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A MECHANISM OF ACTION OF GOLD IN RHEUMATOID ARTHRITIS. A BIOCHEMICAL AND MORPHOLOGICAL INVESTIGATION OF THE DIRECT EFFECT OF GOLD SODIUM THIOMALATE (GST) ON HUMAN SYNOVIAL CELLS IN CULTURE

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ABSTRACT

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Gold sodium thiomalate (GST) effects on cultured rheumatoid synovial cells were used as a model to investigate a mechanism which can explain clinical benefits gained by patients from gold therapy. Synovial tissue obtained from patients with rheumatoid arthritis who were undergoing reconstructive joint surgery was used to obtain explant culture of synovial cells. The experiments described were performed on growing monolayer cultures during the second to fifth passages. Synovial cells were exposed to GST at pharmacologically relevant concentrations (10-100 uM). Vacuoles were found by phase contrast microscopy in synovial cells in culture after 2 days of exposure to 100 uM GST. The vacuoles,aurosomes, were found to contain electron dense material by electron microscopy that contained gold as identified by X-ray probe analysis. The structure of the aurosomes, electron dense lamellae structures in membrane bound vesicles, was found to be similar to those found in synovial tissue of patients with rheumatoid arthritis who had received gold therapy. The time course for gold accumulation in the cell layer paralleled with aurosome formation and the degree of inhibition of [^{3}H]thymidine incorporation into DNA.

DNA synthesis, DNA content and cell number were decreased in a dose dependent manner after synovial cells were exposed to GST containing medium. The synthesis of medium proteins per flask by synovial cells was decreased in a dose dependent manner with collagen decreased to a greater extent. The percentage of collagen to total protein synthesized by synovial cells was decreased in a dose dependent manner. A biphasic response of an increase followed by a decrease of proteins synthesized per cell or per DNA unit was found due to GST exposure. The increase in collagen protein per cell was found to be predominantly type I collagen. The percentage of type III collagen decreased due to synovial cells exposure to GST containing medium. Removal of GST from the medium resulted in recovery of biochemical parameters at low concentrations and a partial recovery at high concentration. Neither aurosomes nor gold could be completely removed from synovial cells after medium changes and subculturing. An important therapeutic mechanism of action of gold in the treatment of patients with rheumatoid arthritis may be by directly inhibiting synovial cells proliferation and altering protein synthesis by synovial cells to a nonconducive matrix for pannus expansion.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

I. INTRODUCTION

Gold has been used for half a century to treat rheumatoid arthritis (RA). Although the rationale for the introduction of gold compounds into therapeutics was based upon gold's bactericidal property, RA has not been established to be bacterial in origin and gold does not eradicate the bacterial infections in laboratory animal models of RA. Therefore, the introduction of gold salts in treating RA is not now believed to be related to the original proposed mechanism of action of the agent. The efficacy of gold therapy, chrysotherapy, has firmly been established in several double blind studies. Theories dealing with the mechanism of action of gold are concerned with the interaction of gold with inflammatory and immunological processes. No single theory can fully explain the clinical benefits RA patients derive from chrysotherapy.

Animal and cell culture models are used to study RA, but no single model can reflect the complex disease of human RA. Because rheumatoid hyperplastic synovium, the pannus, invades the surrounding connective tissue of the joint, gold's therapeutic effect may be related to a direct effect on the proliferating synovial cells. To test this hypothesis, gold sodium thiomalate (GST) at pharmacologically relevant concentrations was added to the medium of synovial cells derived from RA patients' synovial tissue, pannus, explants.

In this model, the synovial cells developed an analogous gold induced morphological change *in vitro* as was found in gold treated patients' synovial cells *in vivo*. The biochemical changes in gold-exposed synovial cells reported in this dissertation may be important factors in the efficacy of gold in RA.

A better understanding of the mechanism of action of a proven therapeutic drug will hopefully lead to the development of a more efficacious, less toxic drug and a better understanding of the pathophysiology of rheumatoid arthritis.

II. LITERATURE SURVEY

A. Rheumatoid Arthritis

Among the diseases which cause chronic loss of functional capabilities, arthritis and rheumatism are second only to cardiovascular diseases. "There is no other group of diseases which causes so much suffering by so many for so long (Hollander, 1972)." Approximately 5% of the total world population are affected and over 11 million Americans suffer from rheumatism and arthritis. Of these, osteoarthritis, degenerative joint disease, is the most common, but rheumatoid arthritis (RA) is a greater problem with regard to severity and prolonged disability. Arthritis rarely kills; it is the life-long crippling rather than mortality that is the problem.

1. Etiology of rheumatoid arthritis.

The etiology of rheumatoid arthritis (RA) is still an enigma. Theories for the origin of RA include infections, metabolic, nutrition, endocrine, autoimmune, trauma, environmental, psychosomatic, genetic and a multifactoral of the above. According to Person and Sharp (1977), RA is a convenient designation to describe a clinically, serologically and etiologically heterogenous group of conditions.

An analogous disease to human RA can be produced in animals by bacterial infection (Cecil et al., 1939 and Timoney

and Berman, 1970). Similarly, mycoplasma, viruses and antigenic proteins can elicit inflammatory joint reactions (Consden et al., 1971; Cooke et al., 1974; Currey, 1970; Stuart et al., 1979 and Chambers and Bywaters, 1963). The infective agent (or the antigen) cannot always be cultured or found in the inflamed joint (Cole et al., 1973 and Person et al., 1973). Therefore, the initial trigger for RA need not be associated with the joint or the patient during the active disease process. The transfer of homologous nodules in laboratory animals does not cause a rheumatoid-like arthritis. Even the transfer of serum or synovial fluid from RA patients to non-RA patients does not transmit the rheumatic disease (Harris and Vaughan, 1961).

There may be a genetic predisposition to RA (Wasmuth et al., 1972). Unlike ankylosing spondylitis which is linked with the HLA-B-27A loci (Pasternack and Tulikaine, 1977), there is only a modest increased frequency of HLA-Dw3 and HLA-Dw4 loci (McMichael et al., 1977) found associated with RA patients (4 times more often than non-RA controls). The classic rheumatoid factor (RF), IgM antibodies directed against IgG, is associated with 70-80% of RA patients but is not specific to RA. Patients with bacterial endocarditis and systemic lupus erythomatosis often are RF positive as is 5% of the normal healthy population (Bartfeld, 1969 and Williams, 1974). These again point to RA as a heterogenous disease with possible different origins and a multifactoral etiology with a common manifestation of RA.

2. Pathogenesis of rheumatoid arthritis.

Although the etiology of RA is unknown, much is known about the pathogenesis of this disease. The defense mechanisms of the body, both humoral and cellular immunity, play important roles in the pathogenesis of RA. An initial synovitis (of unknown origin) evokes an immunological sequel of events that result in antibodies to autoantigens. Antibodies directed against (self-components) nucleic acids, collagens, fibrin and lymphocytes have been found in the serum and synovial fluid of RA patients (Osgood, 1976 and Williams, 1974). The primary offending agent is thought to be antibodies directed against the Fc portion of the γ-globulin (IgG) (Natvig and Munths, 1975). Seropositive RA patients are defined as patients with immunoglobulin M (IgM) antibodies, directed against IgG. These antibodies have been identified as the classical rheumatoid factor. Both seropositive and seronegative patients have a rheumatoid factor consisting of IgG anti-IgG. When these autoantibodies reach a critical titer, they form immune complexes in the synovial fluid and, to a lesser extent, in the synovial tissues (Natvig and Melblye, 1979). Normal synovial fluid usually has the same amount of compliment (C) activity as found in serum, but rheumatoid joint fluid has a depleted level of C activity due to activation of both the classical and alternate C pathways by the immune complexes. High concentrations of the chemotactic factors for granulocytes (C3a, C5a and $\overline{C567}$)

are formed in the activation of the C pathway (Ward and Zvaifler, 1971). A large number of neutrophils polymorphonuclear cells (PMNs), infiltrate the inflamed rheumatoid joint synovium. However, most are found in the synovial fluid where the neutrophils phagocytize large amounts of C3 and immune complexes. Inflammatory mediators and hydrolytic enzymes are released from the phagocytosing neutrophils (regurgitation during feeding or cell death) that perpetuate the inflammation and subsequently degrade the cartilage connective tissues. Attracted mononuclear cells, lymphocytes and macrophages, infiltrate the edematous, hypertrophied and hyperplastic synovial tissue. Lymphocytes proliferate in synovial tissue and sometimes form germinal centers around blood vessels, as in a lymph node, where B-lymphocytes become plasma cells to produce more antibodies (e.g., rheumatoid factors) that perpetuate the disease. Most of the lymphocytes in the synovium are T-lymphocytes (75%).

