachenbrock et al., 1971) or decrease (Ibsen, Coe and Mckee, 1958). It is likely that different kinds of interactions between glycolysis and respiration might exist among different cell types. However, it is also possible that failure to carefully control the physiological conditions of the cells could account for these conflicting results (Bassham, Bissell, and White, 1974). The latter problem can now be overcome by the use of the steady-state tracer technique (Bissell et al., 1973) in which the environmental conditions can be carefully controlled.

In this chapter, I examine the transient changes in glucose and energy metabolism in chick embryo fibroblasts induced by 2-deoxy-D-glucose using this new technique. This work was first initiated by White, Bissell and Bassham (1974, unpublished). The results in this chapter confirm their previous findings.

Effect of 2-deoxy-D-glucose on glucose metabolism

To maximize the effect of 2-deoxy-D-glucose, a high ratio of this sugar to glucose was used. The concentration of glucose was reduced to 0.55 mM in the medium and 20 mM of 2-deoxy-D-glucose was used to assure an effective competition for the transport and phosphorylation of glucose. The kinetic analysis of the pool size of some representative metabolites in the glycolytic pathway and the pentose phosphate shunt is

shown in Fig. 3-1. The addition of 2-doxy-D-glucose caused an immediate drop of the pool size of metabolites in both pathways. The metabolic pools, however, tended to recover to varying degrees after new steady-state levels were reached. The lactate and fructose-1,6-diphosphate pools recovered to at most 50% of the original pool sizes. The pentose monophosphate, on the other hand, returned to more than 90% of its original value.

While other gylcolytic pools reached new steady-state levels, the level of glucose-6-phosphate continued to rise (Fig. 3-2). Since the level of fructose-6-phosphate did not rise, it seemed that the conversion of glucose-6-phosphate to fructose-6-phosphate was partially inhibited.

Fig. 3-3 shows the kinetic analysis of the pool size of metabolites related to the TCA cycle. Although the complete TCA cycle was affected by the addition of 2-deoxy-D-glucose as shown by the drop in citrate and malate pools, the immediate increase in aspartate pool and decrease in glutamate pool showed that cells responded to this stress by quickly operating a partial cycle through glucogenic amino acids.

Effect of 2-deoxy-D-glucose on Energy Metabolism

The most drastic change in the energy metabolism after the addition of 2-deoxy-D-glucose was the immediate drop in the ATP pool (Fig. 3-4). The pool size of ATP remained low after the new steady-state had been reached. Concurrent with the rapid decrease in ATP pool, the ADP and AMP pools rose immediately after 2-deoxy-D-glucose addition. The pool size of ADP, however, dropped to about 50% of the unperturbed level when new steady state was reached. The pool size of AMP, on the other hand,

FIGURE 3-1. Effects of 20mM 2-deoxy-D-glucose on the steady-state pools of lactate, fructose-diphosphate, and pentose-mono-phosphate. The medium of cells used in this experiment was changed to that containing 5.5 mM ^{14}C-glucose (specific activity 10.2 Ci/mole) and 1.25 mM inorganic ^{32}P-phosphate (specific activity 1 Ci/mole) 15 min and again 1 hr before the measurement of the pools to insure complete labeling and steady-state. 20 mM 2-deoxy-D-glucose was added at "0" time. Cell samples were taken 30 sec, 1, 2, 5, 15, and 30 min after the addition. These pools were quantitated by their ^{14}C content (nmoles ^{14}C/mg protein).
●, steady-state pools
o, pools after the addition of 20mM 2-deoxy-D-glucose

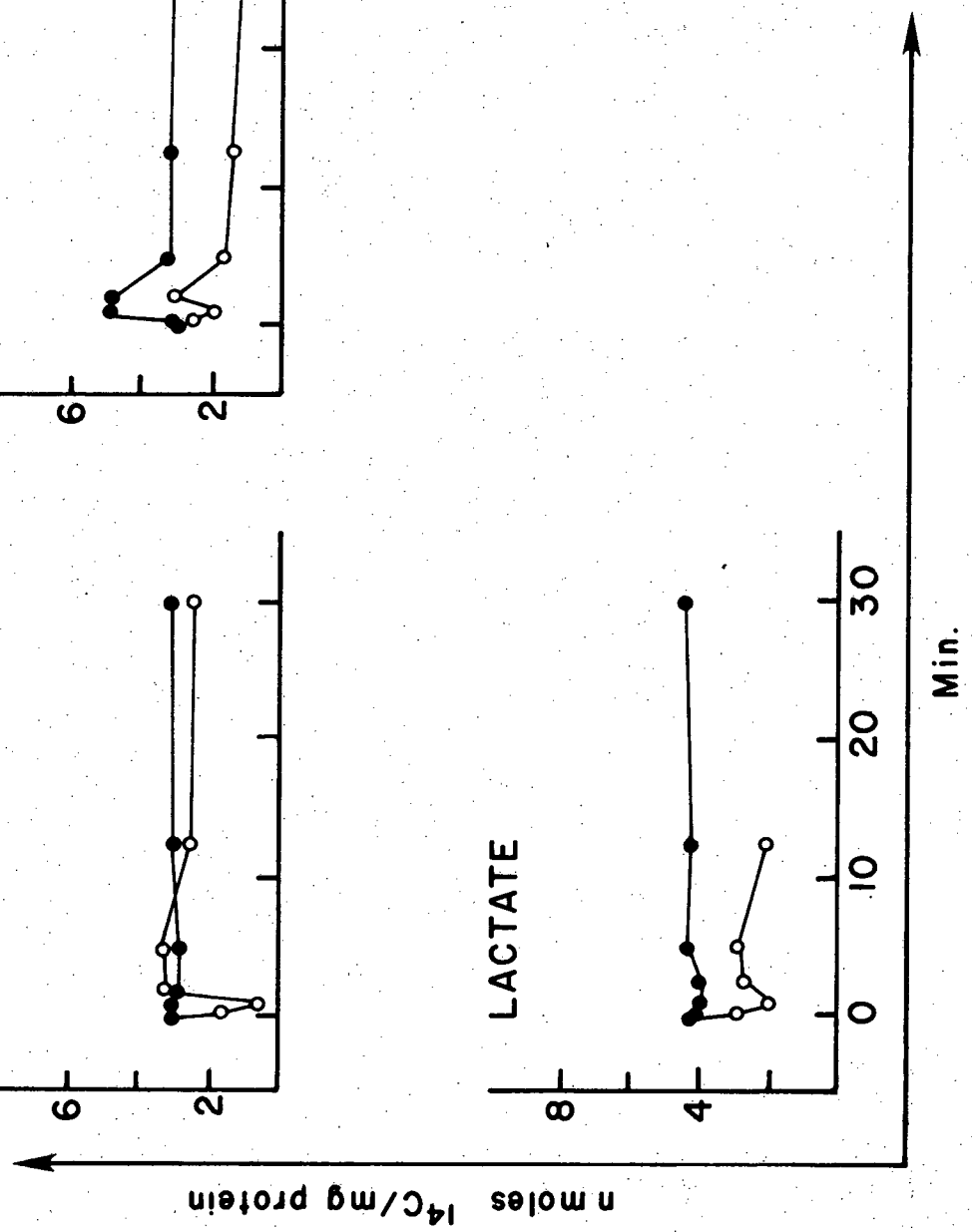


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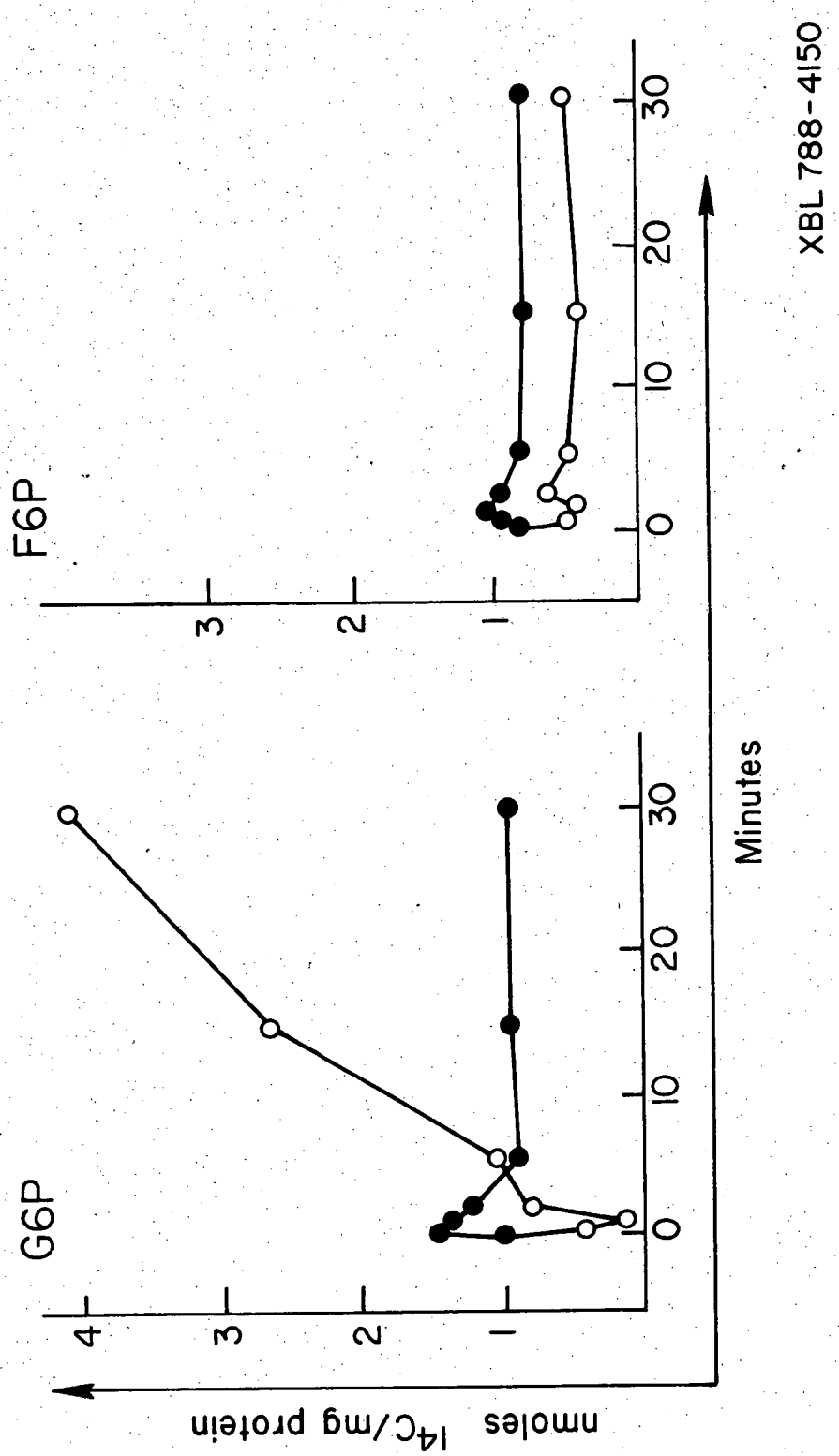
FIGURE 3-2. Effects of 20 mM 2-deoxy-D-glucose on the steady-state pools of glucose-6-phosphate and fructose-6-phosphate. These pools were measured from the same experiment described in Figure 3-1. 2-deoxy-D-glucose was added at "0" time.

●, steady-state pools

o, pools after the addition of 20 mM 2-deoxy-D-glucose

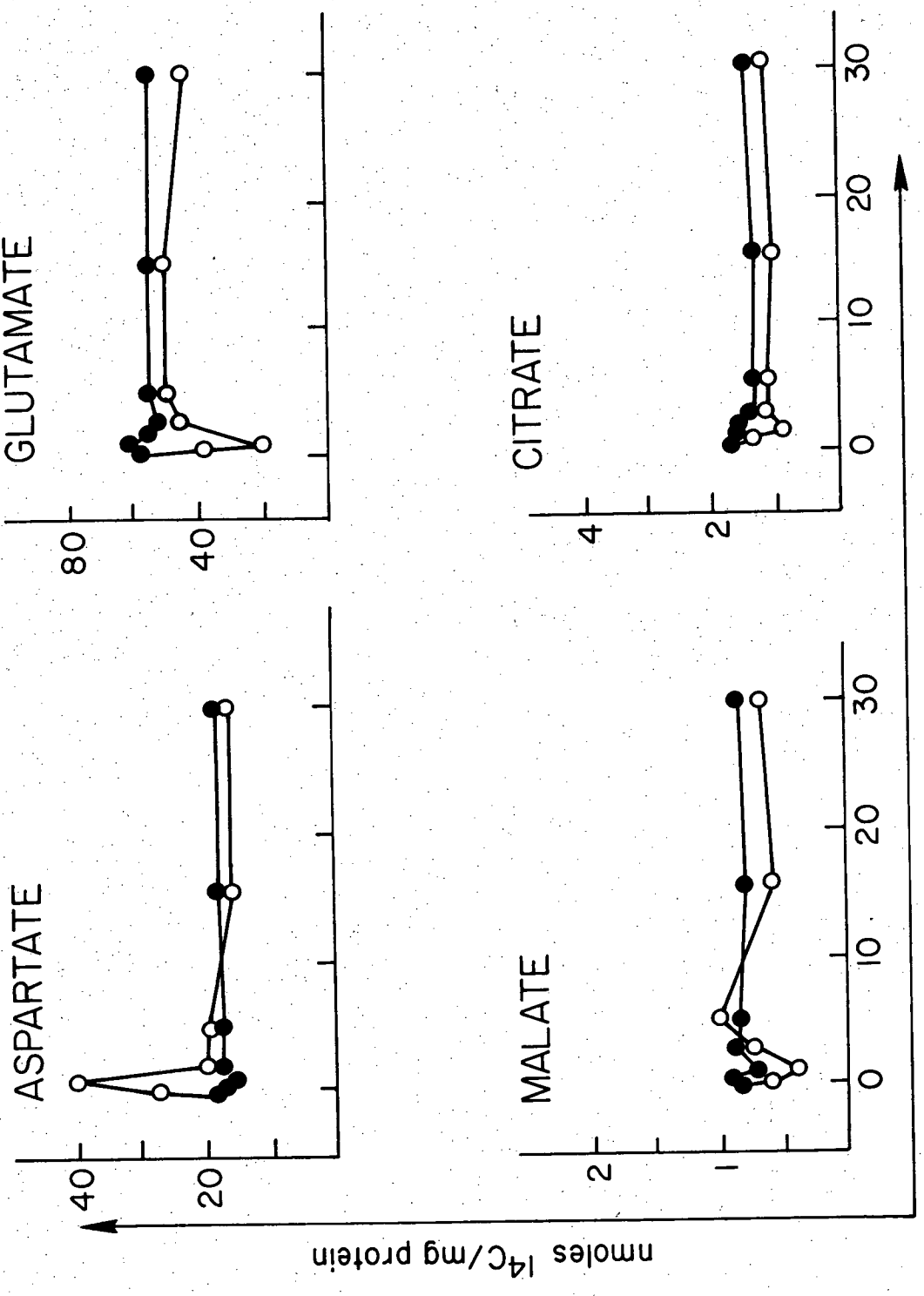


XBL 788-4150

FIGURE 3-3. Effects of 20 mM 2-deoxy-D-glucose on the steady-state pools of aspartate, glutamate, malate, and citrate. These pools were measured from the same experiment described in Figure 3-1. 2-deoxy-D-glucose was added at "0" time.