The cellular immunity, delayed type hypersensitivity, is very important in the pathogenesis of RA (Natvig and Mellbye, 1979). The subpopulation of T-cells and their functions include: k-cells, killer cells which are cytotoxic to other cells by direct contact; h-cells, helper cells that cooperate with B cells in antibody production, and s-cells, suppressor cells that modulate the B cell antibody production. A lack of suppression by the s-cells to allow the production of forbidden clone(s) appear to be an important factor in the development of the RA lesion

(Tannenbaum and Shur, 1974). Lymphocytes elaborate many types of lymphokines. Factors like macrophage inhibitory factor (MIF) keeps macrophages in the inflamed synovium. Lymphokines can stimulate macropages to release factors which cause the lymphocytes to proliferate. Lymphokines injected into a rabbit joint causes a rheumatoid-like arthritis, and lymphokines or mononuclear cell factors can stimulate synovial cells to proliferate and produce collagen in culture (Stastny et al., 1975). Inflammatory mediators and modulators including bradykinins, histamine, seratonin, SRS-A, platelet factors and prostaglandins interact synergistically to perpetuate the inflammation. Positive feedback loops with immunological and inflammatory cells and immunological and inflmmatory factors perpetuate the disease resulting in swelling and pain (Zvaifer, 1970 and 1973). Eventually, the hyperplastic synovium invades other connective tissue compounds (cartilage, bone or ligaments) and is then called a pannus (Harris, 1974, 1976 and Harris et al., 1972). At the edge of the pannus, proteolytic enzymes including mammalian collagenase have been identified by immunofluorescence (Wooley et al., 1975 and 1977). Irreversible damage occurs when the pannus enzymes digest the structural protein collagen. Pannus growth may be stopped by either blocking the stimulatory factors that promote the growth of the granulation type tissue, preventing the formation of the granulation extracellular matrix, blocking enzyme release at the edge of the pannus or inhibiting synovial cell division. Preventing the irreversible

joint damage due to the pannus is important because eventually the damage could lead to loss of function of the joint.

3. Pharmacological management of rheumatoid arthritis.

At present no drugs are known to adequately control chronic inflammatory diseases like RA in all clinical situations. Drugs used today only suppress the disease. Therapeutic management of RA is to reduce inflammation and pain, with attempts to maintain function and prevent deformities. In treating the RA patient, drug therapy is only one component of the management. Physical therapy, nutrition and psychology for chronic, possibly disabling, disease are also important.

a. First line drugs

Nonsteroidal anti-inflammatory agent (NSAIA) are the first line drugs used to treat RA. The NSAIA were thought to work by uncoupling oxidative phosphorylation (Whitehouse, 1963); however, more specific drugs for uncoupling oxidative phosphorylation (e.g., dinitrophenol) are not anti-inflammatory. The present theory most widely accepted for NSAIA mechanisms of action is inhibition of prostaglandin synthesis (Vane, 1971). NSAIA drugs decrease the edema which causes the pain in morning stiffness and are mild analgesics. Salicylate therapy, especially aspirin, is used primarily because of low toxicity, efficacy and minimal expense. If toxicity or lack of efficacy is found with aspirin, other NSAIA such as indomethacin are used. More potent drugs

are used alone or in conjunction with the NSAIA in the more severe cases of RA.

b. Steroids

When the inflammation of RA is not controlled by NSAIA, steroids are sometimes used. The mechanism of action was thought to be via lysosomal membrane stabilization (Symons et al., 1961) but Ignarro (1972) presented evidence that this is an in vitro artifact. Anti-inflammatory steroids as well as hormonal steroids have been shown to bind to a cytoplasm receptor. The steroid-receptor "active complex" is transported to the nucleus resulting in the repression and activation of transcription of mRNAs from DNA (Haynes and Larner, 1975). One of the pharmacological properties of hormonal and anti-inflammatory steroids is selective gene expression for protein synthesis. Inhibition of lymphocyte proliferation and lymphokine production (Weston et al., 1973 and Kayashima et al., 1978) and inhibition of prostaglandin release (Lewis and Piper, 1975) by blocking the phospholipase enzyme have also been proposed to explain the action of anti-inflammatory steroids. A long-term hazard of systemic corticosteroids, Cushing's disease, makes steroid therapy a controversial form of treatment (Hess and Goldman, 1972 and Dayer et al., 1976). The intraarticular injection of steroids are useful and decrease the systemic problems associated with the systemic use of this drug (Hess and Goldman, 1972). Neither steroids nor NSAIA prevent the progression

of the pannus (Harvey, 1975), and therefore cannot prevent irreversible erosion causing the disabling consequence of the illness.

c. Second line drugs

Second line drugs are generally drugs used in chronic erosive disease which are characterized by a delay in onset of therapeutic action and a slow return of symptoms after termination of drug use. Hydroxychloroquine, gold salts and penicillamine all have severe side effects that need to be monitored, but there also is data to suggest these drugs inhibit the erosive consequence from the pannus (Co-operative Clinic Committee ARA, 1973; Multicenter Trial Group, 1973 and Mainland and Sutcliffe, 1962). The early signs of toxicity with gold salts and penicillamine are a pruritic dermatitis and a proteinuria which are reversible. Bone marrow suppression is the most dangerous side effect (Harvey, 1972) and with hydroxycloriquine retinopathy leading to blindness can occur (Zvaifler, 1968 and Rubin et al., 1963).

A subset of second line drugs include cytotoxic drugs. Cyclophosphamide and azathioprine were first used as anti-cancer drugs and are carcinogens. It is thought that these drugs are immunosuppressive and prevent pannus progression (Urowitz, 1974). The use of these drugs is restricted to only the most resistent cases (Cooperative Clinic Committee ARA, 1970).

B. Animal Models for Rheumatoid Arthritis

1. Adjuvant rat model.

No one experimental model has all the characteristics of human RA. The animal model most commonly used is the rodent adjuvant arthritis (Stoerck et al., 1954). This is a polyarthritis that can be induced by a single i.p. injection of killed mycobacteria or tuberculin (Currey, 1970). The antigen responsible is the peptidoglycan of max D (Adam et al., 1972) from the bacteria cell wall. The suppressor T-lymphocytes play a major role in the development of the disease (Kayashima et al., 1978). Even though many similarities exist between this model and human RA, rheumatoid nodules and rheumatoid factor are not present (Pearson, 1972).

2. Antigen rabbit model.

Another model is the antigen-induced arthritis in rabbits. The rabbit is immunized with a protein antigen, e.g. fibrin (Dumonde and Glyn, 1962). Several weeks later the antigen is injected into the joint. A monoarticular arthritis develops with neutrophils and mononuclear infiltrates. Immunoglobulin synthesis by lymphocytes in the synovial tissue are directed against the challenge antigen (Cooke and Jasin, 1972 and Cooke and Richer, 1974). The histology of the synovial tissue in this model is very similar to the pannus, though in human RA there is usually a polyarthritis.

3. Collagen induced arthritis.

Recently, a type of rodent arthritis was found to be caused by injection of collagen. Native but not denatured type II collagen injected subcutaneously caused a polyarthritis in rats (Stuart et al., 1979a, b). A similar model of collagen induced arthritis was also induced in rabbits (Steffen et al., 1979). Collagen induced arthritis might have clinical relevance. Andriopulous et al. (1976) found antibodies against type II collagen in the sera of patients with RA. The antibody titers were highest against denatured rather than native types II and I collagen. It is unlikely that collagen is the unknown trigger antigen in RA. Rather the breakdown of cartilage by the pannus releasing type II collagen acts as an antigen which perpetuate the inflammation.

Gardner (1960) reviewed the experimental models for RA and classified them into five groups, infective, chemical, immunological, endocrine and physical. His conclusion that because of the lack of resemblance to human RA, that future investigations should be conducted with human tissue is still true two decades later with the newer animal models.

C. Human Synovial Tissue and Cells in Culture

1. Tissue.

The diathroid (free moving) joint has two periosteal bones covered at their articular end with hyaline cartilage,

held together by fibrous ligaments, which form a joint capsule. The inner lining of the joint capsule has a one to three cell layer thick membrane called the synovial membrane. Synovial fluid is a viscous translucent yellow-colored liquid within the diathroid joint. The composition of synovial fluid is an ultrafiltrate of plasma plus protein, glycosaminoglycans and a hyaluronic acid secreted by the synovial cells. The synovial cells also keep the synovial fluid clear by phagocytosis of particulate matter and invading organisms.

2. Cells and culture models.

Three cell types have been characterized in the synovial membrane (Barland et al., 1962). Type A cells are phagocytic macrophage like cells with many vacuoles. Type B cells are fibroblast-like cells with abundant rough endoplasmic reticulum. Type C cells are either precursors or an intermediate between type A and B cells.

Two techniques are currently used for initiating synovial cell cultures. One is the enzymatic release adhesion method; the other is the explant method. Both methods result in heterogeneous cell populations in culture (Marsh et al., 1978).

a. Enzymatic release-adhesion

The enzyme release adhesion method results in primary cultures of cells which are dissociated from the tissue with collagenase and trypsin. The cells are inocu-

lated into the medium in a cell culture flask. After an hour the medium is removed and the cells attached to the flask are used for the cultures. These cells produce relatively large quantities of collagenase and prostaglandins (Dayer et al., 1976). Some adherent synovial cells (ASC) assume a stellate morphology in culture but do not possess the immunoglobulin (EA) or compliment (EAC) receptors (Dayer and Krane, 1978).

b. Explant

The explant culture method first described by Vaubel (1933a, b) produces heterogenous populations of cells that grow out on the culture plate from a small minced piece of synovial tissue. These cells produce mucopolysaccaride, hyaluronic acid and collagen (Castor, 1957 and 1970). These observations are compatible with the conclusion that for the study of enzymatic activity, the proteolytic release-adherent cell culture model would be used, and that for the synthesis of connective tissue components, the explant method would be a preferred cell culture model.

Frazer et al. (1979) described a type 1 and 2 pattern of changes with synovial cells derived from primary culture explants. The type 1 response consists of increased vacuoles, increased degradation enzymes and decreased hyaluronic acid obtained with sucrose (8mM), adenosine (0.4mM) and serum (50% v/v). The type 2 response consists of increased hyaluronic acid synthesis and dendrite formation obtained

by increasing intracellular c-AMP (cholera toxin and dbc-AMP) and carrageenin (Clarris *et al.*, 1979).