●, steady-state pools

o, pools after the addition of 20 mM 2-deoxy-D-glucose.

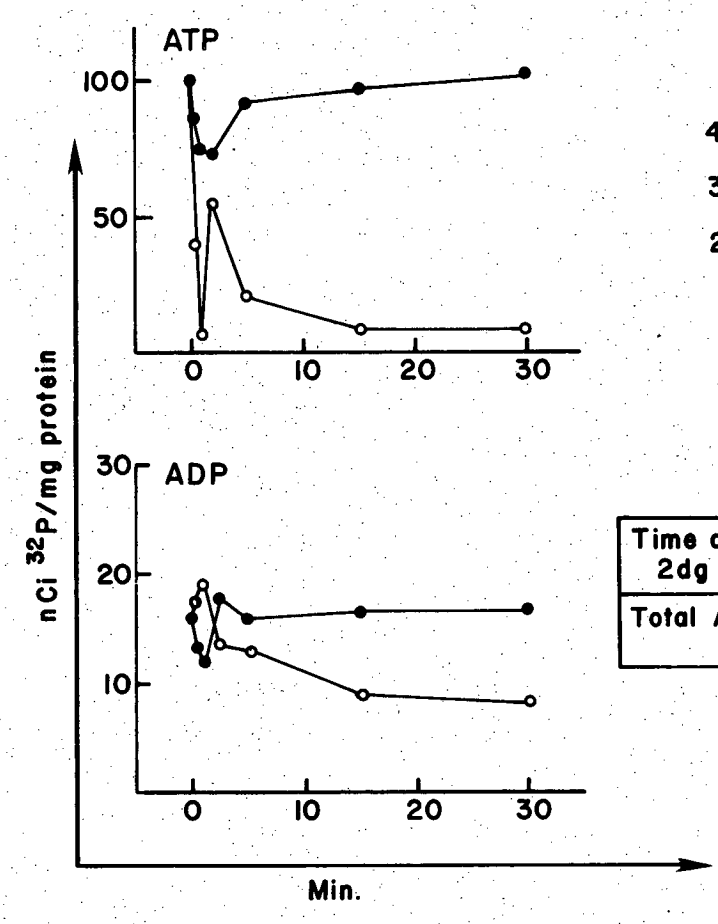

GLUTAMATE
ASPARTATE
CITRATE
MALATE
80
40
40
20
4
2
2
1
0
10
20
30
nmoles ^{14}C/mg protein
Minutes

XBL 788-4149

FIGURE 3-4. Effects of 20 mM 2-deoxy-D-glucose on the steady-state pools of ATP, ADP, AMP, and total adenylate. These pools were measured from the same experiment described in Figure 3-1 by their ^{32}P content (nCi ^{32}P/mg protein). 2-deoxy-D-glucose was added at "0" time.

●, steady-state pools

o, pools after the addition of 20 mM 2-deoxy-D-glucose



Time after 2dg addition	0"	30"	1'	2'	5'	15'	30'
Total Adenylate pool	126	72	42	84	47	29	28

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dropped back and stayed at the unperturbed level. When the total adenylate pool was considered, it was found that the pool size decreased to 21% of the unperturbed level after the new steady-state was reached. This observation agreed with those reported by McComb and Yushok (1964) and by Overgaard-Hansen (1965) that 2-deoxy-D-glucose induced ATP degradation by dephosphorylation and deamination to form inosine and hypoxanthine which were then released into the medium by the cells.

Discussion

A direct effect of 2-deoxy-D-glucose on glucose metabolism is that it strongly inhibits the uptake and phosphorylation of glucose. Inhibition of these two steps leads to an immediate drop in the level of glucose-6-phosphate after 2-deoxy-D-glucose addition. The low level of glucose-6-phosphate then limits glycolysis and the pentose phosphate cycle so that the pool size of intermediates in these pathways is also reduced. Phosphorylation of 2-deoxy-D-glucose gives rise to 2-deoxy-D-glucose-6-phosphate (Kipnis and Cori, 1959; Tsuboi and Petricciani, 1975) which seems to inhibit the conversion of glucose-6-phosphate to fructose-6-phosphate since the former but not the latter sugar phosphate is accumulated inside the cell. The *in vitro* inhibition of hexose phosphate isomerase by 2-deoxy-D-glucose-6-phosphate has been reported by Wick et al. (1957). 2-deoxy-D-glucose-6-phosphate, however, probably does not inhibit glucose-6-phosphate dehydrogenase and 6-phosphogluconolactonase very effectively, so that the serious block to glycolysis could be bypassed to some extent by the pentose phosphate shunt in the presence of normal or elevated levels of glucose-6-phosphate. This is evidenced by the greater recovery for pentose monophosphate and less recovery for

intermediates in glycolytic pathway after the initial drop. It seems that the inhibition of glycolysis is partially relieved by the conversion of some glucose-6-phosphate to glyceraldehyde-3-phosphate through the pentose phosphate cycle.

The quick rise in the aspartate pool and drop in glutamate pool after 2-deoxy-D-glucose addition suggested that a glutamate-aspartate partial cycle has to be operated temporarily to maintain the TCA cycle activity. Due to a limited supply of acetyl CoA from glycolysis, a partial cycle has to function via the glutamate-aspartate transaminase and conversion of α-ketoglutarate to oxalacetate to produce electron flow for the production of ATP via oxidative phosphorylation. As glycolysis partially recovers after the first minute, aspartate and glutamate pools returned nearly to original levels and the normal TCA cycle is operating. It is not clear how the normal flow of carbon from glucose through the TCA cycle is restored despite the fact that glycolysis is only partially recovered.

The degradation of adenylates represents another interesting effect of 2-deoxy-D-glucose. The exact mechanism by which 2-deoxy-D-glucose can induce this degradation is still unknown. One possibility, however, is that the degradation represents one way to restore the "energy charge" of the cell. The energy charge, defined by Atkinson (1968) as $(\frac{ATP+1/2\ ADP}{ATP+ADP+AMP})$, has been suggested to play a role in regulating both glycolysis and TCA cycle (Shen et al., 1968). Immediately after the addition of 2-deoxy-D-glucose, the energy charge drops from 0.68 to 0.33. In order to restore the energy charge, AMP has to be degraded since the alternative, the increase in ATP pool, is not possible due to the

continuous phosphorylation of 2-deoxy-D-glucose. The change in energy charge is closely associated in time with that in the pool size of metabolites in both glycolysis and TCA cycle after perturbation by 2-deoxy-D-glucose. In addition, the energy charge is partially restored when the new steady-state is reached. These results suggest that energy charge might indeed serve as a "messenger" for the communication between glycolysis and TCA cycle activities. However, a more specific inhibitor which acts only on the energy charge is needed to further define the role of this ratio in the regulation of cellular metabolism.

CHAPTER 4 EFFECT OF 2-DEOXY-D-GLUCOSE ON THE REPLICATION OF ROUS SARCOMA VIRUS IN CHICK EMBRYO FIBROBLASTS

Introduction

Rous sarcoma virus offers several advantages for the study of the effect of 2-deoxy-D-glucose on the replication of tumor virus. First, both the virus and its host cell, chick embryo fibroblast, are well-characterized. Second, the effect of 2-deoxy-D-glucose on the cellular metabolism of chick embryo cells is known. In addition, most of the previous studies that examined the effect of this sugar analogue on the replication of cytophathic viruses used chick embryo cells as the host cells. A direct comparison bewteen effects of 2-deoxy-D-glucose on the replication of cytopathic and tumor viruses is therefore possible.

The mechanism by which 2-deoxy-D-glucose interferes with the replication of Rous sarcoma virus in chick embryo fibroblasts is studied in this chapter.

Effect of 2-deoxy-D-glucose on the Production of Infectious and of Physical Particles of Rous Sarcoma Virus

The effect of two concentrations (5 and 20 mM) of 2-deoxy-D-glucose on the production of Rous sarcoma virus in transformed chick fibroblasts was tested. Secondary transformed cells were seeded in 60 mm Petri dishes at the density of 2×10^6 cells per dish and allowed to grow for 36 hr. The medium was removed and replaced by 5 ml of fresh medium 199 containing 0, 5, or 20 mM 2-deoxy-D-glucose. Media were then collected at hourly intervals for the first four hours and every four hours thereafter, and fresh media, containing the same concentrations of 2-deoxy-D-glucose, were again added. The media were clarified of

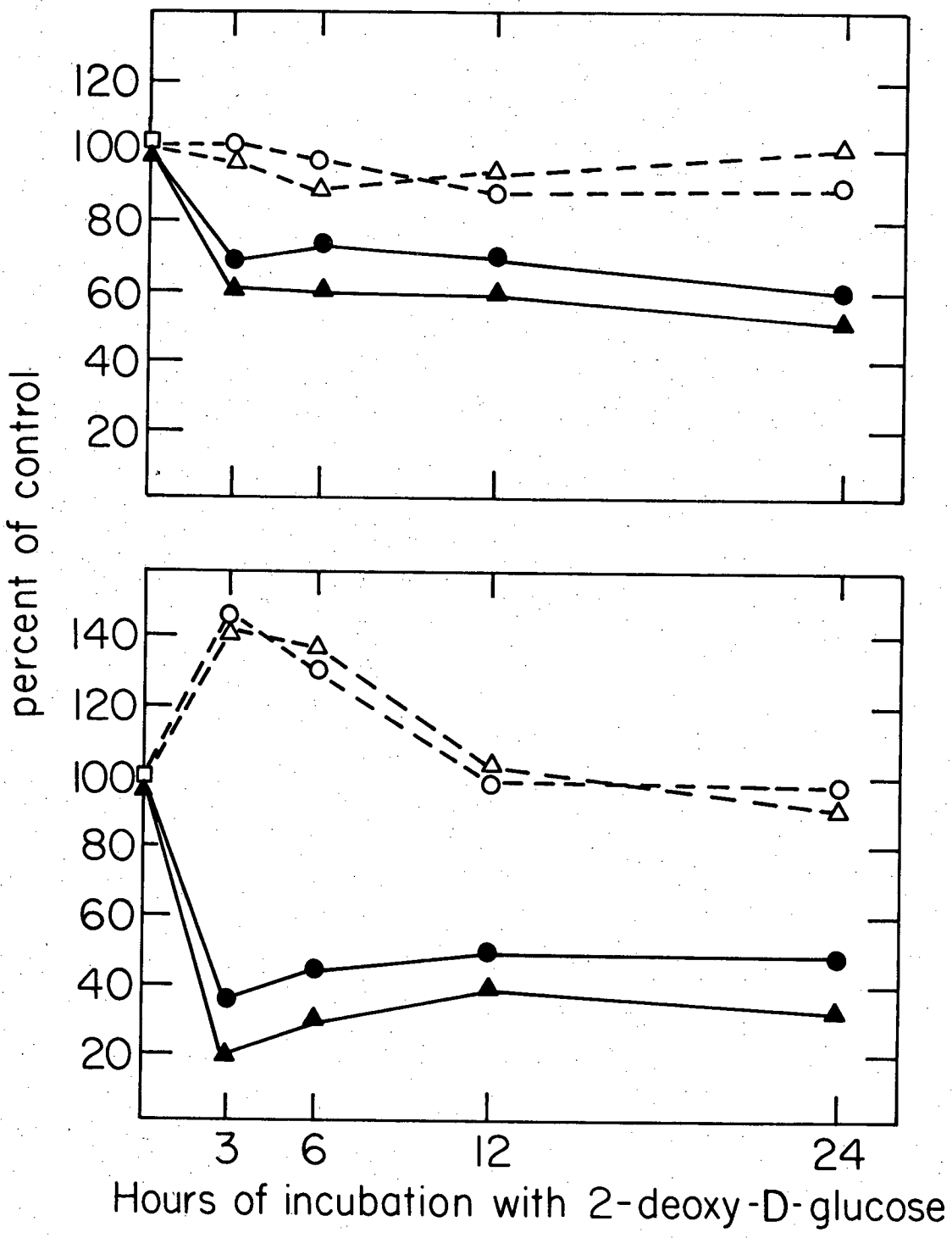
cell debris by centrifugation, and titered by focus assay for the infectious virus and by RNA-dependent-DNA polymerase assay for physical particles of the virus. Both concentrations of 2-deoxy-D-glucose reduced the titer of infectious virus by more than 50% as early as 3 hr after the addition of the sugar analogue, but only reduced the titer of physical particles of the virus by 25% at the same time. The production of viral particles was thus less inhibited than that of infectious virus. This difference was even more dramatic at 12 hr after 2-deoxy-D-glucose addition when the titer of infectious virus was reduced by more than 98% and that of physical particles was reduced by only 60% (Fig. 4-1).

The production of viral particles in the presence and absence of 2-deoxy-D-glucose was also estimated by labeling the virus with ^{3}H-uridine. The labeled virus was precipated from the medium, purified and banded on the sucrose gradient, and the total radioactivity of the virus band was estimated. Fig. 4-2 represents a typical example of such experiments. Between 8 and 12 hr after the addition of 20 mM 2-deoxy-D-glucose, the amount of labeled virus produced by 2-deoxy-D-glucose-treated cells was roughly 30% of that produced by non-treated cells during the same period of time. This result agreed with the estimation using RNA-dependent-DNA polymerase assay as shown in Fig. 4-1. Furthermore, the observation that the viruses produced in the presence and absence of 2-deoxy-D-glucose were banded at the same density (1.165 g/ml) on the sucrose gradient suggested that the sugar analogue did not cause any major structural change in the virus.

Effect of 2-deoxy-D-glucose on Cellular RNA and Protein Synthesis

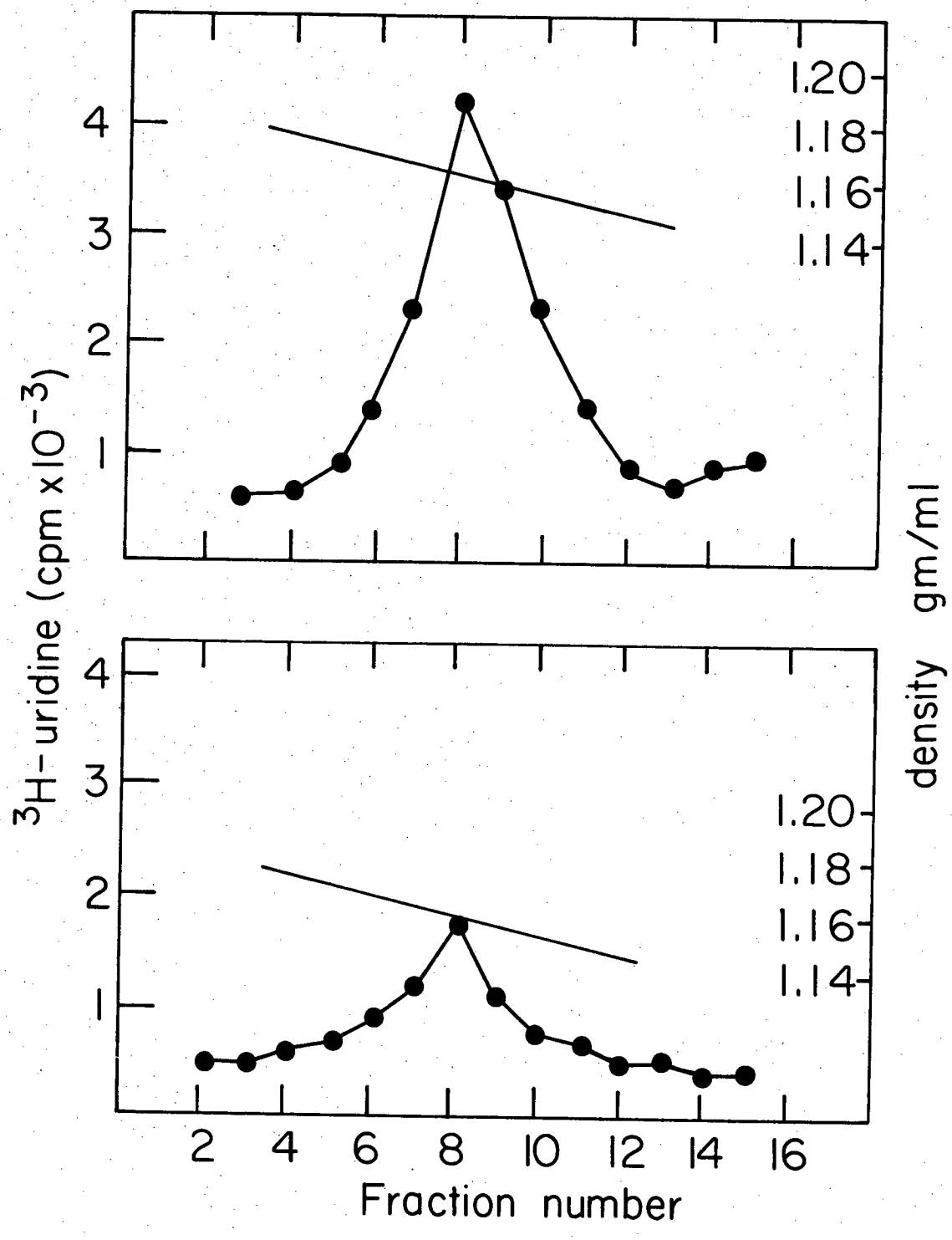
The production of physical particles of the virus from 2-deoxy-D-glucose-treated cells might have been inhibited because of a reduction of macromolecular synthesis. To test this assumption, secondary

FIGURE 4-1. Effects of 2-deoxy-D-glucose on the production of infectious and physical particles of Rous sarcoma virus. Secondary cultures were seeded at 2.0 x 10^6 cells per 60 mm dish and cells were allowed to grow for 36 hr. The medium was then removed and two-thirds of the plates received fresh medium 199 (2-2-1) containing either 5 or 25 mM 2-deoxy-D-glucose. The remaining one-third of the plates received only medium 199 (2-2-1) and served as controls. Media were then collected at hourly intervals for the first four hours and every four hours thereafter. The titer of infectious virus was assayed by Focus Assay and that of physical particles of the virus by RNA-dependent DNA polymerase assay as described in Materials and Methods. To correct for different rates of cellular macromolecular synthesis during the course of the experiment, the titers were adjusted to be the number of foci or enzyme activity per mg of protein in each plate. Each point represents the percentage of decrease in foci or enzyme activity compared to medium from control cultures. Symbols: closed, focus forming units; open, enzyme activity; ^{o}, 5 mM 2-deoxy-D-glucose; Δ, 20 mM 2-deoxy-D-glucose.


120
100
80
60
40
20
140
120
100
80
60
40
20
percent of control
3
6
12
24
Hours of incubation with 2-deoxy-D-glucose

XBL 789-4211

FIGURE 4-2. Effect of 20 mM 2-deoxy-D-glucose on the incorporation of ^{3}H-uridine into Rous sarcoma virus. Secondary transformed cells were seeded as described in Figure 4-1. 36 hr after seeding, cells were labeled with ^{3}H-uridine and 8 hr later, either 20 or 0 mM 2-deoxy-D-glucose was added. The cultures were then continuously labeled with uridine for another 24 hr and the media were collected. The virus was purified from the medium as described in Material and Methods. (A) Rous sarcoma virus labeled with ^{3}H-uridine in the absence of 2-deosy-D-glucose, (B) Rous sarcoma virus labeled with ^{3}H-uridine in the presence of 20 mM 2-deoxy-D-glucose.


1.20
1.18
1.16
1.14
4
3
2
1
^{3}H-uridine (cpm x10^{-3})
density gm/ml
2 4 6 8 10 12 14 16
Fraction number

XBL789-4212

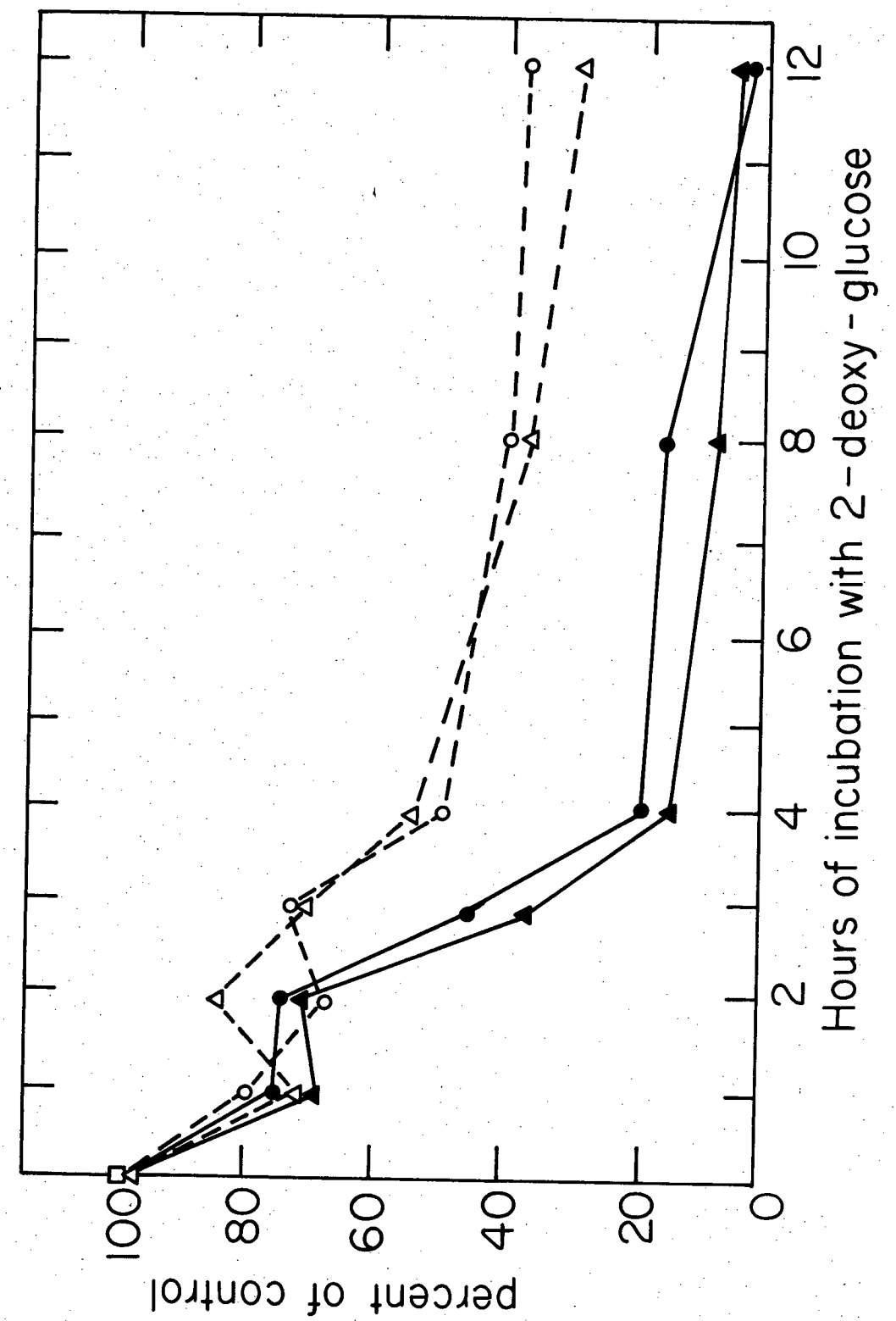
transformed cells were again treated with 0, 5, and 20 mM 2-deoxy-D-glucose and the incorporation of ^{3}H-uridine and ^{3}H-leucine into acid-soluble and acid-precipitable pools was measured as described in Chapter 2. The result is shown in Figure 4-3.

Depending on the concentrations of the sugar analogue added (5 or 20 mM), the incorporation of ^{3}H-uridine into acid precipitable material was reduced by 30% or 40% within 3 hr after the addition of 2-deoxy-D-glucose and remained at this low level thereafter. Actual RNA synthesis was inhibited, more or less, to the same extent since the ^{3}H-uridine pool inside the cell was not significantly changed by either concentrations of 2-deoxy-D-glucose.

The incorporation of ^{3}H-leucine into acid-precipitable material was decreased by 60% and 80% within 3 hr after 2-deoxy-D-glucose was added. The intracellular pool size of ^{3}H-leucine, however, was increased by 40% during the same period of time, so that actual protein synthesis was thus inhibited by more than 70% and 85% respectively. The pool size of ^{3}H-leucine was back to the control level by 12 hr after 2-deoxy-D-glucose addition when the incorporation into acid-precipitable material was slightly increased. The inhibition of actual protein synthesis was thus gradually lessened between 3 and 12 hr after the addition of 2-deoxy-D-glucose.

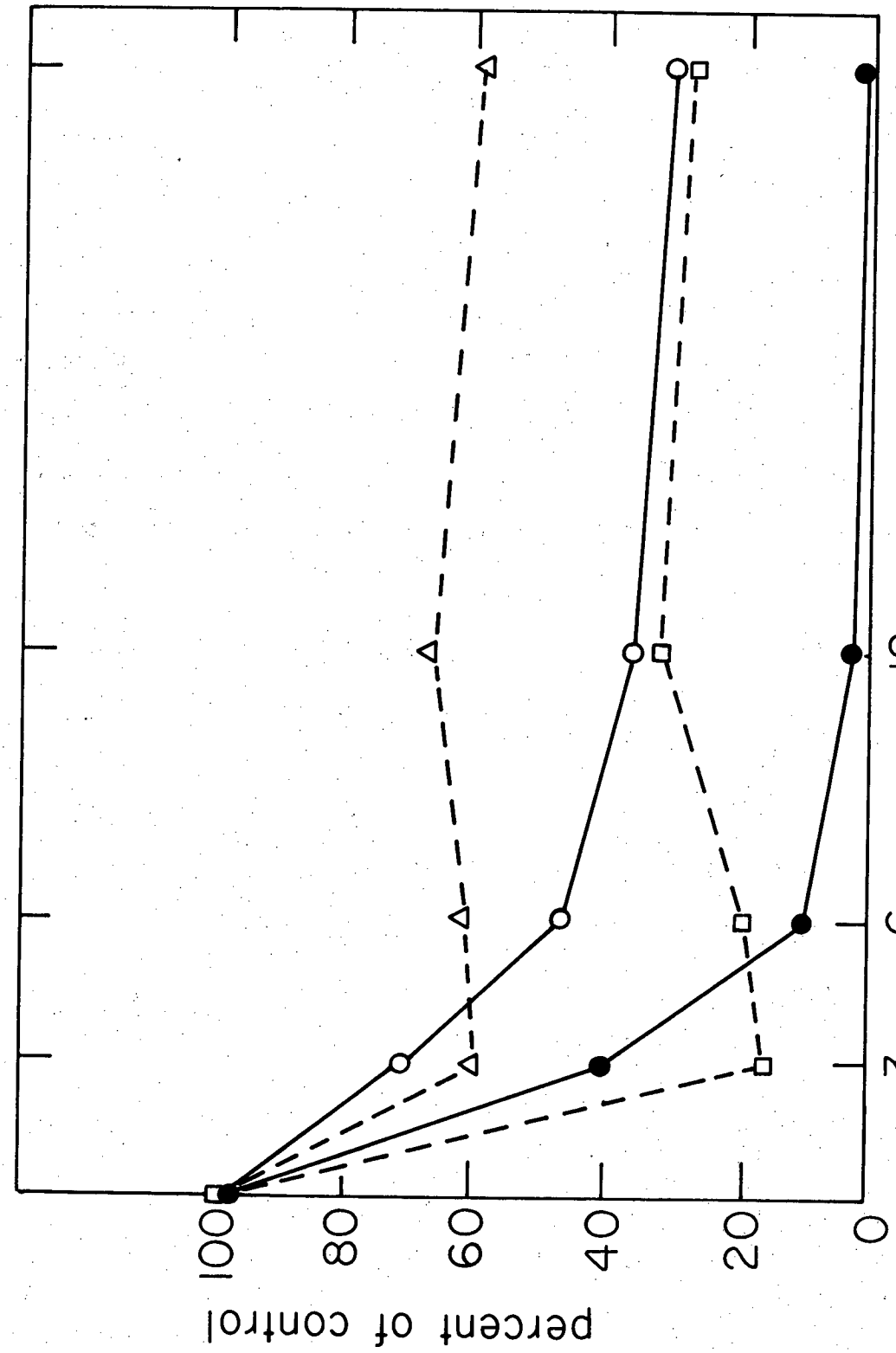
The effect of 20 mM 2-deoxy-D-glucose on the production of infectious and of physical particles of Rous sarcoms virus is compared to that on cellular RNA and protein synthesis in Figure 4-4. Early after the addition of the sugar analogue, the production of the physical particles of Rous sarcoma virus was inhibited less than cellular protein