Glen-Bott (1972) examined the ultrastructure of synovial cells *in situ* and in culture. Because of the substantial difference, he concluded that culture cells do not reflect their function *in vivo*. There are certain functions that synovial cells do retain in culture as stated above, but still one should be cautious in the interpretation.

By definition, synovial cells are cells derived from synovial tissue. At present, no biochemical, morphological, or cell surface markers are used to define a culture as synovial cells. The relationship of synovial cells in culture may be related to the synovial cell *in situ* as follows. The explant culture method may select for the type B synoviocytes because in culture these cells produce structural proteins such as collagen and carbohydrates such as hyaluronic acid. These cells also have a fibroblast-like morphology with abundant rough endoplasmic reticulum and golgi apparatus. The adherent culture method may select for the type A synoviocyte because in culture these cells produce proteolytic enzymes like collagenase, and have abundant vacuoles typical for a phagocytic cell. The type 1 and 2 response in culture may be the commitment or differentiation of a type C cell to either an A or B synoviocyte which is thought to occur *in vivo*.

c. Rheumatoid disease in culture:

The human synovial cell culture model has been used for over forty years to study and better understand rheumatoid arthritis. It is controversial, however, whether rheumatoid disease can be brought into culture. The comparison of normal versus rheumatoid-derived synovial cell lines results in conflicting results. No morphological difference was found in the rheumatoid synovial cells by Castor and Dorstewitz (1966), Waynne-Roberts and Castor (1972) and Ghose et al. (1975). An increase in multinucleated cells, cytoplasm granulation and media particles in RA synovial cell was reported by Bartfeld (1965), Goldfisher et al., (1968) Smith and Hamerman (1969) and Marsh et al. (1974 and 1977). Castor and Dorstewitz (1966), Smith and Hamerman (1969) and Marsh et al. (1974) reported slower doubling and shorter life span in culture for rheumatoid cells, whereas, Ghose et al. (1975) found no difference. Castor (1973) found an increase in low molecular weight hyaluronic acid in the medium of the rheumatoid synovial cells relative to the non-rheumatoid. This particular change is characteristic of the synovial fluid in RA. Vuorio (1977) confirmed the above changes in rheumatoid and nonrheumatoid synovial cells and also found a 75,000 dalton membrane protein only in rheumatoid synovial cell membranes. Smith (1970) reported a growth inhibitor (GI) in rheumatoid synovial cells. Possibly the growth inhibitor is reponsible for slower doubling times

of rheumatoid cultures. The heterogenity of rheumatoid disease and the heterogenity of synovial cells make it difficult, so far, to have a parameter as an indicator for RA synovial cells.

3. Peptide modulators effects in culture.

The addition of endogenous peptides can modulate cell metabolism. Castor (1971) first described connective tissue activating peptide (CTAP) which was derived from leukocytes. CTAP increased glucose uptake, lactic acid formation and hyper-production of low molecular weight hyaluronic acid. Castor et al. (1979) have described at least four different types of CTAP: CTAP-I from lymphoid tissue, CTAP-II from a tumor cell line, CTAP-III from platelets, and CTAP-ex from neutrophils.

Korn et al. (1980) found that mononuclear supernates (MCS) stimulated PGE_2 production and suppressed proliferation of human dermal fibroblasts. Wahl et al. (1978) with fibroblasts and Parrott et al. (1980) with synovial cells found MCS to stimulate proliferation and collagen synthesis. Jimenez et al. (1979) found MCS inhibited collagen synthesis of fibroblasts. There are (presumably) both inhibitors and activators in the MCS. Inhibition or activation depends upon the preparation and the cells used.

Burke and Ross (1977), Kohler and Lipton (1974) and Castor et al. (1979) found platelet factors to stimulated synovial cells and fibroblast proliferation. Collagen

synthesis was increased with platelet factors (Burke and Ross, 1977).

One of the problems of bringing the rheumatoid disease into culture is the possibility that "normal" synovial cells placed into an environment conducive to proliferation may behave like rheumatoid cells. Factors (peptides) found in fetal calf serum may have many of the stimulatory and inhibitory factors described above. The only differences in rheumatoid and nonrheumatoid cell lines may be the enrichment of a certain cell population in the cultures derived from RA patients.

4. Effect of Antirheumatic drugs on cells in culture.

The addition of antirheumatic drugs to cells in culture is a means of studying changes that may be related to their mechanisms of action. Castor (1972) found the effect of CTAP was blocked by corticoid, NSAIA and adrenergic blocking drugs (Castor, 1975). Colchicine, choroquine and gold sodium thiomalate were ineffective. Rheumatoid cells were reported to be less responsive than normal synovial cells to hydrocortisone stimulation of proliferation and inhibition of hyaluronic acid synthesis (Castor and Drowitz, 1966). Ponec et al. (1979) reported that glucocorticosteroids decreased collagen synthesis without effecting hydroxylation. Bayer et al. (1979) reported that indomethacin reversibly blocked cell proliferation in the G_1 phase. Schorn et al. (1979) reported that penicillamine suppresses collagen

biosynthesis which may be related to the patient's clinical improvement of rheumatoid arthritis. The effects of drugs in culture or in vitro are a good indication of mechanism but are not proof (i.e., membrane stabilization with steroids (Ignarro, 1972)).

D. Role of Collagen

1. Structure and synthesis.

Collagens are the structural proteins of the extracellular matrix. They consist of three polypeptides, α chains, circa 1000 amino acid long twisted into a helix, 1.5nMX300nM. Only short sequences at each end are nonhelical. In the helical region the molecular formula is (gly-X-Y)333. When Y is proline, it is usually hydroxylated. The hydroxylation of proline is important for a stable helix at body temperature. Imino acids account for 25% of the residues in collagen, limiting the rotation of the polypeptide backbone (Ramachandran and Ramakrishnan, 1976, and Kivirikko and Risteli, 1976).

Collagens are first synthesized as 40% larger procollagens α chains. After the message is transcribed from the DNA, the mRNA is translated on polyribosomes. While the nascent chain is being elongated into the rough endoplasmic reticulum cisterne, some proline and lysine residues are hydroxylated by prolyl and lysl hydroxylases. Some hydroxylation also occurs as the procollagen is transported to the smooth endoplasmic reticulum and the golgi apparatus. Sites

of glycosylation are on hydroxylated lysine residues. The carboxy terminal procollagen extensions have cystein residues that hold three procollagen together for helix formation (Byers et al., 1975). Helix formation is necessary for rapid secretion of functional protein. Some of the procollagen produced may be aggregated and degraded intracellularly (Prockop et al., 1979, Brenkowski et al., 1978).

Once the procollagens are secreted, extracellular-post-translational modifications take place (Goldberg et al., 1975). The procollagen peptides are cleaved at both the amine and carboxy terminal by procollagen peptidases. The collagen aggregates are assembled in a quarterstagger model into fibrils. Lysl oxidase oxidatively deaminates lysine residues leaving aldehyde moieties which participate in intra- and intermolecular covalent crosslinks. Crosslinking decreases the solubility, increases the tensile strength and decreases the susceptibility of collagen fibers to degradation by collagenases (Prockop et al., 1979; Fuller and Mann, 1980 and Tanzer, 1976 and Bailey et al., 1974).

2. Types of collagen.

Five types of collagens requiring seven different genes have been described. Primary structure differences underlies differences in the extent of posttranslational modifications, and therefore, the function of each collagen type. Only recently are the relationship of collagen types to tissue differentiation and disease processes being elucidated (Eyre, 1980; Fuller and Mann, 1980, and Lapiere and

Nusgens, 1976).

Collagen has a nonconventional protein nomenclature. The Greek letter α, β and γ mean monomer, dimer and trimer. The tertiary structure of the collagen molecule is a trimer but separation of denatured collagen results in α, β, γ and higher molecular weight forms due to lysine and histadine derived covalent intra- (holds together the tertiary structure) and inter- (holds together the quatenary structure) crosslinks (Tanzer, 1976). The arabic numbers (1,2) following the Greek letter α indicate the order of the α chain elution from a carboxymethyl cellulose column for that type of collagen. The subscript arabic number is the number of chains found in the collagen molecule. The Roman numeral (I, II...) indicates the collagen type.

Type I collagen, $[\alpha 1(I)]_2\alpha 2$ is found ubiquitously and forms thick fibrils. Only a few of the hydroxylysines are glycosylated. Bone, tendon, dentin, ligaments and scars are predominantly type I collagen (Prockop et al., 1979).

Type III collagen, $[\alpha 1(III)]_3$ is usually associated with type I collagen and is found in greatest abundance in more elastic tissues such as lung, blood vessels, fetal skin and granulation tissue. There is a shift from a type III to a type I collagen predominance with the maturation of skin from fetal to adult as well as in the transformation from granulation tissue to scar tissue (Epstein, 1974, Epstein and Munderlok, 1975, and Bailey et al., 1973 and 1975).

Fine reticulin fibers contain type III collagen but this collagen is not the basis for the histological silver staining (Norwalk et al., 1974 and Timple et al., 1977). Type III collagen has a low hydroxylysine and carbohydrate content but a high hydroxyproline content. The three α1(III) chains are held together by disulfide bonds at the end of the helical region of the carboxy terminal (Chung and Miller, 1974).

Type II collagen is found in only a few tissues like cartilage, vitreous body and cornea which are soft tissues with a high water content. It consists of three α1(II) chains $[\alpha 1(II)]_3$ which are highly glycosylated and form thin fibrils (Miller, 1976).