FIGURE 4-3. Effects of 2-deoxy-D-glucose on the incorporation of ^{3}H-uridine and ^{3}H-leucine into acid-soluble and acid-precipitable pools of Rous sarcoma virus-transformed chick fibroblasts. Petri plates were seeded with 2.0×10^{6} cells and incubated for 36 hr. The medium was removed and replaced with fresh medium 199 (2-2-1) containing 0, 5, or 20 mM 2-deoxy-D-glucose for various lengths of time. Pool sizes were determined as described in Materials and Methods. Each point represents the average of three plates. (A) Incorporation of ^{3}H-uridine, (B) Incorporation of ^{3}H-leucine. Symbols: closed, acid-precipitable radioactivity; open, acid-soluble radioactivity; ^{o}, 5 mM 2-deoxy-D-glucose, Δ, 20 mM 2-deoxy-D-glucose.


percent of control
100
80
60
40
20
0
2
4
6
8
10
12
Hours of incubation with 2-deoxy-glucose

XBL 789-4208

FIGURE 4-4. Comparison of effects of 20 mM 2-deoxy-D-glucose on the replication of Rous sarcoma virus and on the RNA and protein synthesis of chick embryo fibroblasts. Effects of 20 mM 2-deoxy-D-glucose on the production of infectious and physical particles of the virus were determined as described in Figure 4-1 except that media were cellected every 3 hr for analysis after 2-deoxy-D-glucose addition. Data from Figure 4-3 were used to calculate the inhibition of cellular RNA and protein synthesis as described in the text. Symbols: o, RNA-dependent DNA polymerase activity; ●, focus forming units; △, RNA synthesis of transformed chick fibroblasts; □, protein synthesis of transformed chick fibroblasts.


percent of control
100
80
60
40
20
0
3
6
12
24
Hours of incubation with 2-deoxy-D-glucose

XBL 789-4213

synthesis. It is possible that viral protein synthesis was less sensitive to the effect of 2-deoxy-D-glucose than cellular protein synthesis. This concept of differential sensitivity, however, could not explain why at a later stage by 12 hr after the addition of 2-deoxy-D-glucose and thereafter, the production of viral particles was inhibited to the same extent as cellular protein synthesis. A better explanation would be that the decrease in the production of viral particles by 2-deoxy-D-glucose was a direct consequence of the cytotoxic effect of this sugar analogue in general and a reduction in cellular RNA and protein synthesis in particular. The smaller inhibition of the production of viral particles at an earlier stage would then reflect a lag period during which the viral proteins already synthesized were assembled into mature viral particles, a process not likely to be inhibited by 2-deoxy-D-glucose.

Incorporation of 2-deoxy-D-glucose into Rous Sarcoma Virus

To account for the drastic inhibition of the titer of the infectious virus by 2-deoxy-D-glucose, a separate mechanism has to be proposed. One possibility is that 2-deoxy-D-glucose interferes with the proper glycosylation of viral glycoproteins by being incorporated into the carbohydrate moiety of these proteins as an analogue of mannose and terminates further elongation of the carbohydrate chain. This could render the resulting glycoproteins non-functional and the virus non-infections.

This idea was tested first by determining if 2-deoxy-D-glucose could be incorporated into Rous sarcoma virus. To increase the chance of 2-deoxy-D-glucose incorporation, cells were grwon in a medium in which

glucose was replaced by 5 mM pyruvate. The cells were labeled with ^{3}H-2-deoxy-D-glucose for 18 hr before the virus was collected from the medium. Figure 4-5 shows that Rous sarcoma virus could indeed be labeled with ^{3}H-2-deoxy-D-glucose. Although the exact structure and location of the labeled compound in the virus was not determined, it was most likely that intact 2-deoxy-D-glucose was incorporated into the viral glycoproteins since this sugar analogue could not be metabolized to any significant extent by chick cells (Plageman, 1973; Schmidt, Schwarz and Scholtissek, 1974).

Effect of 2-Deoxy-D-Glucose on the Incorporation of Labeled Sugars into Rous Sarcoma Virus

If 2-deoxy-D-glucose indeed blocks the glycosylation of viral glycoproteins, most of the non-metabolizable sugars should not be incorporated into the virus in the presence of this sugar analogue. This was tested by labeling the virus with ^{3}H-fucose, ^{3}H-galactose and ^{14}C-glucosamine in the presence and absence of 2-deoxy-D-glucose, As shown in Fig. 4-6, 20 mM 2-deoxy-D-glucose effectively blocked the incorporation of all three sugars into Rous sarcoma virus. That virus was indeed produced in the presence of 20 mM 2-deoxy-D-glucose was shown by a peak of RNA-dependent-DNA polymerase activity on the sucrose gradient.

Reversal of the Effect of 2-deoxy-D-glucose by Glucose, Pyruvate and Mannose

If 2-deoxy-D-glucose inhibits the production of infections virus via its interference with glycosylation by acting as an analogue for mannose, it should be possible to reverse this inhibition with high

Figure 4-5. Incorporation of ^{3}H-2-deoxy-D-glucose into Rous sarcoma virus. 36 hr after secondary transformed cells were seeded, the medium was removed and replaced with a special one in which 5 mM pyruvate was used instead of glucose. At the same time, ^{3}H-2-deoxy-D-glucose was added. Cells were incubated for another 24 hr and the medium was collected and the virus purified as described in Materials and Methods. For most of the fractions of the sucrose gradient, RNA-dependent DNA polymerase activity was also assayed. Symbols: ●, ^{3}H-2-deoxy-glucose radioactivity; ^{o}, RNA-dependent DNA polymerase activity.

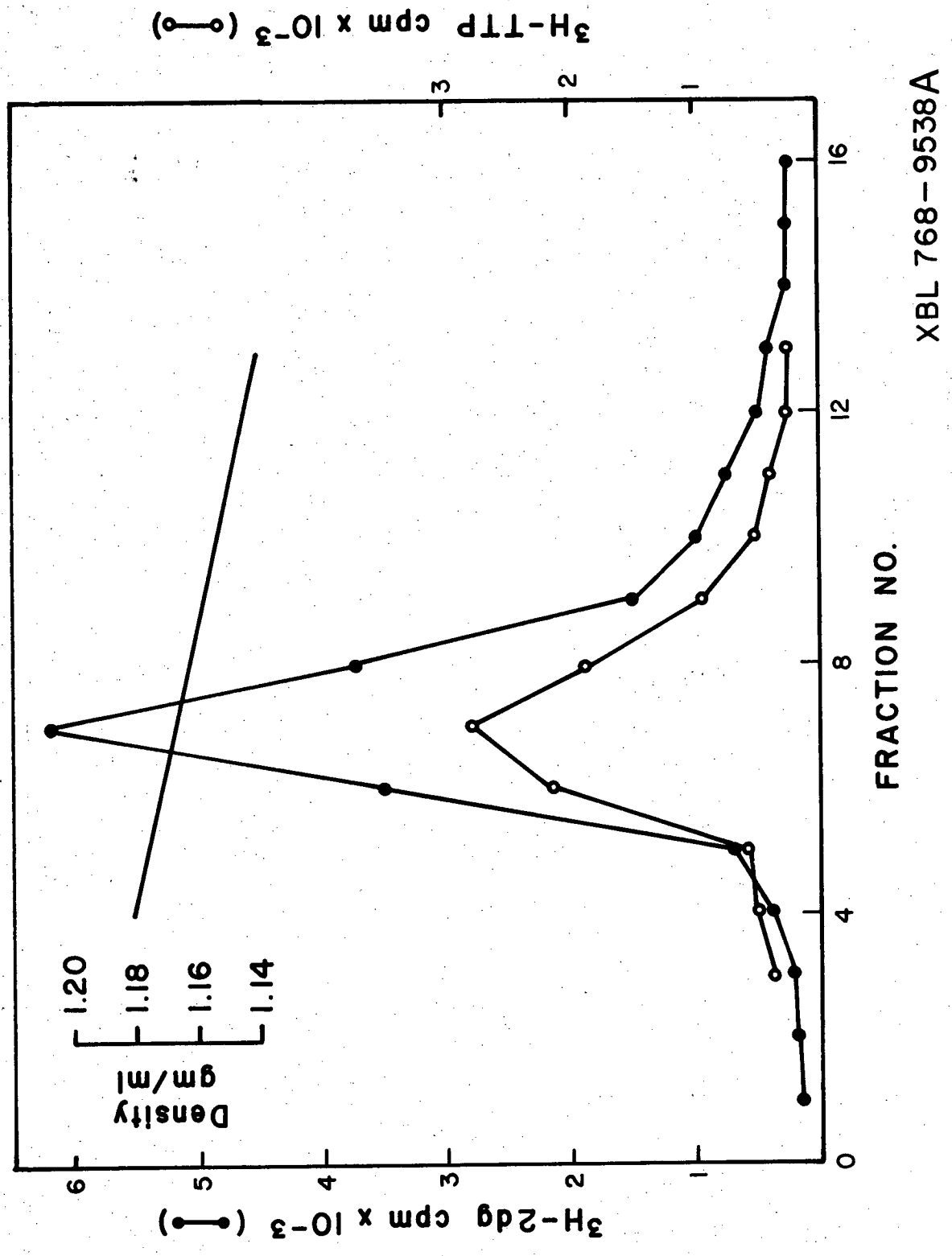
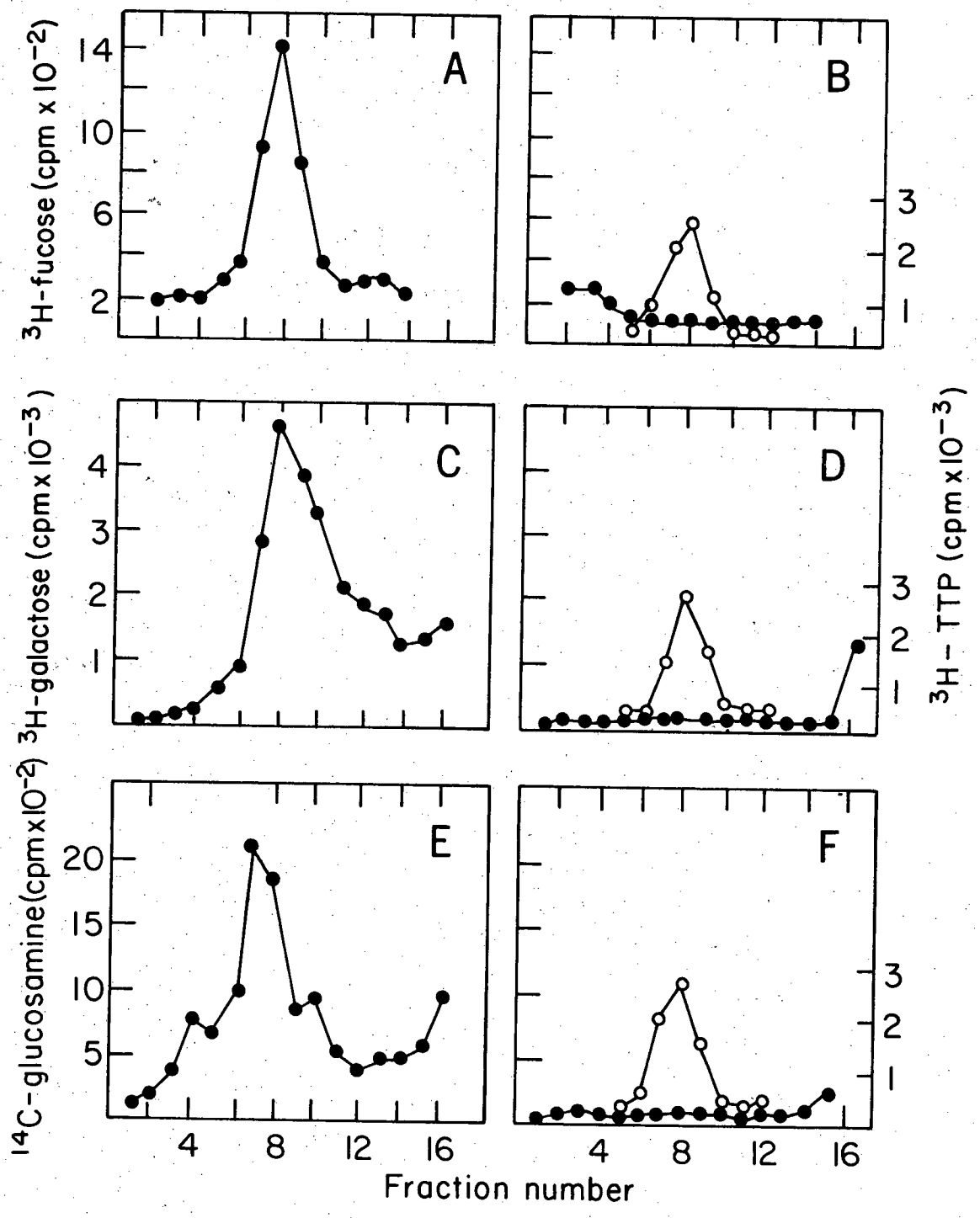

^{3}H-TTP cpm x 10^{-3} (o—o)
^{3}H-2dg cpm x 10-3 (•—•)
Density gm/ml
1.20
1.18
1.16
1.14
FRACTION NO.
XBL 768-9538A

FIGURE 4-6. Effects of 20 mM 2-deoxy-D-glucose on the incorporation of labeled sugars into Rous sarcoma virus. 36 hr after secondary seeding, the medium was removed and replaced with a fresh one containing either 0 or 20 mM 2-deoxy-D-glucose. 18 hr after the medium change, either ^{3}H-fucose, ^{3}H-galactose or ^{14}C-glucosamine was added to the cultures. The cells were incubated for another 18 hr and the media were collected and the virus purified. For some of the gradients, most of the fractions were also assayed for RNA-dependent DNA polymerase activity. (A) Rous sarcoma virus labeled with ^{3}H-fucose in the absence of 2-deoxy-D-glucose, (B) Rous sarcoma virus labeled with ^{3}H-fucose in the presence of 20 mM 2-deoxy-D-glucose, (C) Rous sarcoma virus labeled with ^{3}H-galactose in the absence of 2-deoxy-D-glucose, (D) Rous sarcoma virus labeled with ^{3}H-galactose in the presence of 20 mM 2-deoxy-D-glucose, (E) Rous sarcoma virus labeled with ^{14}C-glucosamine in the abosence of 2-deoxy-D-glucose, (F) Rous sarcoma virus labeled with ^{14}C-glucosamine in the presence of 20 mM 2-deoxy-D-glucose. Symbols: $\bullet$, radioactivity of the sugars; ^{o}, RNA-dependent DNA polymerase activity.


A
B
C
D
E
F
^{3}H-fucose (cpm x 10^{-2})
^{3}H-galactose (cpm x 10^{-3})
^{14}C-glucosamine (cpm x 10^{-2})
^{3}H-TTP (cpm x 10^{-3})
Fraction number

XBL 789-4205

concentration of mannose. On the other hand, because the accumulated 2-deoxy-D-glucose-6-phosphate inside the cell could block the conversion of glucose-6-phosphate to mannose-6-phosphate, glucose, without being converted to mannose, should be much less effective in reversing the effect of 2-deoxy-D-glucose on glycosylation. Furthermore, since 2-deoxy-D-glucose-6-phosphate blocks the conversion of both glucose-6-phosphate and mannose-6-phosphate to fructore-6-phosphate, neither glucose nor mannose should be as effective as pyruvate in reversing the 2-deoxy-D-glucose inhibition of glycolysis and thus cellular macromolecular synthesis and the production of physical particles of the virus.

To test these assumptions, cells were treated with 20 mM 2-deoxy-D-glucose for 24 hr, and 5.5 mM glucose, 10 mM pyruvate and 5.5 mM mannose were used to reverse the effect of 2-deoxy-D-glucose for the next 24 hr. In the presence of 20 mM 2-deoxy-D-glucose for 24 hr, the titer of viral particles was reduced to 34% of the control level and that of infectious virus to 2.2% as shown in Table 4-1.

Mannose was highly capable of reversing the effect of 2-deoxy-D-glucose on the production of infectious virus and increased the titer of infectious virus from 2.2% to 40% of the control value. Since it also increased the titer of viral particles from 34% to 39%, the resulting virus population would have the same infectious/non-infectious (or focus-forming units/reverse transcriptase activity) ratio as the control population which was not treated with 2-deoxy-D-glucose. These results suggested that although mannose was not very effective in reversing the effect of 2-deoxy-D-glucose on cellular metabolism,

TABLE 4-1. Reversal of 2-deoxy-D-glucose inhibition by glucose, pyruvate, and mannose[a]

Sugar used	FFU per 10^6 cells	% of control	RDDP activity[b]	% of control
Control	8.6×10^6		8014	
2-deoxy-D-glucose (20 mM) 1st 24 hr	1.9×10^5	2.2 %	2727	34%
2nd 24 hr	1.0×10^4	0.12%	2310	29%
Glucose (5.5 mM)	2.9×10^5	3.4 %	3760	47%
Pyruvate (10 mM)	2.3×10^5	2.7 %	4078	51%
Mannose	3.5×10^6	39.6 %	3155	39%

[a]Secondary transformed cells were treated with 20 mM 2-deoxy-D-glucose for the first 24 hr. The medium was removed and replaced with fresh medium containing 20 mM 2-deoxy-D-glucose with 5.5 mM glucose, or special medium without glucose but with either 10 mM pyruvate or 5.5 mM mannose for the next 24 hr. Media were collected at the end of this second 24 hr incubation and assayed for the titer of infectious virus by focus assay and of physical particles of the virus by RNA-dependent DNA polymerase assay. Control cells were not treated with 2-deoxy-D-glucose. Media from control cells and from cells treated with 20 mM 2-deoxy-D-glucose for the first 24 hr were also assayed.

[b]Expressed as ^{3}H-thymidine 5'-triphosphate incorporated counts/min/25 ul of medium as described in Materials and Methods.

it fully reversed the effect of this sugar analogue on glycosylation. Both glucose and pyruvate were more effective than mannose in reversing the effect of 2-deoxy-D-glucose on the production of viral particles and increased the titer from 34% to 47% and 51% of the control value respectively. These nutrients, however, were not effective in reversing the inhibitory effect of 2-deoxy-D-glucose on the production of infectious virus since the titer only increased from 2.2% to 3.4% by glucose and to 2.7% by pyruvate.

Discussion

The results in this chapter show that 2-deoxy-D-glucose inhibits the replication of Rous sarcoma virus by two different mechanisms.

As an analogue for glucose, it interferes with glycolysis, depletes the energy source, and reduces the macromolecular synthesis of the host cell. Consequently, the corresponding synthesis of viral RNA and proteins and thus the production of physical particles of the virus are also reduced to the same extent. These results are in sharp contrast to the observations of Prochownick, Panem and Kirsten (1975), who reported that the release of physical particles of Kirsten murine sarcoma-leukemia virus was not affected by 20 mM 2-deoxy-D-glucose even though the RNA and protein synthesis of the host rat kidney cell were reduced by 30% and 60% respectively. If one assumes that RNA tumor viruses replicate by the same mechanism, it is not clear why such discrepancies could exist. These investigators, however, later reported (Prowchownick, Panem and Kirsten, 1976) that 2-deoxy-D-glucose could induce production of type-C virions in normal rat kidney cell. It is possible that 2-deoxy-D-gluclse also induced the production of type-C virons in murine sarcoma-

leukemia virus-infected rat kidney cell and these authors failed to distinguish the induced virions from sarcoma-leukemia viral particles.

That 2-deoxy-D-glucose could act as an analogue for mannose was first suggested by studies showing that the antimetabolite could be activated inside the cell to the corresponding nucleoside diphosphate derivative by GTP, similar to mannose (Biely, Kratky and Bauer, 1972; Schmidt, Schwarz and Scholtissek, 1974). Later study with Semliki Forest virus suggested that 2-deoxy-D-glucose could be incorporated into the carbohydrate moiety of the viral glycoprotein using GDP-2-deoxy-D-Glucose as an intermediate and prematurely terminate the further elongation of the carbohydrate chain. This suggestion is now further supported by the results in this chapter that 2-deoxy-D-glucose can be incorporated into Rous sarcoma virus and this incorporation effectively blocks the incorporation of other sugars into the virus. If the blockage of the proper glycosylation indeed renders the virus non-infectious, one explanation would be that the carbohydrate moiety of the viral glycoprotein is important for the infectivity of the virus. This would, however, disagree with the study by Bolognesi et al. (1977) who found that the removal of most of the carbohydrate from the glycoprotein of Friend murine leukemia virus did not reduce the interference potential of the glycoprotein for the virus and concluded that carbohydrate did not contribute to the infectivity of the RNA tumor virus. Since the endoglycosidase used by these authors in their experiments only removed 70% of the sugars from the glycoprotein of the virus, the remaining portion of the carbohydrate chains could still be responsible for the biological properties of the glycoprotein. Krantz, Lee and Hung (1976)

have suggested that the glycoprotein of Rous sarcoma virus contains two types of oligosaccharide chain and, in both types, mannosyl residues are located in the core portion of the carbohydrate chains. It is possible that the replacement of mannose by 2-deoxy-D-glucose results in a glycoprotein with even less carbohydrate and lacks the major ologosaccharide structure which is responsible for the infectivity of the virus. This might be the portion that cannot be removed easily by the endoglycosidase used by Bolognesi et al. (1977). This possibility warrants further experimentation.

Carbohydrate, on the other hand, might be only indirectly responsible for the biological functions of the glycoprotein. Many intracellular precursors of viral glycoproteins require proteolytic cleavage and most of them are glycosylated (Halpern, Bolognesi and Lewandowski, 1974; Klenk et al. 1974; Arcement, et al. 1976). Schwarz and Klenk (1974) and Klenk, Scholtissek and Rott (1972) have suggested that the structural positioning of the carbohydrate can play a central role in determining the specific cleavage of these precursors into proper glycoproteins. In the presence of 2-deoxy-D-glucose, the glycosylation is inhibited and the cleavage becomes random so that a heterogeneous population of non-functional glycoproteins is obtained. In the case of cytopathic viruses, these abnormal glycoproteins could no longer be incorporated into the virions so the production of viral particles is greatly reduced. It is possible that the requirement is not so stringent in the case of Rous sarcoma virus so that the abnormal glycoproteins could still be used for the assembly of the virus. Virions that contain these abnormal glycoproteins, however, would be rendered non-infections. The less

stringent requirements for the assembly of Rous sarcoma virus is suggested by the fact that the defective Bryan high-titer strain of this virus lacks glycoprotein but is, nevertheless, still produced (De Giuli et al. 1975). This idea could be further tested by a detailed analysis on the carbohydrate side chains as well as the protein backbone of the glycoprotein of Rous sarcoma virus released in the presence of 2-deoxy-D-glucose.

In addition to its effect on glucose metabolism and glycosylation, 2-deoxy-D-glucose could potentially also inhibit the conversion of glucose to many other sugars. The suggested mechanism by which 2-deoxy-D-glucose might interfere with these conversions is summerized in Fig. 4-7. It is possible that the formation of derivatives of mannose, frucose, glucosamine and sialic acid from glucose could all be affected. A direct consequence of this inhibition would be a drastic reduction of the pool size of these sugar derivatives. This in turn could aggravate the effect of 2-deoxy-D-glucose in inhibiting the process of glycosylation. Although this idea is not tested in the present study, it deserves special consideration in future.

FIGURE 4-7. Possible steps at which 2-deoxy-D-glucose might inhibit the conversion of glucose to other sugar derivatives.

Abbreviations: Enzymes- PMM, phosphomannomutase

PMI, phosphomannose isomerase

PGI, phosphoglucose isomerase

PGM, phosphoglucomutase

UDPG-Epi, UDP-glucose epimerase

Sugar derivatives:

M or Man, mannose

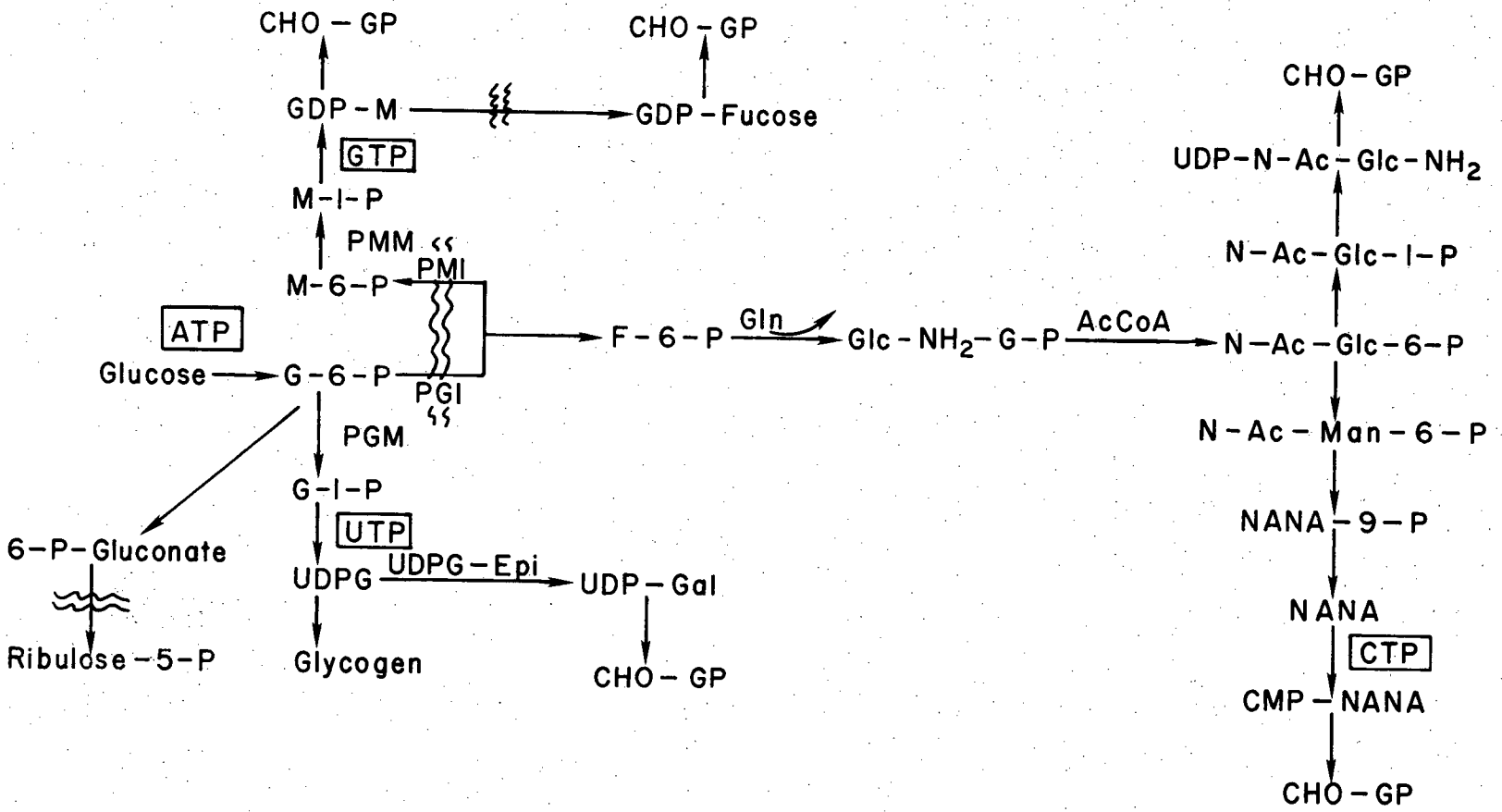
G or Glc, glucose

Gal, galactose

F. fructose

NANA, N-acetylneuraminic acid

CHO-GP, carbohydrate moiety of the glycoprotein


CHO – GP
CHO – GP
GDP – M
GDP – Fucose
GTP
M – 1 – P
PMM
M – 6 – P
PMI
ATP
Glucose
G – 6 – P
PGI
F – 6 – P
Gln
Glc – NH2 – G – P
AcCoA
PGM
G – 1 – P
UTP
6 – P – Gluconate
UDPG
UDPG – Epi
UDP – Gal
Ribulose – 5 – P
Glycogen
CHO – GP
CHO – GP
UDP – N – Ac – Glc – NH2
N – Ac – Glc – 1 – P
N – Ac – Glc – 6 – P
N – Ac – Man – 6 – P
NANA – 9 – P
NANA
CTP
CMP – NANA
CHO – GP

XBL 787-4078

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APPENDIX II PUBLICATIONS

ABSTRACTS

1. Soo, W.-J., Bissell, M. J., and Bassham, J. A. (1977). Inhibition of the Multiplication and the Infectivity of Rous Sacroma Virus in Chick Embryo Fibroblasts by 2-deoxy-D-glucose and Glucosamine. Fed. Proc. 36, 741.
2. Soo, W.-J., Bissell, M. J., and Bassham, J. A. (1977). Mechanism(s) of Reduction in Collagen Synthesis upon Oncogenic Transformation. J. Cel. Biol. 75, 398a.
3. Schwarz, R. I., Farson, D., Bissell, M. J. and Soo, W.-J. (1977). Avian Tendon Cells: A New Culture system for Understnading Differentiation and Cell-Virus Interaction. J. Cell Biol. 75, 398a.

PAPER

Schwarz, R. I., Farson, D. A., Soo, W.-J., and Bissell, M. J. (1978). Primary Avian Tendon Cells in Culture--An Improved System for Understanding Malignant Transformation. J. Cell Biol. In press.