Type IV collagen $[\alpha 1(IV)]_3$ is a basement membrane collagen. This collagen has a high hydroxylsine content nearly completely glycosylated, a low alanine content and retains much of the procollagen nonhelical extension pieces (Jaffe et al., 1976 and Eyre, 1970). Metastatic tumors produce collagenases specific for this collagen (Liotta et al., 1977 and 1979).

A type V collagen has recently been characterized (Burgeson et al., 1976). The molecular composition may be $\alpha A[(\alpha B)]_2$ or $(\alpha A)_3$ and $(\alpha B)_3$ (Rhodes and Miller, 1978; Deyl et al., 1979). (Type V collagen has not to date been given the typical collagen nomenclature.) This collagen is found ubiquitously but in small amounts. Type V collagen is thought to be a cell surface collagen and may be important in cell migration (Eyre, 1980 and Stenn et al., 1979).

A type I trimer, $[\alpha(I)]_3$ has been reported in inflamed gingiva and is produced by cells in culture (Little et al., 1977, Benya et al., 1977 and Narayanan et al., 1978). This may be an altered type I collagen or a different gene product of $\alpha 1(I)$ with similar composition (Eyre, 1980).

3. Alteration of collagen types with disease.

In some diseases there is an alteration of the tissue composition of collagen types. In some cases the alteration may be the primary lesion as in Ehlers-Danlos syndrome and osteogenic imperfecta or part of the pathogenesis as in cirrhosis and arteriosclerosis. In Ehlers Danlos syndrome type IV, there is a lack of type III collagen synthesis (Pope et al., 1976). In osteogenesis imperfecta, skin cells synthesize a greater proportion of type III collagen (Penttinin et al., 1975 and Sykes et al., 1977). In mitral valve prolapse, ruptured chordae tendinae, Hammer et al., (1979) reported an absence of type III and type V collagens.

In diabetes melatus, an increase in the relative synthesis of type III collagen in skin has been reported (Kern et al., 1979). In liver cirrhosis, there is an increase in both type I and III with type I increasing more than type III collagen (Rojkind and Martinez-Palomo, 1976 and Seyes et al., 1977). Similarly, in arteriosclerosis there is an increase in aorta collagen synthesis with type I collagen predominating (Fisher et al., 1980 and McCullagh and Balian, 1975). With both cirrhosis (Rojkind and Martinez-Palomo,

1976) and arteriosclerosis (Burke and Ross, 1979),as with granulation tissue (Gay et al., 1978 and Bailey et al., 1973 and 1975) there is first an increase in type III collagen followed by an increase in type I collagen. Gay et al. (1978) proposed that the mesenchymal cells predominate early in wound healing and produce predominantly type III collagen, while later mature fibroblasts predominate and synthesize more type I collagen.

4. Synovial collagen.

The data implicating a change in the collagen heterogeneity in the rheumatoid synovium are conflicting. Weiss et al. (1975) found a higher proportion of type III collagen relative to type I collagen in the rheumatoid versus the nonrheumatoid synovium. Their results were criticized by Eyre and Muir (1975a) in that less than 20% of the total normal synovium collagen was pepsin-acetic acid extracted. Using a cyanogen bromide extraction, which extracts more than 80% of the collagen, Eyre and Muir (1975b) found the ratios of type I/III collagen to be similar in rheumatoid and nonrheumatoid synovial tissue. Both groups agreed the rheumatoid collagen was more susceptible to pepsin extraction. Brown et al. (1979) extracted type V collagen from synovial tissue. This collagen represented less than 2% of the total collagen. Normal and rheumatoid synovium have relatively similar amounts.

Subcultured synovial cells in culture produce a similar ratio of type I/III collagen (Parrott et al., 1980 and

Vuorio, 1979). The rheumatoid primary cell cultures produced a greater percentage of type III collagen than the subcultured cells; also, the rheumatoid cell lines maintained a greater propensity for synthesis of type III collagen than nonrheumatoid cell lines (Parrott et al., 1980). Again the rheumatoid disease is heterogenous, as are patients. The factors influencing the types of collagen could be relative to the amount of different cells brought into culture. The age of the patient and the stage of the disease in the synovium at the time of the operation, may also influence the cell culture population.

E. Gold in Rheumatoid Arthritis

1. Efficacy and Toxicity

Forestier (1929) reported beneficial effects of gold therapy in RA patients. Numerous reports followed confirming the efficacy of gold in RA but most were not controlled or double blind studies. Extensive double blind studies were reported by the Research Subcommittee of the Empire Rheumatism Council of Great Britain (1960, 1961) and the Cooperative Clinics Committee of the American Rheumatism Association (1973). Both groups found that gold treated patients had stronger grips, decreased erythrocyte sedimentation rates (ESR) and fewer involved joints. The cooperative clinic did not find radiological improvement but a longer (2 year) double blind study (Sigler et al.,

1974) reported significant radiological improvement with regard to the progression of bone and cartilage destruction. In some patients the disease process was arrested. The percentage of patients benefiting from chrysotherapy is 40-75% depending upon the study (Freyberg, 1972).

The first sign of gold toxicity is pruritis followed by dermatitis. More dangerous common side effects are blood dyscrasias-thrombocytopenia, leukopenia agranulocytosis and aplastic anemia. Patients receiving chrysotherapy may develop a proteinuria which on occasion will warrant discontinuance of gold treatments. There is no correlation between toxicity and therapeutics (Harvey, 1975 and Freyberg, 1972).

2. Pharmacokinetics

a. Whole body-blood and tissue

Gold sodium thiomalate (GST) does not follow the classical drug pharmacokinetics. The gold moiety of the drug is not entirely eliminated (Gottlieb et al., 1972) and the blood levels do not correlate with therapeutics (Gerber et al., 1972) or toxicity (Mascarehas et al., 1972). The accumulation of gold in the tissues is a more important parameter than plasma levels (Smith et al., 1958). The peak plasma gold level, after an intramuscular injection of GST, is 4-6 hours. The half time of elimination is 2-3 weeks, though higher doses and longer duration of treatment will reduce the rate of elimination (Rubinstein and Dietz, 1973).

The thiomalate dissociates from gold sodium thiomalate and is eliminated more rapidly (Jellum et al., 1980). The mean blood gold levels attained in chrysotherapy with GST are between 2-5 ug Au/ml serum (Mascarehas et al., 1972 and Lorber et al., 1973).

There are reversible and irreversible deposits of gold in body tissues. The kidney, liver adrenals and reticuloendothelial system have the highest concentrations (Gottlieb et al., 1972). The synovial tissue concentration of gold is 20-50 ug/Au/g tissue. An inflamed synovium accumulates more gold than a noninflamed synovium (Grahame et al., 1974).

b. Cellular-aurosomes.

The gold deposited in the tissues is internalized by the cells and sequestered in membrane-bound vesicles. The gold containing lysosomes have been named "aurosomes" by Oryschak and Ghadially (1974). Norton et al. (1968) described membrane bound electron dense material in patients lysosomes after intra-articular injection of GST. They reported similar electron dense material after thiomalic acid injection, though Ghadially (1978) has refuted these later findings with thiomalic acid. The organic thio linked gold compounds, gold sodium thiomalate (GST) and gold thioglucose (GSG) Au(I), form lamellar-like structures in the lysosomes and gold chloride, $HAuCl_4$, Au(III), form aggregates in lysosomes (Ghadially, 1979). Electron probe X-ray analysis has confirmed the electron dense material to be gold

(Ghadially et al., 1976). Stone and Galle (1969) observed gold deposits in kidney tubule mitochondria but further work is necessary to confirm this observation (Ghadially, 1978).

Penney et al. (1976) quantitated the subcellular distribution of ^{195}Au in Kupffer cells. The centrifuged fractions corresponding to the nucleus and mitochondria contained the largest percentage of radioactivity. The interpretation of these findings is obscured because gold may alter the density of the organelle, and the aurosomes may become as large as a nucleus. Most of the gold in the cell is bound (Penney et al., 1975, and Lawson et al., 1977). The distribution of gold binding to uncharacterized lysomal "macromolecules" was spread over a wide range. In the cytoplasm, "macromolecules" of 300,000, 40,000, 10,000 and 3,000 dalton bound gold (Lawson et al., 1977).