INHIBITION OF THE MULTIPLICATION AND THE INFECTIVITY OF ROUS SARCOMA VIRUS IN CHICK EMBRYO FIBROBLASTS BY 2-DEOXY-D-GLUCOSE AND GLUCOSAMINE. Whai-Jen Soo, Mina J. Bissell and James A. Bassham. Laboratory of Chemical Biodynamics, and Department of Biochemistry, University of California, Berkeley, CA 94720.

2-deoxy-D-glucose (2-DG) and glucosamine inhibited the replication of Rous sarcoma viruses (RSV) (Schmidt-Ruppin, subgroup A and Prague, subgroup C) through different mechanisms. Both sugars were cytotoxic and reduced the rate of protein, RNA, and DNA synthesis in RSV-transformed chick embryo cells to the same extent. 20mM glucosamine inhibited the replication of the viruses to more than 25% in 3 hrs. and more than 99% in 6 hrs. which was in excess of the cellular toxicity. However, 5mM and 20mM 2-DG reduced the yield of the viruses only to the same extent that it reduced the rate of protein and RNA synthesis in RSV-transformed cells. Furthermore, the released virus in the presence of 2-DG was non-infectious and it could no longer be labeled by radioactive fucose or galactose. ^{3}H-2-DG was found associated with the virus purified by the sucrose gradient. Thus this sugar may incorporate into the viral glycoprotein and interfere with its normal glycosylation. This could lead to a premature termination of the elongation of the carbohydrate chain and could render the virus non infectious. (Supported by the U. S. Energy Research and Development Administration and, in part, by NCI Grant CA-14828-03).

MECHANISM(S) OF REDUCTION IN COLLAGEN SYNTHESIS UPON ONCOGENIC TRANSFORMATION

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Transformation of collagen-producing cells in general and primary avian tendon cells (PAT) in particular by oncogenic viruses resulted in a drastic reduction in collagen synthesis. To explore the mechanism(s) by which viral transformation leads to reduced collagen synthesis, we infected PAT cells with a temperature-sensitive mutant of Rous sarcoma virus (Ts-RSV) and compared the rate of collagen synthesis at permissive (35°) and nonpermissive (41°) temperatures. Collagen synthesis of non-infected cells and cells infected with wild-type virus was not sensitive to temperature change. However, there was an 8 to 10-fold difference in collagen synthesis between ts-RSV infected cells grown at 41° (25-20% of total protein synthesis) and at 35° (3% of total protein synthesis). Since these cells release the same amount of infectious virus at both temperatures, the result supports the conclusion that the decrease in collagen synthesis is not merely the result of virus replication but a direct consequence of oncogenic transformation. It also rules out one possible mechanism in which viral RNA is postulated to complete with collagen m-RNA during translation. Another possible mechanism for such reduction may be the elaboration of collagenolytic and/or proteolytic activity in transformed cells. To test this, we designed a mix-culture experiment in which ^{3}H-proline prelabeled normal cells were mixed with either unlabeled transformed cells and the level of labeled collagen measured. While there was a possible small increase in the level of collagenolytic

activity after transformation, it was too small to account for the magnitude of the drop in collagen synthesis in transformed cells. We are currently studying other possible mechanism(s) in which the reduction in collagen synthesis is controlled either at transcriptional or translational level. The reduction appears to be an early event after transformation since in a typical shift-down experiment using ts-RSV infected cells the decrease in collagen synthesis occurred within 4 hrs after temperature shift when morphological changes were barely detecatble.

AVIAN TENDON: A NEW CULTURE SYSTEM FOR UNDERSTANDING DIFFERENTIATION AND CELL-VIRUS INTERACTION. Richard I. Schwarz, Deborah Farson, Mina J. Bissell and Whai-Jen Soo. Laboratory of Chemical Biodynamics, Lawrence Berkeley Laboratory, University of California, Berkeley, CA

Most culture systems used in studying viral-transformation either contain mixed cell populations and/or are relatively undifferentiated. Cultures of primary avian tendon (PAT) cells have the advantage of both being a single cell type (fibroblasts) and having the potential of retaining their full differentiated state. At high density and in the presence of ascorbate, PAT cells devote 25-30% of their total protein synthetic machinery to the synthesis of collagen, a rate which is comparable to the in vivo level (Schwarz and Bissell, PNAS, in press). We now show that PAT cells are capable of undergoing malignant transformation by Rous sarcoma viruses as demonstrated by altered morphology, increase in the rate of 2-deoxyglucose uptake, loss of growth control and production of infectious virus particles. The amount of virus produced by PAT cells, as measured by reverse transcriptase activity and focus forming units, is roughly comparable to that produced by secondary chick embryo fibroblasts infected under the same conditions. Unlike many other cellular functions, the rate of collagen synthesis is decreased drastically after transformation. In addition, the cells lose their ability to modulate collagen synthesis and in the presence or absence of ascorbate synthesize collagen at a rate of less than 4% of their total protein synthesis. The above alterations are due to transformation rather than viral infection because cells infected with a transformation-defective virus behave normally. These studies document

the fact that in addition to loss of growth control, the ability of a differentiated cell to respond correctly to environmental effectors is also lost upon transformation. It is possible that the study of loss of a single prominant function-collagen in this case-may prove fruitful in understanding how the viral genome interferes with gene expression of host cell in malignant transformation. (Supported in part by U.S. Energy Research & Development Administration. RIS is supported by an NIH post-doctoral fellowship).

PRIMARY AVIAN TENDON CELLS IN CULTURE--AN IMPROVED SYSTEM FOR UNDERSTANDING MALIGNANT TRANSFORMATION

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Abstract

Primary avian tendon (PAT) cells which maintain their differentiated state in culture are rapidly transformed by Rous sarcoma virus. By criteria of morphology, increased rate of 2-deoxyglucose uptake, and loss of density dependent growth control, PAT cells transform as well as their less differentiated counterpart, chick embryo fibroblasts. Furthermore, the percentage of collagen produced by PAT cells drops on transformation by an order of magnitude, from 23 to 2.5%, but is unaffected by viral replication of a transformation-defective mutant.

The responsiveness of normal and transformed PAT cells to various environmental factors, changes dramatically upon transformation. Normal PAT cells respond to the presence of ascorbate and high cell density by raising the level of collagen synthesis from 5 to 23%. Transformed PAT cells are totally unresponsive. These and previously reported results, lead us to relate the breakdown in the normal mechanisms used by the cell to maintain the differentiated state to the onset of transformation.

INTRODUCTION

Chick fibroblasts, derived from the body wall of chick embryos (chick embryo fibroblasts, CEF) and their Rous transformed counterparts have been used as models for normal and malignant states for many years. There can be no doubt that this system has brought advances in our understanding of viral-transformation; nevertheless, a significant improvement could be obtained by using highly differentiated fibroblasts, which possess tissue-specific functions. A basic assumption in using mixed fibroblast cultures from many tissues is that they all behave the same. While no one would expect epithelial cells from liver, breast, and kidney to behave the same, fibroblasts from the above tissues are treated as if they are all equivalent. Using mixed fibroblast cultures has the added disadvantage of reducing one's ability to define with any specificity the differentiated state of the cells since no clear in vivo reference point exists. Furthermore, cells are grown in complex medium without regard as to whether or not there are detrimental factors present or beneficial factors absent which are necessary for maintaining the differentiated state (20,21). Using a mixed fibroblast population in a medium which is most likely detrimental to the expression of differentiated function, hinders our ability to resolve differences between the normal differentiated state and the transformed state of the cell (18). This may be extremely important if the mechanisms used by the cell to maintain its differentiated state are the ones blocked by the virus to create the transformed phenotype.

To answer the objections above, we have made use of primary avian tendon (PAT) cells (20,21). These cells have several distinct advantages over CEF cells. First, they are derived from a tissue which is composed almost exclusively of a single type of fibroblast. Therefore, we are dealing

with a homogeneous population. Second, PAT cells in the right environment will maintain their differentiated state in culture (21). In vivo, or in culture, PAT cells devote 25-30% of their total protein to collagen (8,21) (the percentage increases by 1.5 times when one takes into account that collagen is first synthesized as a procollagen precursor [6]). This extremely biased synthesis in the direction of a differentiated function makes for an easy assay of changes that occur after transformation.

In the present communication, we have two aims: One, to show that highly differentiated PAT cells could be transformed and studied in the same way as less differentiated cell types. By using standard criteria of morphology, loss of density dependent growth control, increase in 2-deoxy-glucose uptake and decrease in collagen synthesis, PAT cells can be transformed as well as chick embryo fibroblasts. Two, to study the similarity and differences in the normal and transformed PAT cells' response to environmental factors which affect the stability of the differentiated state (22). An abstract of the work has appeared (23).

MATERIALS AND METHODS

Cell Culture. PAT cells were isolated from a modification (20,21) of the Dehm and Prockop procedure (8). PAT cells (8×10^5 cells in 25 cm^2 flasks; Falcon, Oxnard, CA) were allowed to attach in 5 mls of F12 medium (12) for 40 min. The medium was then changed and the cells were grown in F12 with 0.2% fetal calf serum (Gibco, Grand Island, NY; deactivated 1/2 hr at 56°C) with subsequent daily changes of medium. When ascorbic acid was used in the medium (50 µg/ml), it was added daily from 100x stock. The stock solution of ascorbic acid was freshly prepared every other day.

Virus Infection and Focus Assay. After PAT cells had attached, the medium was changed to 5 mls of F12 with 0.2% serum plus Rous sarcoma virus cloned from single focus. The cells were incubated for 1 hr and then an

additional 5 mls of medium was added. The ratio of virus to cell varied from 1:1 to 1:20 as specified in the figure legends. The rate at which infection spread appeared to be more a function of the strain of the virus than the size of the initial innoculum. Focus assays were performed as described previously (4).

2-deoxyglucose Uptake and Reverse Transcriptase Activity. These methods have been described elsewhere (5,24).

Collagen Assay. The cells were labeled with ^{3}H proline for 3 hr and were assayed using a purified collagenase as described (20,15).

Cell Counts. Cells were removed from the flask with trypsin (0.05%) and gently pipetted to eliminate clumps. They were then counted in a Coulter counter.

RESULTS

Morphology. One of the most dramatic effects of transformation of mixed chick embryo fibroblasts by Rous sarcoma virus is a change in the morphology of the cells: cells round up, pile up, and are more refractile under a phase microscope (4). With PAT cells the effect of transformation is even more pronounced. Normal PAT cells in medium which promotes the differentiated state, low serum (0.2%) and ascorbic acid (50 µg/ml), assume a very round and flat morphology at high density. Under the phase microscope this gives the appearance of a continuous sheet of cells (although under the electron microscope the distinct separation of cells is clear). PAT cells innoculated with Rous sarcoma virus on the day of isolation show a radical change in the morphology at the end of a week in culture. This is shown in Fig. 1. Transformed PAT cells pile up and are more spindle shaped than their normal counterpart. In addition there is an abundance of rounded cells. By the criteria of morphology PAT cells are transformed.

Growth Control. Another common criteria of transformation is a lack of density dependent growth control. Almost by definition a tumor *in vivo* has to have an impaired sensitivity for cell density, although the rate and the range of cell overgrowth can vary immensely depending on the type of tumor. In culture, transformed cells tend to exhibit what appears to be an exaggerated loss of density dependent growth control. In this regard PAT cells are no exception: after infection by Rous sarcoma virus, PAT cells do not show the normal sharp decline in generation time on reaching a confluent monolayer (3). The growth curves for normal and virus infected PAT cells (Fig. 2) shows that normal cells go from a generation time of about 1 day in the early part of the week, to one which approaches zero growth at the end of the week. The transformed PAT cells are much less inhibited and the generation time only increases from 1 day to 2 days as the cells reach high density. Also shown in Fig. 2 is the fact that PAT cells infected with a transformation defective virus grow at a rate which is similar to normal cells. This common control indicates that loss of density-dependent growth inhibition is a property of viral transformation and not viral infection and replication. To assure that infection by the defective virus has indeed occurred, we assayed for the presence of virus particles in the medium by looking for the presence of reverse transcriptase (23). On a per cell basis, on day 7, the level of enzyme activity as measured by incorporation of deoxythymidine triphosphate was approximately the same for the wild type and the mutant virus (data not shown).

By exhibiting loss of density dependent growth control, PAT cells appear to be well transformed.

2-deoxyglucose Uptake. The rate of uptake of glucose is a frequently used measure of transformation: transformed cells transport glucose much more readily than their normal counterpart (4,10). By using the

non-metabolizable analog, 2-deoxyglucose, uptake (plus the first step in phosphorylation) canbe easily measured by accumulation of the label within the cell. In this respect also, PAT cells respond to being transformed in a typical fashion. The time course of 2-deoxyglucose uptake per µg of protein of normal and transformed PAT cells is presented in Fig. 3A. The rate is 15-fold greater in the transformed cells than in the normal cells.

As has been shown with CEF (10,25) and other cell types, in the rate of 2-deoxyglucose uptake varies both with the ratio of growth of normal cells and also after transformation. To test the effect of growth on 2-deoxyglucose uptake, this parameter was measured on normal and transformed PAT cells over a three day period at the end of the week in culture where initially both cell types were growing at the same rate. In this experiment, only one time point on each day was taken (5 min), and a plot of this data is presented in Fig. 3B. The level of uptake on day 4, where the generation times were equal (Fig. 2), showed a clear difference, but it was a third the difference in magnitude for normal versus transformed cells as on day 6. With the use of primary cultures, the increased difference in uptake observed at the end of the week reflects both the slowdown in growth of the normal cells and the greater percentage of transformed cells present in the culture. Despite this complexity, 2deoxyglucose uptaken remains a good indicator of transformation for two reasons. Even when growth rate are equal (day 4) substantial differences still persist. Also, when normal PAT cells slow down their division rate, they also slow down by half their uptake of 2-deoxyglucose; transformed cells, on the other hand, at high cell density slow down their growth rate (although to a far less extent [Fig.2]) but they increase their level of 2-deoxyglucose uptake by two-fold. Taken together, the data refects the fact that a large component of the uptake mechanism for glucose is directly transformation sensitive, as has also been shown

previously for CEF (10,24).

Collagen Synthesis. While a drop in the level of collagen synthesis is not a widely accepted criterion of transformation, over the past fifteen years several laboratories have shown a correlation between transformation and a decline in the percentage of collagen synthesis (11,13,14,17). However, the "normal" cells utilized in these studies synthesized from 6 to 30 fold less collagen than normal PAT cells. With the low level of differentiated synthesis, the question has been raised as to whether or not the changes observed on transformation reflect the actual process in vivo (17). With PAT cells, we can test the action of Rous sarcoma virus on a cell which approximates the in vivo situation much more closely (21).