3. Pharmacodynamic-mechanism of action.

a. Protein interaction

i. Albumin. - Heavy metals have a high affinity for sulfhydryl groups (Harvey, 1975). Ninety-five percent of the serum gold is bound to albumin (Mascarehas et al., 1972). Gerber (1964) found that denatured albumin from the exposure to GST resembles a denatured albumin formed in the presence of reagents that prevent sulfhydryl disulfide interaction. Albumin binding is most likely related to the pharmacokinetics of gold. The pharmacodynamic binding or inter - action of gold is still to be answered.

ii. Collagen. - Adam et al. (1965) found that after 8 weeks of gold thiosulfate (0.2 mg/Kg-rat/weekly) an in vivo staining of collagen occurred in which the collagen banding could be observed with the electron microscope. When the thiosulfate is cleaved, the gold moiety hardens the collagen by forming new crosslinks as demonstrated by the swelling of collagen fibrils and the contraction relaxation time (Adam and Kuhn, 1967). The solubility of collagen and the lathyritic effect of β-amino propionitrile were decreased due to gold thiosulfate (Adam et al., 1967). Bismuth, copper and mercury compounds also cause intra-vital crosslinks (Adam et al., 1968). The concentrations used in these studies are much higher than those used clinically but local concentrations of gold due to dying macrophages loaded with aurosomes may have a hardening effect on the collagen of the pannus.

iii. Lysosomal enzymes. - The inhibition of lysosomal enzymes was proposed by Persellin and Ziff (1966) as a mechanism of action of gold sodium thiomalate. High concentrations of gold (mg/ml) were used to inhibit acid phosphatase, β-glucoronidase and malic dehydrogenase. Tissue concentrations are 50 ug/g but the concentrations of gold in lysosomes, i.e. aurosomes, are much higher (Ghadially, 1979), though most gold in the aurosomes is protein bound (Lawson, 1980).

iv. Compliment. - Schultz et al. (1974) reported the inactivation of C1 (compliment-classical pathway)

with serum concentrations of gold. A lack of serum gold levels and therapeutic effect (Gerber et al., 1972) might indicate Cl and Cl esterase inactivation as a secondary rather than a primary mechanism of action of gold.

b. Cell interaction.

i. Neutrophils. - Mowat (1978) found that neutrophil chemotaxis was inhibited in vitro by gold. Both non-RA patients and RA patients who gained clinical benefit from gold therapy, responders, have neutrophils that showed a gold induced, dose dependent, reduction in chemotaxis. Nonresponders did not exhibit a good dose dependent reduction compared to responders, suggesting that this assay may be a good means to screen for responders and nonresponders to chrysotherapy. More specific blockers of neutrophil chemotaxis have not been shown to be effective anti-rheumatic drugs.

ii. Macrophages. - Jessop et al. (1973) reported the phagocytic activity of neutrophils and macrophages was increased in patients with RA. Patients responding to chrysotherapy have suppressed phagocytic activity due to GST whereas nonresponders did not. Ugai et al. (1979) found the incubation of GST (25 ug/ml) with monocytes in vitro caused a morphological change, aurosomes, and that the phagocytic activity was decreased without significant effect of viability or adherence. The suppression of phagocytosis by gold salts and prednisolone were qualitatively similar

though quantitatively gold is less potent, has a slower onset of action and longer duration of action (Roberts et al., 1973). Even though both gold and steroids are used to treat RA, steroids do not prevent the progression of the disease (Harvey, 1975).

iii. Lymphocytes. - GST, at pharmacologically relevant concentrations, inhibited mitogen and antigen induced proliferation of T-lymphocytes (Lipsky and Ziff, 1977). T-Lymphocytes previously stimulated with concanavalin A were not inhibited by GST. Monocytes reversed the inhibition but preincubation of gold with monocytes interfered with the functional capabilities of the monocytes.

The fact that organic gold therapy impinges on several target systems involved in the generation of the rheumatoid process may be a critical factor accounting for its tolerable or acceptable therapeutic index. An overwhelming effect on any one of these critical functions would be associated with unacceptable toxicity to the patient. Thus, the proven therapeutic benefit derived from chrysotherapy in the management of rheumatoid arthritis may be dependent on a spectrum of pharmacological effects expressed on the different biological components contributing to the rheumatoid process.

4. Other gold compounds.

Metallic gold is ineffective as an antirheumatic drug. Gold chloride is effective but too toxic (Freyberg, 1972). The thio-gold compounds were originally used because

of their solubility (Jaffe, 1977) and have since been found to be more effective and less toxic than nonsulfur containing gold compounds (Freyberg, 1972).

a. Gold thioglucose.

Gold thioglucose is used to treat RA. In mice and guinea pigs gold thioglucose accumulates in the ventromedial hypothalamus and causes obesity (Dalferes et al., 1975 and Chang and Persellin, 1968). Herman et al. (1972) found gold thioglucose added to mouse fibroblast cultures increased anphagic vacuoles with lipid droplets.

b. Auranofin.

A new gold compound, still in phase III clinical trials, SK&F D39162, S-triethylphosphine gold 2,3,4,6 tetra-O-acetyl-1-thio-B-D-glucopyranoside, auranofin, can be taken orally rather than the intramuscular injection required for GST. Like GST, auranofin has been shown to be efficacious in the adjuvant rat model (Walz et al., 1976) and with RA patients (Finkelstein et al., 1976 and 1980). GST was shown to inhibit lysosomal enzyme activity (Presellin and Ziff, 1966 and Ennis et al., 1968) but not enzyme release (Ennis et al., 1968 and Pepper et al., 1974). Auranofin blocks release of leukocytes lysosomal enzymes (DiMartino and Walz, 1977 and Finkelstein et al., 1977). DNA and protein synthesis in lymphocytes (Finkelstein et al., 1977) and Hela cells (Simons et al., 1979) were inhibited by auranofin. With lymphocytes, the transport mechanism was inhibited as demonstrated by [^{3}H]-2-deoxy-D-glucose but, not with Hela

cells. Morphological changes with auranofin include aurosome formation (Thomas and Ghadially, 1977) and surface blebbing on Hela cells (Simon et al., 1979). Thus, the spectrum of auranofin effects reveals both similarities and differences compared to GST. It is not known if both GST and auranofin have the same mechanism of action in RA.

III. METHODS

A. Materials

All chemicals used in this investigation were reagent grade or better. For cell culture: Dulbecco's modified eagles medium (DME), fetal calf serum (FCS), penicillin-streptomycin, fungizone, trypsin and trypan blue were all obtained from Grand Island Biological Co., Grand Island, N.Y. Tissue culture flasks,test tubes and pipets were from Corning Glass Works, Corning, N.Y. Gentamycin was from Schering Corp., Kenilworth, N.J. Ascorbate β-aminopropionitrile (BAPN) and ethylenediaminetetracetic acid (EDTA) were all from Calbiochem, Los Angeles, Ca. Isotope [^{14}C] proline (260 Ci/mole, 2-3 [^{3}H]proline (25 Ci/mole) and [^{3}H] thymidine [^{3}H] TdR(542 Ci/mole) were all from New England Nuclear, Boston, Ma. The scintillation cocktail, Hydromix,^{TM} was from Yorktown Research, Miami, Fl. Enzymes used were: ribonuclease (bovine pancrease, A grade, 65000 U/mg) from Calbiochem, Los Angeles, Ca., pepsin (2500 U/mg) from Worthington Biochemical Corp., Freehold, N.J., and bacterial collagenase (2600 U/mg) from Advance Biofacturers Corp., Lynbrook, N.Y. For the nucleic acid assay DNA (calf thymus, type I), RNA (yeast, type XI) and ethidium bromide were all from Sigma Chemical Co., St. Louis, Mo. Carboxymethyl cellulose (CM-52) was from Whatman, Kent, England. For

polyacrylamide gel electrophoresis, electrophoretic grade: sodium dodecylsulfate (SDS), acrylamide, N,N' methylenebis-acrylamide (BIS), N,N,N',N'tetramethylenediamine (TEMED) and ammonium peroxydisulfate were all Eastman Kodak Co., Rochester, N.Y. Acetone (spectrometric grade) and B-mercaptoethanol were both from Aldrich Chemical Co., Milwaukee, Ws. The fluor 2-methoxy-2,4-diphneyl 3(2H) furanone (MDPF) was from Hoffman La Roche, Diagnostic Division, Nutley, N.J. Glutaraldehyde for cell fixation was from Eastman Kodak Co., Rochester, N.Y. Gold sodium thiomalate (GST) was from Merck, Sharp & Dohme, West Point, Pa.

B. Culture Method

Synovial tissues were obtained from patients with RA who were undergoing reconstructive joint surgery at the Roger Williams General Hospital, Providence, R. I. Synovial tissue identified as rheumatoid was obtained from patients fulfilling the American Rheumatism Association criteria for rheumatoid arthritis. The rheumatoid patients had advance erosive stages of the disease that required surgical intervention. In all cases, histological examination of the excised tissue revealed chronic synovitis consistent with rheumatoid arthritis.

The tissue obtained during surgery was dissected free of any excess fat, capsular and cartilage tissue, and finely cut into approximately $1mm^3$ pieces; about fifteen tissue pieces were placed in each 100 x 20 mm tissue culture dish.

The cultures were incubated at 37° in the presence of 95% air and 5% carbon dioxide. The cells were grown in Dulbecco's Modified Eagles (DME) medium containing 10% (v/v) heat denatured fetal calf serum (FCS) plus 100 units penicillin and 100 ug streptomycin/ml medium or gentamicin sulfate (50 ug/ml medium) and Fungizone (2.5 ug/ml medium). After one or two weeks the cells could be seen migrating outwards from the tissue pieces and after approximately four weeks the cells became confluent and were subcultured into 75 cm^2 tissue culture flasks. To subculture (passage) the cells, the medium and original tissue fragments were removed by suction and the cell layer removed from the dish by incubation for five minutes in isotonic phosphate buffered saline (PBS) (ph 7.0) containing 0.1% trypsin 0.2 mg/ml glucose and 0.2 mg/ml ethylenediaminetetraacetic acid (EDTA). After detachment, the cells were resuspended in fresh medium and replated. Two weeks after the first passage the synovial cells were subcultured again and this time replated at one-third of their confluent density. The cells were usually confluent again and subcultured after a further two-week period. The cell medium was normally replaced with fresh media every 4-6 days. All experiments were performed with cells during second to fifth passages. The cell cultures were tested for and found to be free of mycoplasma contamination using a fluorescent assay (Bioassay Systems, Cambridge, MA).

Gold sodium thiomalate (GST) was diluted in sterile distilled water. A 0.01 vol. of drug solution was added to culture medium.

C. Cell Number Determinations

1. Hemocytometer.

With the hemocytometer determinations, cells were diluted with medium and both sides of a hemocytometer (American Optical) were filled. The four corner squares and center square from each side was counted. To determine the viability of the cells a 1:1 (v/v) of 0.04% trypan blue was added to diluted cells which were then counted as described above. Dead cells retained the dye and appeared dark blue under the microscope.

2. Coulter counter.

For Coulter counter determinations, cells were diluted with filtered PBS and the total number of particles in the channels calibrated from 10-60 microns diameters were used for cell counts. Mean cell size was determined by multiplying the number of particles in each channel by the mean particle size of that channel. The product, the total cell layer volume, was divided by the total number of cell particles.

D. DNA Assays

1. Ethidium bromide.

The DNA content of the cell layer was assayed by a modified method of Prasad et al. (1972). The cell suspension was sonicated twice at 50 watts for 10 seconds. A 0.1-0.5 ml aliquot of the cell sonicate was added to 10X75

disposable test tubes. The total volume was brought up to 0.5 ml with PBS. An equal volume (0.5 ml) of 20 ug ethidium bromide/ml PBS (pH 7.5) was added. The fluorescence in the test tubes was determined in an American Bowman spectrophotometer at an excitation of 360 nm and an emission of 590 nm. The RNA was digested by adding 10 ul of 20 mg ribonuclease H_2O then incubating at 50° for 30 min. The fluorescence due to the RNA in the cells was found to be minimal in the assay. Therefore, the RNA digestion step was eliminated. DNA was dissolved in phosphate buffered saline (PBS) pH 7.5 and 1-5 ug/tube were used as standards.

2. [^{3}H]Thymidine

The rate of DNA synthesis was measured by adding 0.5 uCi[^{3}H]thymidine([^{3}H]TdR)/ml medium 4 hr before harvesting the cell layer. Cells were released from the dishes using the trypsin solution described above and precipitated twice in a 10% (v/v) trichloracetic acid (TCA) solution. Precipitent was separated from supernate by 3000g centrifugation for 30 min. The TCA pellet was dissolved in 1 ml of 0.05 N NaOH, and radioactivity was determined in a Packard Tricarb liquid scintillation counter.

E. Gold Determination-Atomic Absorption

The amount of gold in the sonicated cell layer was determined using a Perkin Elmer model 460 atomic absorption spectrophotometer. Gold sodium thiomalate (GST), 50% (w/w)

gold was diluted with water as the standard. The linear range was from 5-50 uM. The cell sonicates were diluted with water and absorption measured at 242.8 nM. A quantitative increase in absorption was found by spiking the biological samples with known quantitates of GST as an internal standard.

F. Collagen and Noncollagen Protein Synthesis

Flasks of synovial cultures were incubated for 24 hrs with DME medium containing 2,3[^{3}H]proline 10 mci/ml β-aminopropionitrile (BAPN) 100 ug/ml ascorbate 100 uM. The medium was removed and dialyzed exhaustively at 4° against 0.1M tris-HCl buffer pH 7.4. The cell layers were trypsin released and cell number or DNA content determined as described above.

The measurement of [^{3}H]collagen and [^{3}H]noncollagen protein synthesis was determined by the collagenase digestible protein assay as described by Peterkofsky and Diegelman (1971). Bacterial collagenase was confirmed to be protease free by the failure of the enzyme solution to degrade ^{14}C-tryptophan-labeled Echerichia coli protein. (Collagen does not contain tryptophan.) From the dialyzed medium a 0.1-0.5 ml aliquot, ±100 units bacterial collagenase were incubated for 3 hrs at 37°. The incubation medium, 0.8 ml total volume, contained 0.25 mM $CaCl_2$, 2.5 mM NEM and 0.2M Tris HCl pH 7.4. At the end of the incubation, 0.1 ml 1%

bovine serum albumin (BSA) and 0.1 ml TCA (50%) were added. The precipitates and solutions were chilled at 4° for 1-24 hrs. After centrifugation, 3000g for 30 min, the supernate was removed and 0.1 ml BSA (1%) was added for a second precipitation. After a second centrifugation of 30 min at 3000g the supernates were placed in 10 ml of Hydromix™ and radioactivity determined.

The percent collagen was calculated according to the following formula:

$$\% \text{ Collagen} = \frac{(\text{CPM}[^3\text{H}]\text{proline in collagen}) \times 100}{5.4 \times (\text{CPM}[^3\text{H}]\text{proline in noncollagen}) + (\text{CPM}[^3\text{H}]\text{proline in collagen})}$$

where the total protein equals the dialyzed medium CPM, the collagen protein equals the CPM of supernate with collagenase minus the supernate CPM without collagenase and the noncollagenase protein equals the total protein minus collagen protein. When the pellets were analyzed total protein equals CPM of pellet with collagenase and collagen protein equals the total protein minus noncollagen protein. The pellet method is variable because the quantitation of collagen is a small difference between two large numbers, as opposed to the supernate method. The above formula accounts for the 5.4 times greater amount of proline in collagen than in noncollagen protein.

G. Medium Collagen Assay for Type I and III Collagen

1. Extraction.

Cells in 75 cm flasks were incubated with 10 ml of

fresh medium containing B-aminoproprionitrile (BAPN) (10^{-4} g/ml) and ascorbate (10^{-4}M). Ascorbate was added to the flasks every day during the incubation period. [^{14}C]proline or 2,3[^{3}H]proline (1 uCi/ml medium) was added 48 to 72 hrs before the medium was harvested. Labeled proline incorporation in this system was found to be linear over a 72 hr period. In the presence of BAPN to inhibit collagen crosslinking and ascorbate to ensure hydroxylation, the amount of radioactive collagen present in the cell layer was below the limit of detection of the collagenase digestion assay (Kuttan et al., 1979). In view of this, these studies were conducted on collagen harvested only from the medium; the cell layer was used for the cell proliferation studies described above. Medium protein was precipitated by adding ammonium sulfate crystals to 45% saturation. After overnight incubation at 4°, the precipitate and solution were centrifuged at 3000 g for 30 min. The pellet (which contained more than 95% of the nondialyzable hydroxyproline) was dissolved in 1 ml of 0.5 M acetic acid and then dialyzed in the cold at 4° against 0.5 M acetic acid. Pepsin (100 ug/ml) was added to the dialysis bags to digest non-collagen protein and convert procollagen to collagen. Dialysis was continued for another 2 days and the samples were then lyophilized or dialyzed against .05M sodium phosphate pH 7.4.

2. Sample preparation for electrophoresis.

Samples were prepared for electrophoresis as

described by Goldberg and Fuller (1978) by dissolving them in 0.05 M sodium phosphate (pH 7.2) containing 0.2% sodium dodecylsulfate (SDS) and 10% sucrose. The pH was adjusted with sodium hydroxide and the proteins were made fluorescent by the addition (with mixing) of 0.2 vol. of a 2-4 mg MDPF*/ml acetone solution. Disulfide bonds of type III collagen were reduced (R) by heating the alkaline samples (pH = 10-11) at 56° for 15 min. Nonreduced (NR) samples were prepared by returning the fluorescent samples to neutrality (pH = 7-8) with hydrochloric acid before heating.

3. Electrophoresis procedure (MDPF-SDS-PAGE)

Samples were electrophoresed according to the procedure of Neville (1971). This is a discontinuous Tris-borate buffer system with SDS only in the upper buffer and sample preparation buffer. The upper buffer is 0.5% Tris, 0.25% boric acid and 0.1% SDS. The lower buffer is 0.15M Tris-HCl pH 8.5. The gels (5mm x 55mm) used to separate collagen components were 6% acrylamide, 1% N,N'-methylene-bisacrylamide(BIS), 0.05% ammonium peroxy-disulfate, 0.1% N,N,N',N'-tetramethylendiamine (TEMED) and 0.15M Tris HCl pH 8.5. The stacking gels were 4% acrylamide, 3.3% Bis, 0.1% TEMED, 0.05% ammonium peroxydisulfate and 0.04M Tris-HCl pH 6.1. The samples were electrophoresed (Buchler Instruments) into the stacking gels (0.5-1.0 h), at 0.2 mAmps per tube; the current was increased to 1.0 mAmp per

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*MDPF=2-methoxy-2,4-diphenyl-3-(2H)-furanone.

tube for electrophoresis in the running gel for 2.5-3.5 at room temperature.

4. Quantitation of collagen types I and III.

a. Scanning procedure.

The quantitation of fluorescence in the gels was determined as described by Goldberg and Fuller (1978). After electrophoresis the gel tubes (containing gels) were either sealed with parafilm and stored at 4°C or placed in the gel tube holder of the Gilford linear transport gel scanner. Fluorescence scanning was done with a Gilford 250 spectrophotometer equipped with a Corning 840 recorder, a Gilford 2520 gel scanner, and the Gilford 2515 fluorescence gel accessory. The Model 2515 has a light source and excitation filter to provide an excitation energy at 390 nm at right angles to the optical path of the spectrophotometer; the emission filter placed between the gel tube and the photometer allows emission light to pass through at 480 nm. The Model 2515 also includes a signal converter which performs an antilog conversion of the photometer signal. Therefore, the recorded signal is proportional to the light received. To standardize measurements, standard quinine solutions in gel tubes sealed with parafilm were placed in the linear transport. By adjusting the recorder span control the pen of the recorder was set to record the output of the spectrophotometer in accord with the following equation:

$$RFI = \log^{-1} (2 - Abs.)$$

where RFI is the relative fluorescent intensity measured by the percentage of pen movement. Abs is the absorbance units read from the Gilford spectrophotometer Model 250.

b. Fluorescence of band.