To study the ability of PAT cells to synthesize collagen after transformation, cells were infected with Rous sarcoma virus and the level of collagen synthesis was measured over a one week period. This was compared to normal cells and cells infected with a transformation defective virus. Under this protocol little change was expected until 4-5 days when infection has spread to a majority of the culture. We therefore concentrated our analysis to the latter part of the week. The data is presented in Fig. 4. By the fifth day, as the cells began to show alteration in morphology, collagen synthesis began to drop. Over the next two days, transformation proceeded swiftly and collagen synthesis declined steadily. In this experiment, by the end of the week there was a drop in collagen synthesis on transformation from 23% to 7.5% [in other experiments, where complete transformation of the culture was achieved within the week, collagen synthesis declined to 2.5% (Fig. 5)]. This drop was a function of transformation and not just virus replication since cells infected with a transformation-defective virus synthesized the normal percentage of collagen.

In addition, it should be pointed out that the sharp slow down in

growth rate observed in the normal cells has at most only a minor positive effect on the percentage of collagen synthesis (20). This is shown by the fact that normal cells on day 4 can grow with a rapid 24 hr generation time (similar to transformed cells) and yet produce almost 20% collagen (Fig. 2 and 4). Therefore, the drop in collagen synthesis upon transformation is an effect which is independent of the release from density dependent growth control. From this experiment, we can conclude that transformation of the cells has a decisive effect on collagen synthesis of PAT cells which agrees with most of the observations reported in the literature with other fibroblasts (11, 13,14,17).

Responsiveness to External Factors. While a decline in collagen synthesis parallels the degree of transformation in PAT cells, their interrelationship is unclear from the above experiments. We would like to distinguish between two possibilities. One, that the control of collagen synthesis after transformation is still normal but at a reduced level. Two, that normal control of collagen synthesis has been disrupted and what remains is a residual synthesis which does not respond to "normal" control mechanisms. These two mechanisms are basically distinguished by whether or not a quantitative loss in collagen synthesis reflects a radical change in the responsiveness of the cell. Normal tendon cells *in vivo* or in culture are sensitive to their environment and only synthesize a higher percentage of collagen when ascorbate is present and the cells are at high density (21). The question can then be raised as to whether transformed PAT cells are also sensitive to these same factors.

To test this possibility, we looked at the ability of normal and transformed PAT cells to modulate the level of collagen synthesis when ascorbate was added to ascorbate-deficient cultures. Normal PAT cells

responded dramatically to ascorbate by increasing their collagen synthesis 3-fold from 8 to 23% (Fig. 5). Transformed PAT cells on the other hand, were insensitive to ascorbate, making approximately 2.5% with or without the addition of the vitamin. Transformed PAT cells were not only insensitive to concentrations of ascorbate and to density dependent inhibition of growth (Fig. 2), but also to cell density stimulation of collagen synthesis. As has been shown before (20), and is displayed again in Fig. 5, normal PAT cells seeded at a low cell density respond to a small degree to a density increase even in the absence of vitamin C. If one compares normal and transformed PAT cells which were not given vitamin C, then one sees that as the cells reach high cell density at the end of a week in culture, the normal cells respond and raise their synthesis of collagen slightly from 4.5 to 8%; the transformed cultures remain unresponsive within experimental error. Thus, the difference between the sensitivity of the normal PAT cell to its environment and the lack of sensitivity of its transformed counterpart is clearly established.

DISCUSSION

In this paper we have shown that a highly differentiated fibroblast culture can be transformed by RNA-tumor viruses as easily as its less differentiated predecessors. By several of the accepted criteria of transformation: altered morphology, increased rate of 2-deoxyglucose uptake, and loss of density dependent growth control--PAT cells can be transformed by oncogenic viruses. Because the gap between the "normal" differentiated state and the transformed state is broader in PAT cells than in CEF or in cell lines, several of the virusinduced changes have been magnified and are thus easier to study.

In addition we have shown that the synthesis of collagen, the major differentiated product of PAT cells, is impaired after transformation by over an order of magnitude. This result appears to agree with a number of previous studies which have shown several fold drops in collagen synthesis upon transformation by oncogenic viruses (1,11,13,14). While in these studies the relative change in the level of collagen synthesis upon transformation was similar to that in PAT cells, the initial level of synthesis of the "normal" cells was either less than, or only slightly higher than a transformed PAT cell. Comparing in several cell systems the ratio of collagen synthesis before and after transformation becomes confusing when such large differences exist in the "normal" cells. Two perspectives can be taken with respect to the large quantitative difference between the "normal" level of collagen synthesis in PAT cells and other cell systems (17). First is that the ability to respond is critical, while the actual quantitative level achieved is of only minor importance. The second view is that the type of response that a cell has to transformation can be radically different depending on its initial state. In the latter perspective one would argue that a change in collagen synthesis from 23% to 2.5% (for PAT cells) may be by quite a different mechanism than a drop observed from 2% to 0.5% (for 3T3 [16,17]). While we have no direct evidence that the changes which occur when PAT cells are transformed are different from that of various cell lines, we do know that the response of the respective normal cells is different towards various external factors. For example, in trying to mimic scurvey in cell culture, PAT cells respond to vitamin C by a mechanism which is similar to cells <u>in vivo</u> (2). They lower the percentage of collagen produced (21) and reduce the level of hydroxylation of proline (Schwarz and Bissel unpublished [23]). Cell lines appear only to alter the level of hydroxylation (16). Therefore,

changes in the level of synthesis can affect the type of response as well. PAT cells approximate the "normal" differentiated state more accurately and they may also approximate the transformation process more faithfully.

One reason why PAT cells respond more dramatically to the transformation process is that they will grow at very low serum concentration (0.2%). High serum concentrations (1%) cause the same changes as viral transformation only to a lesser degree: collagen synthesis declines, the cells no longer respond to ascorbate or as strongly to density dependent growth control, and morphology is changed in the direction of virally transformed cells (19,20,21, and unpublished results). Therefore, starting with ""normal" cells in high serum could significantly reduce the effect of viral transformation.

The similarity of effects of serum and viral transformation on PAT cells raises the question as to whether their mode of action is similar. What these two entirely different agents appear to have in common is that they desensitize the normal cell to its environment. PAT cells which can no longer translate high cell density into a slowdown in generation time, will no longer be able to detect cell density and ascorbate in order to make high levels of collagen. This multifacited action of both serum and the virus could be because they have individual components (or branching steps) which interact with the cell in a variety of ways; or, on the other hand, these two effectors could disrupt a common pathway of the cell which is essential for maintaining its differentiated state. The fact that serum causes the same changes to occur within the cell as viral transformation but only to a lesser degree makes the latter case more probable. The value of the PAT cell system is that one can approach the problem of viral transformation from two directions. One can use the common approach of trying to decipher the primary action of the src gene against a background of a myriad

of secondary characteristics acquired after transformation. An additional approach is to study the control mechanisms used by the normal cells to maintain the differentiated state. In the case of PAT cells, these include an understanding of the mode of action of cell density and ascorbate on collagen synthesis. If the virus does interfere with an essential step that the cell uses to maintain its differentiated function, then these two approaches will come together to a single and more clearly defined focus on the mechanism of transformation.

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FIGURE LEGENDS

Figure 1: PAT Cells grown for one week in F12, 0.2% fetal calf serum and 50 μg/ml ascorbate with daily change of medium. A. Normal cells. B. Cells infected on the day of isolation with Schmidt-Ruppin A subgroup of Rous sarcoma virus. The ratio of virus to cells was 1:20 as determined by focus forming units.

Figure 2: Growth cruves for normal cells (o), cells infected with wild type Prague C, Rous sarcoma virsu, (Δ); cells infected with transformation defective mutant of Prague C, Rous sarcoma virus. (o). Cells were grown in F12, as described in the legend to Fig. 1. The ratio of wild type virus to the cells was 1:1.

Figure 3: The uptake of 2-deoxyglucose in normal (o) and transformed (Δ) PAT cells. A) Shows the time course of uptake on day 6 after isolation. B) Shows the variation in the level of uptake in a 5 min pulse on days 4-6. The culture conditions are the same as those described in the legend to Figure 1.

Figure 4: The percentage of collagen synthesis over a one week period for normal cells (o); cells infected with wild type Rous sarcoma virus (Δ); and cells infected with a transformation defective mutant (o). The conditions were the same as described in the legend to Figure 2. The left ordinate expresses the percentage of radioactive proline which was incorporated into collagen relative to total protein. The right ordinate corrects for the fact that proline occurs 5.2 times more often in collagen than in the average protein (9,21). The corrected value is the

one referred to in the text.

Figure 5. The effect of addition of ascorbic acid to the medium of normal (o) and virally transformed (Δ) PAT cells. Normal PAT cells and cells infected with Schmidt-Ruppin Rous sarcoma virus (20:1, respectively), were grown for 5 days in medium deficient in ascorbic acid (-). On the 5th day, half the cultures of each set were switched to medium containing vitamin C (--).

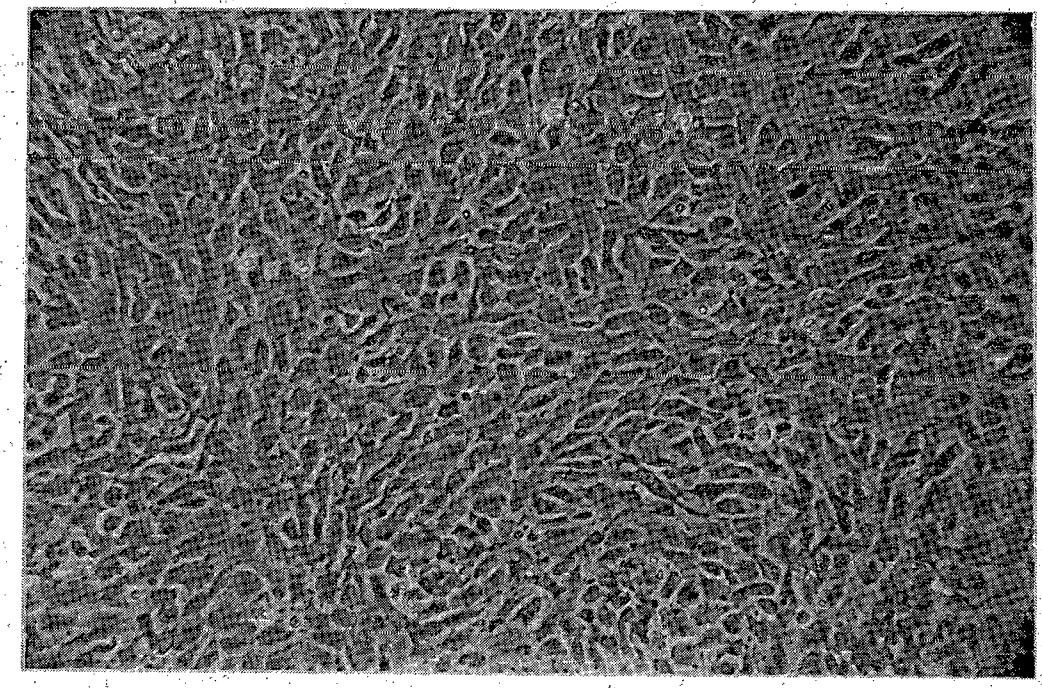


Fig. A

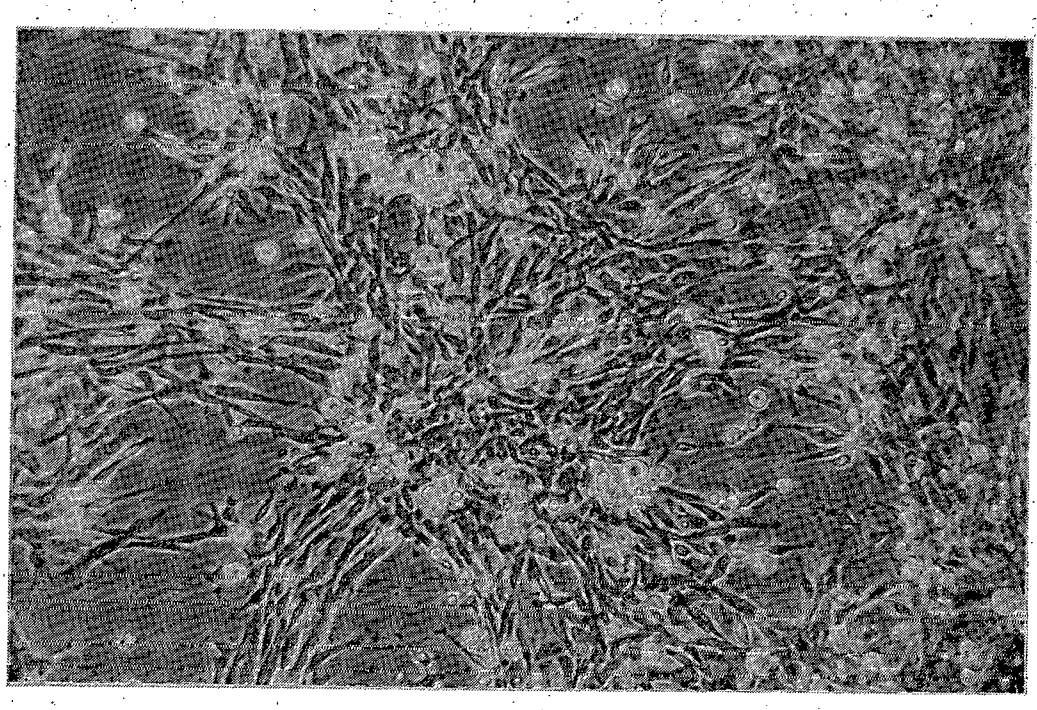
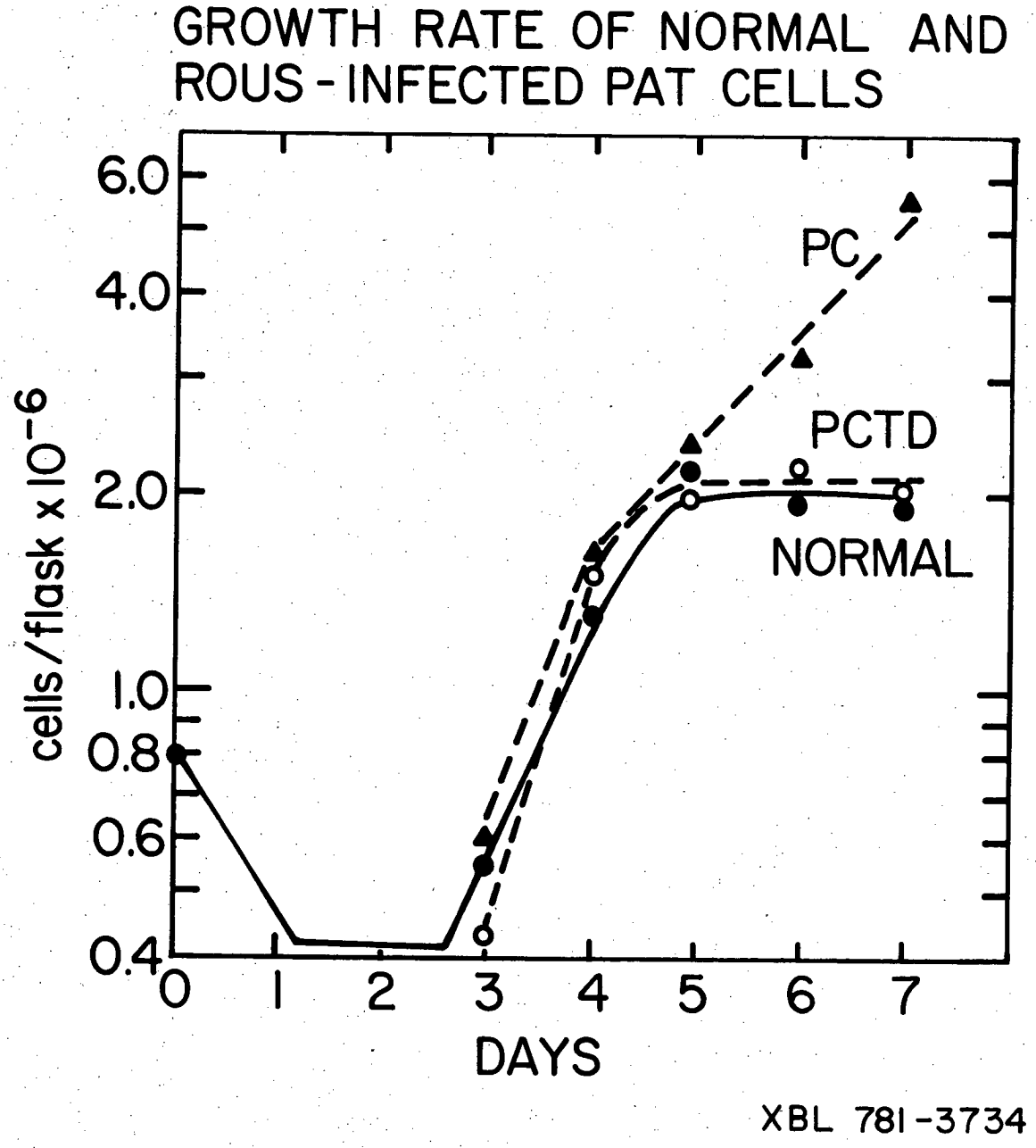
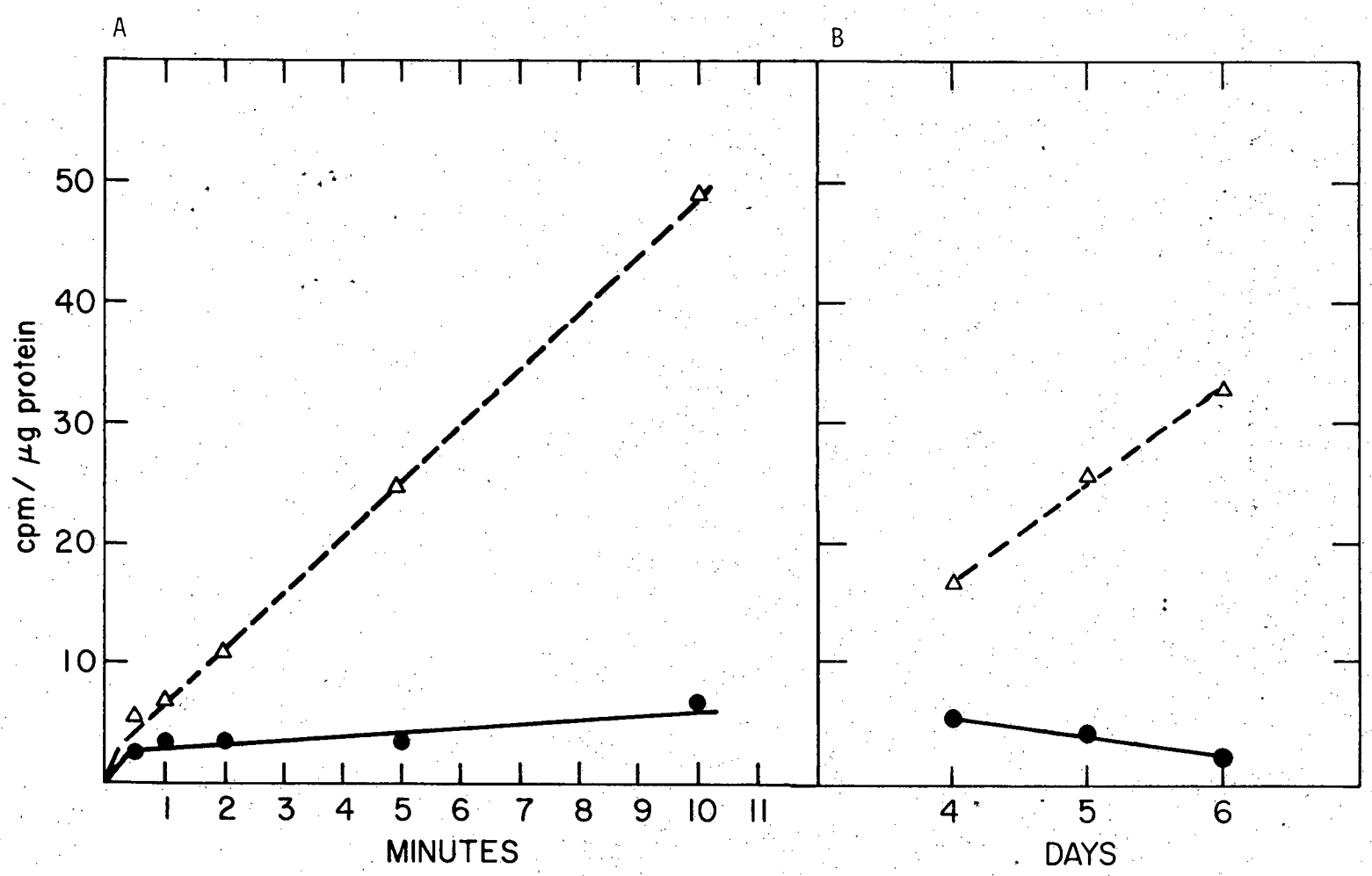


Fig. B

XBB 770-11262


GROWTH RATE OF NORMAL AND
ROUS-INFECTED PAT CELLS
6.0
4.0
2.0
1.0
0.8
0.6
0.4
cells/flask x 10⁻⁶
PC
PCTD
NORMAL
0
1
2
3
4
5
6
7
DAYS
XBL 781-3734


A
B
50
40
30
20
10
cpm / μg protein
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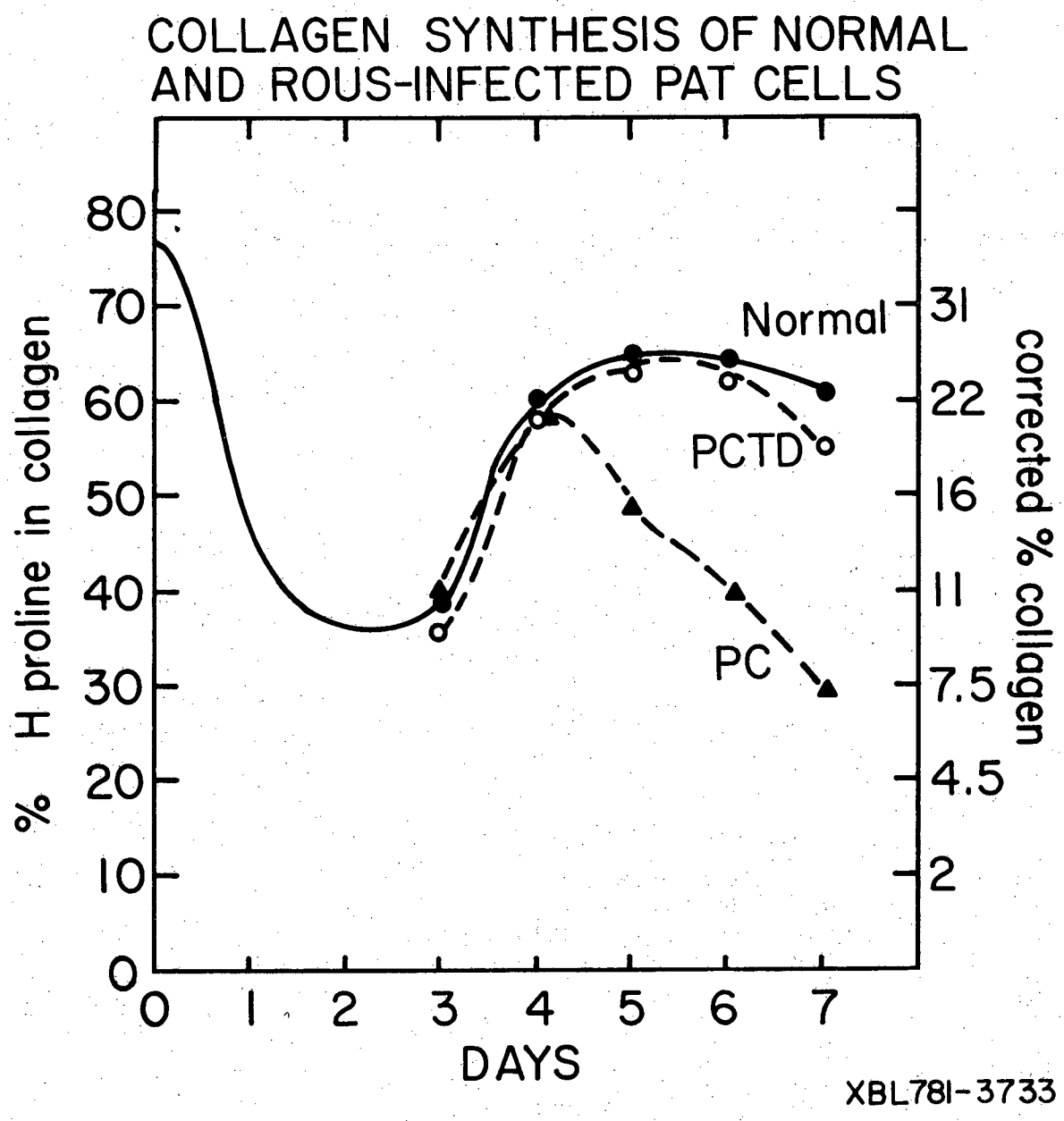
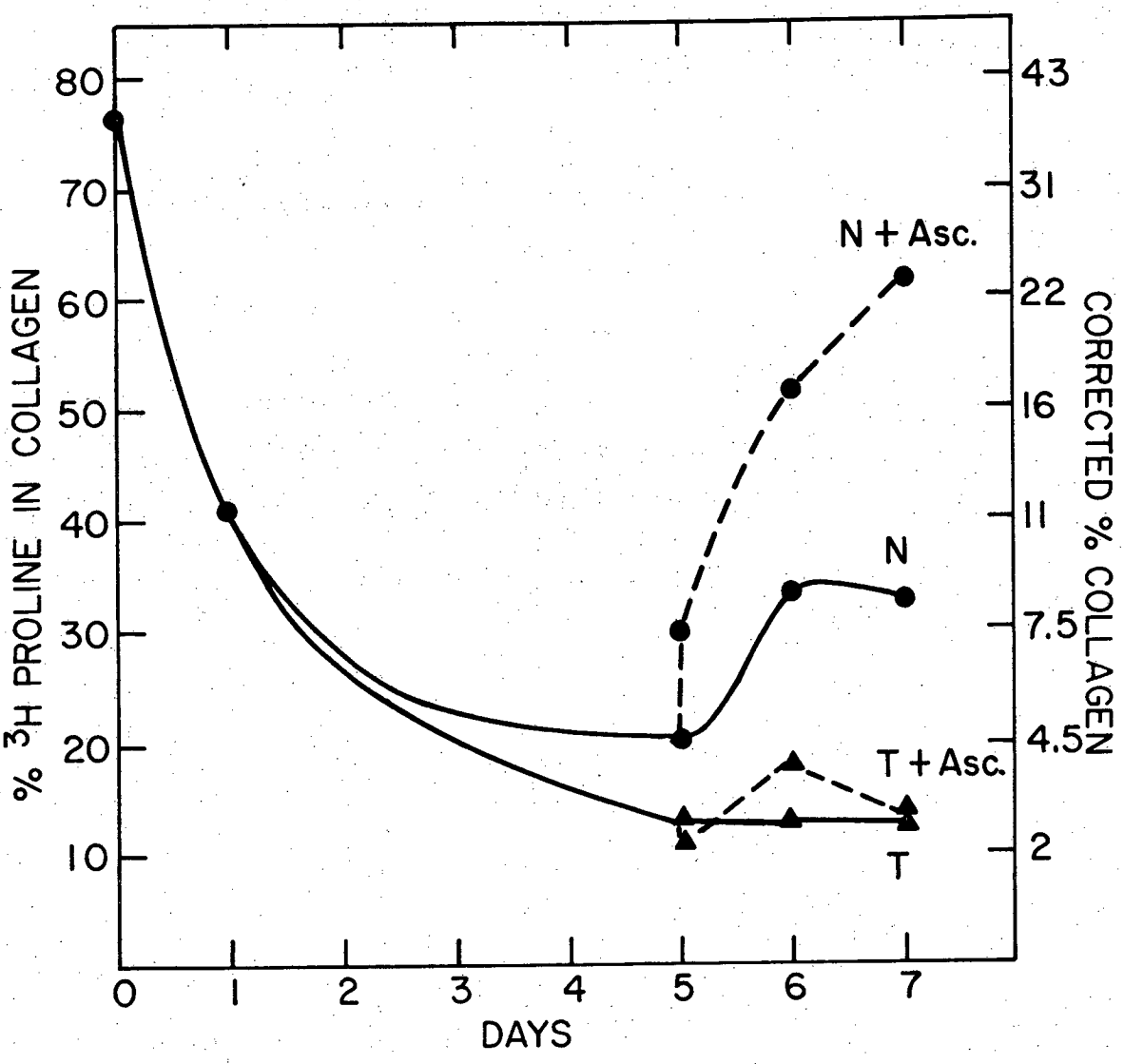
MINUTES
4 5 6
DAYS

XBL 786-4015


COLLAGEN SYNTHESIS OF NORMAL
AND ROUS-INFECTED PAT CELLS
% H proline in collagen
corrected % collagen
Normal
PCTD
PC
DAYS
0
1
2
3
4
5
6
7
80
70
60
50
40
30
20
10
0
31
22
16
11
7.5
4.5
2
XBL781-3733

MODULATION OF COLLAGEN SYNTHESIS IN NORMAL AND TRANSFORMED PAT CELLS



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