The gels were scanned at 2 cm/min with a chart speed of 4 cm/min. The areas under the peaks were quantitated using a Numonic Electronic Planimeter Model 210-117, with peak area given in square centimeters. (Full-scale pen deflection on the Corning recorder was 25 cm.) The fluorescence in the gels was quantitated by the following equation:

$$RFI \times mm = \frac{\text{area } (cm^2) \times \text{gel scan rate (cm/min)} \times 10 \text{ (mm/cm)}}{\text{scale factor (cm/RFI)} \times \text{chart speed (cm/min)}}$$

where RFI x mm is the relative fluorescent intensity corrected for band width on the gel. Increasing the instrument scale factor results in a linear increase in sensitivity of the recorder thereby increasing pen deflection (cm) per unit of RFI. The area under the peaks recorded from fluorometric scanning of MDPF-labeled $\alpha 1(I)$, $\alpha 2$, and $\alpha 1(III)$ was linear from 10^{-5} to 10^{-8} g. The standard curves for the three α chains were similar. The results from nonreplicate determinations had an SE of $\pm$ 6. Using a standard curve from purified $\alpha 1(I)$ calf skin collagen, the fluorescent area (RFI X mm) of synovial cell medium MDPF-labeled collagen was converted to amount of collagen α chains.

c. Radioactivity of bands.

The radioactivity in the bands corresponding to chains was determined by dissolving 5 mm slices in 0.2 ml hydrogen peroxide and then heating at 60° for 4 hr or at 37° overnight. Ten milliliters Hydromix™ were added to the vials, and the radioactivity was determined in a Packard liquid scintillation counter.

The percentages of type I and type III collagen in SDS polyacrylamide gels were calculated from the following formulas:

$$\% \text{ Type I} = \frac{3}{\frac{\alpha 1(R)}{\alpha 2(R)} + 1} \times 100$$

$$\% \text{ Type III} = \frac{\frac{\alpha 1(R)}{\alpha 2(R)} - \frac{\alpha 1(NR)}{\alpha 2(NR)}}{\frac{\alpha 1(R)}{\alpha 2(R)} + 1} \times 100$$

where $\alpha 1(NR)$ and $\alpha 2(NR)$ are CPM found in the gel slices or fluorescence found in the peaks at the $\alpha 1$ and $\alpha 2$ chain positions, respectively, after electrophoresis of a nonreduced sample; and where $\alpha 1(R)$ and $\alpha 2(R)$ are the CPM found in the gel slices or fluorescence found in the peaks at the $\alpha 1$ and $\alpha 2$ chain positions, respectively, after electrophoresis of a reduced sample.

H. Carboxymethyl Cellulose Chromatography (CMC) of Collagen

Collagen [^{14}C]labeled samples were analyzed by carboxymethyl cellulose chromatography, using the procedure described by Miller (1976). A 1.8 x 10 cm column (Pharmacia,

Uppsala, Sweden) was filled with carboxymethyl cellulose equilibrated in 0.06 M sodium acetate buffer (pH 4.8) and maintained at 42°. The ^{14}C-labeled collagen samples, containing 2-4 mg of type I and type III calf skin collagen (prepared as described previously (Fujii and Kuhn, 1975)) as carrier, were denatured by heating at 56° for 30 min in the presence of 10 M urea. The samples were reduced by the addition of 1% (v/v) B-mercaptoethanol and applied to the column. The collagen α chain components were eluted from the column using a linear salt gradient; the starting buffer consisted of 200 ml of 0.06 M sodium acetate buffer (pH 4.8), and the final buffer consisted of 200 ml of starting buffer containing 0.1 M sodium chloride. The eluant was collected in 12-ml fractions; 1 ml of each fraction was mixed with 10 ml Hydromix^{TM} and the radioactivity was counted. The percentages of type I and type III ^{14}C-labeled collagen were calculated from the radioactivity eluted in the peaks that correspond to α1(1), α1(III) and α2 chains. The α1(I), α1(III) and α2 peaks were confirmed by their electrophoretic migration on SDS-polyacrylamide gels.

I. Purification of Type I and III Collagen Standards

The procedure adopted for the purification of collagen was that of Fujii and Kuhn (1975). Skin from a newborn calf was extracted in cold 0.05M Tris-HCl pH 7.5, 1M NaCl for several days. The insoluble material was extracted in

the cold, with 10% acetic acid for several more days. The remaining skin and extract were both lyopholized. Freeze-dried skin, 100g, was rehydrated in 25 l of 0.1% acetic acid pH 2.0 (adjusted with HCl), 25.0 g pepsin was added slowly with stirring, after 24 h 17.5 g pepsin was added for a further 24 h digestion at room temperature.

The solubilized material was filtered through sintered glass wool. Sodium chloride crystals were added to the filtrate to give a final concentration of 0.9M sodium chloride; the mixture was allowed to stand overnight at 4° C. The collagen that precipitated out was collected by continuous centrifugation at 15,000Xg for 1 hr. The pellet was resuspended in 0.5M Tris-HCl pH 7.5, to inactivate the pepsin for four days, and then centrifuged at 35,000Xg for 1 hr. The supernatant was lyopholized.

Differential salt precipitation (DSP) was performed to harvest the pepsin released Type I and Type III collagen; 30g of the freeze-dried supernatant was dissolved in 10 l of .05M Tris-HCl pH 7.5, 1M sodium chloride. Sodium chloride-(4M) was added to make a final salt solution of 1.7M sodium chloride. The volume of 4M NaCl added was determined by the following equation:

$$C_i V_i + C_a V_a = C_f (V_i + V_a)$$

where C_i is the initial salt concentration (1M), V_i is the initial volume (10 l), C_a is the added concentration of salt (4M) and C_f is the final desired salt concentration (1.7M).

The equation is solved for V_a which is the volume of 4M sodium chloride added. The solution was allowed to stand for 24 hr at 4° and then centrifuged at 35,000Xg for 2 hr. The precipitant was washed by centrifugation three times in 0.5 M Tris-HCl pH 7.5, 1.7M NaCl. It was resuspended in 1% acetic acid and dialyzed against 1% acetic acid, and then centrifuged at 55,000Xg for 1 hr. The supernatant was lyopholized and characterized as type III collagen by further purification gel electrophoresis, and cyanogen bromide peptide mapping. The salt concentration of the supernatant was then increased as described previously, again this time from 1.7M sodium chloride to 2.5M sodium chloride with 4M sodium chloride. The resulting precipitant was allowed to settle for 24 hr and harvested by centrifugation at 35,000Xg for 2 hr. The pellet was resuspended in 1% acetic acid dialyzed against 1% acetic acid and then centrifuged at 55,000Xg 1 hr. The supernatant yielded 10g of Type I collagen. The collagen was characterized by further purification, gel electrophoresis, and cyanogen bromide peptide mapping.

J. Phase Contrast Photomicrographs

Phase contrast photomicrographs of cells in growing monolayers were obtained using an American Optical inverted microscope equipped with Expo star automatic shutter control format 35mm (2.8X), ASA 16 and Reciprocity B. The film used was Kodak high contrast copy film HC135-36.

K. Electron Microscope (EM)

Three types of preparations were used to examine synovial cells and tissue with the EM. Osmium stained preparations which stained membranes were used to examine ultrastructures and for X-ray probe analysis.

1. Nonosmium procedure.

For the nonosmium treated preparation,[1] cells were fixed in the culture dishes with 1.6% (v/v) gluteraldehyde-PBS pH 7.3 for 30 min at 4°. The cell layers were scraped off with rubber policemen, transferred to a 15 ml conical centrifuge tube and centrifuged at 2,000 RPM for 5 min. The pellets were resuspended in 200 ul of PBS pH 7.3 and transferred to a 400 ul microcentrifuge tube (Eppendorf). The pellet was washed three times by centrifugation, 8000 RPM for 5 min. A drop of PBS, approximately half the volume of the pellet, was added after the last wash to maintain moisture. The samples were dehydrated with alcohol and epon embedded. Sections, using a Sorvall MT-1, were stained with uranyl acetate and lead acetate and examined with a Siemens 101-TEM.

2. Osmium procedure.

For osmium fixed cells,[2] the cells were first fixed

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[1]The EM work was conducted by Dr. Elizabeth LaDuc at Institut de Rechurches sur le Cancer, Boite #8, 9480 Villejif, France.

[2]The EM work was conducted by Grant Jolly at the Roger Williams General Hospital, Department of Pathology, Providence, Rhode Island.

with 1.5% gluteraldehyde in 0.1M sodium cacodylate buffer and then post fixed in 1% osmium tetroxide in 0.1M sodium cacodylate buffer. The cell pellet was embedded in epon and 1 micron sections were stained with toladine blue. Ultra-thin sections (60 nM) were cut using a LKB ultratome III then stained with uranyl acetate and lead citrate. Representative photomicrographs were taken with an RCA EMU-4C.

3. X-ray probe analysis.

For X-ray probe analysis[3] cells were fixed in 2% gluteraldehyde, 2% parafermaldehyde-Cacodylic acid buffer. An energy level of 75 KeV in a Hatachi H500-1 was used to obtain the L and M shells for gold.

L. Statistics

Linear regression lines for plots were drawn using a Hewlett Packard XY plotter model 9862A connected to a Hewlett Packard model 10 calculator. A Texas Instrument SR-52 programmable calculator was used for statistical analysis.

1. Mean: $\bar{X} = \frac{\Sigma X_i}{n}$ $\quad i=1,2,\ldots,n$

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[3]The X-ray probe analysis was conducted by Philip McGrath and John Ewell at the Bureau of Biologics, Division of Pathology, Food and Drug Administration, Building 29, National Institute of Health, Bethesda, Maryland.

where n = sample size, X_i = value for each sample

2. Standard error: $S.E. = \sqrt{\frac{\Sigma (X_i - \bar{X})^2}{n(n-1)}}$

3. Linear regression: $Y = \hat{b}_o + \hat{b}_1 X + e$

a. Slope: $\hat{b}_1 = \sqrt{\frac{\sum (X_i - \bar{X})(Y_i - \bar{Y})}{\sum (X_i - \bar{X})^2}}$

b. Intercept: $\hat{b}_o = \bar{Y} - \hat{b}_1 \bar{X}$

c. Correlation coefficient $r = \sqrt{\frac{\Sigma (X_i - \bar{X})(Y_i - \bar{Y})^2}{\sum (X_i - \bar{X})^2 \Sigma (Y_i - \bar{Y})^2}}$

4. Student t test

$$t_{(n-2)} = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{\left(\frac{ss_1 + ss_2}{(N_1 - 1) + (N_2 - 1)}\right)\left(\frac{1}{N_1} + \frac{1}{N_2}\right)}}$$

d,f. = $(N_1 + N_2 - 2) = (n-2)$

5. One way analysis of variance (ANOVA) with completely randomized design.

a. ANOVA TABLE

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F
Treatment	K-1	$\sum^{K} T\cdot j^2 n - T..^2/n$	MSTr	MSTr/MSE
Error	n-K	$\sum\sum Y_{ij}^{Kn\ 2} - \sum^{K} T\cdot j^2/N$	MSE	
Total	n-1	$\sum\sum Y_{ij}^{km\ 2} - T..^2/n$		$F_{k-1,n-K,1-\alpha}$

NK=n i=1,2,...,N j=1,2,...,K

b. Post Hoc ANOVA was Duncan's multiple range test

$$R_{p=r_p} \sqrt{\frac{MSE}{n}} \quad p=2...K$$

6. A $P < 0.05$ was considered significant.

IV. EXPERIMENTAL RESULTS

A. Morphology

1. Light microscope.

When synovial cells were examined by phase contrast microscopy, perinuclear vacuoles were observed in the synovial cells which had gold in the medium. In order to determine the time and concentration dependence on vacuole formation, synovial cells were grown in medium containing 250, 125, 100, 60, 50, 30, 25, 15, 10, and 7.5 uM gold sodium thiomalate (GST) containing medium. Cells were observed daily and representive photomicrographs were taken (Fig. 1). The onset of vacuole formation occurred between 2-3 days for concentrations between 250-100uM. The number and the size of the vacuoles increased with time of incubation to a maximum observed at 5-7 days when most of the cells had vacuoles. There was also a decrease in the observed number of cells in the cultures. If the gold (250-100uM) was removed from the medium the observed number of cells remained the same and the cultures did not grow to confluency even after subculturing. The number of cells retaining trypan blue did not increase due to the GST exposure (Table 1).

Vacuole formation was found at 60 and 50uM GST after 5-7 days and an increased number and size of vacuoles was observed over the following week. The size and number of

Fig. 1. Phase contrast photographs of synovial cells exposed to GST (200X). (a) control (b) 30 uM GST for 14 days (c) 125 uM GST for 4 days (d) 125 uM for 7 days.

TABLE 1

EFFECT OF GOLD SODIUM THIOMALATE (GST) ON CELL VIABILITY

GST[a] (uM)	Cell Counted[b] per flask	Percent[c] Viable
0	157 ± 20 (4)[d]	98.8 ± 0.5
10	119 ± 7 (3)	98.3 ± 1.2
30	48 ± 6 (4)[e]	95.3 ± 1.4
100	31 (23-39)[f]	89 (83-95)

[a]Cells were exposed to GST for one or two weeks.

[b]Cells were counted with a hemocytometer.

[c]Viability was determined by trptan blue exclusion.

[d]Mean ± S.E. (n = number of flasks) from two experiments.

[e]$P < 0.01$.

[f]Mean (range) for two flasks.

vacuoles after two weeks of 60 and 50uM GST were less than those obtained for 100uM GST for one week. Only a minimal amount of vacuole formation was obtained from the 30 and 25 uM concentrations after two weeks of exposure. Synovial cells did not develop vacuoles after one month exposure to the lower concentrations.

Exposure of the synovial cells for one week to 100uM of mercury, lead, cadmium, silver, copper, zinc, sodium thiomalate and gold chloride did not reveal the characteristic vacuole formation observed with gold sodium thiomalate (GST).

2. Electron microscope (EM).

In order to determine if the vacuoles contained gold, cells were fixed with gluteradlehyde at various times after exposure to GST. In cultures (100uM for one week) that presented numerous vacuoles by phase contrast microscopy, many such vacuoles were found with electron dense material by EM (Fig. 2). At the low GST concentration (10uM for one week) that did not have any evident vacuoles by light microscopy, only a few electron dense vacuoles were found after examining many cells. X-ray probe analyses confirmed that the electron dense material in the membrane bound vesicles is gold.

a. In vivo and in vitro aurosomes.

Oryshak and Ghadially (1976) named the membrane bound vesicles of gold found in cells aurosomes. Fig. 3 is a comparison of aurosomes observed both in vivo and in vitro.

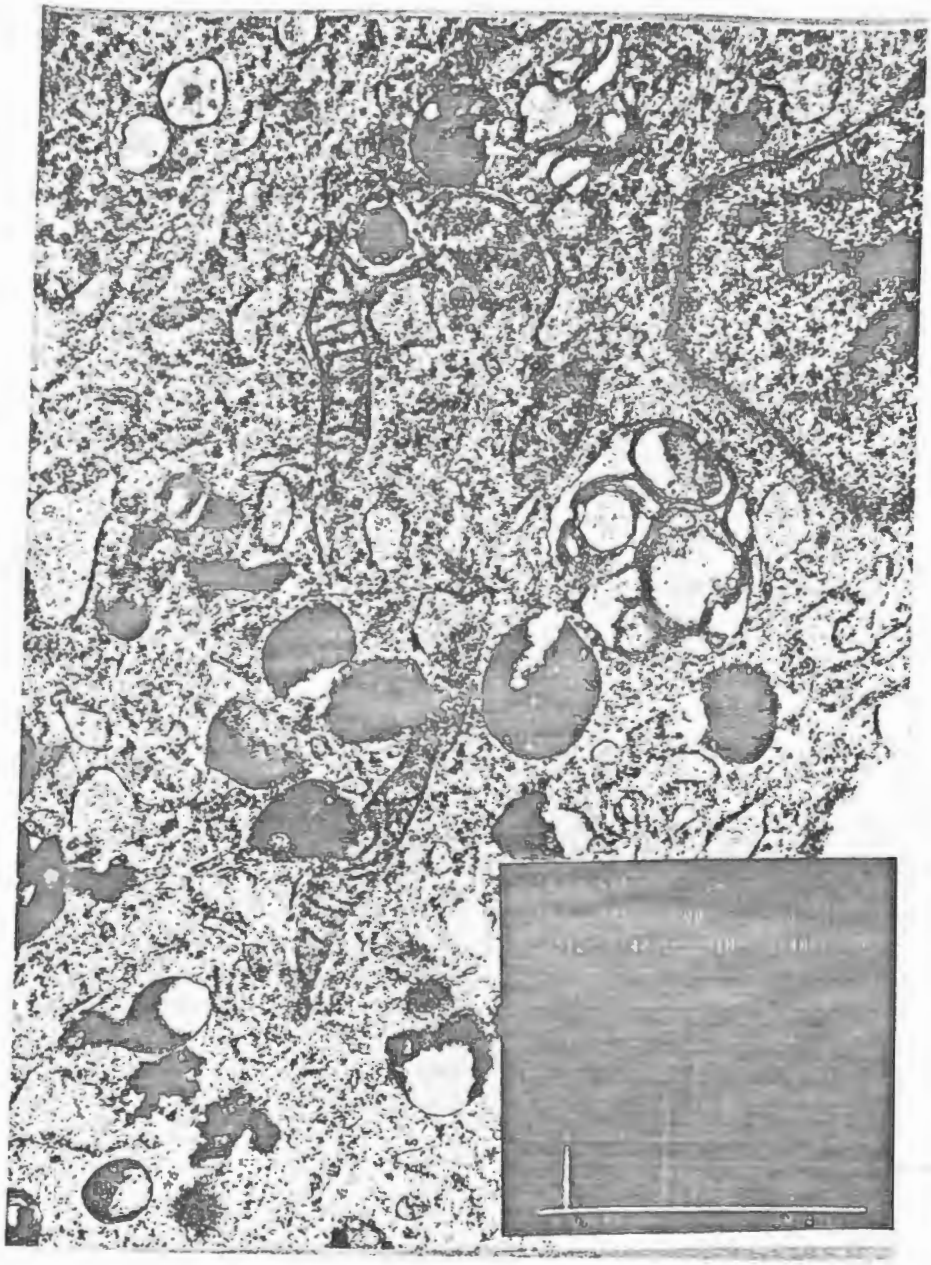


Fig. 2. EM photograph of aurosome and X-ray probe analysis. Cells were stained with osmium. A portion of the nucleus is in the upper right-hand corner. Beneath the nucleus is a golgi apparatus. Three longitudinal sections of mitochondrias are to the left of the golgi. Electron dense areas in the membrane bound vesicles had an X-ray probe analysis for gold, lower left. The bars in the center are the theoretical lines for the L&M shell of gold. Beneath the bars is the scan of an electron dense area. The large peak in the center is from the copper in the copper grid.

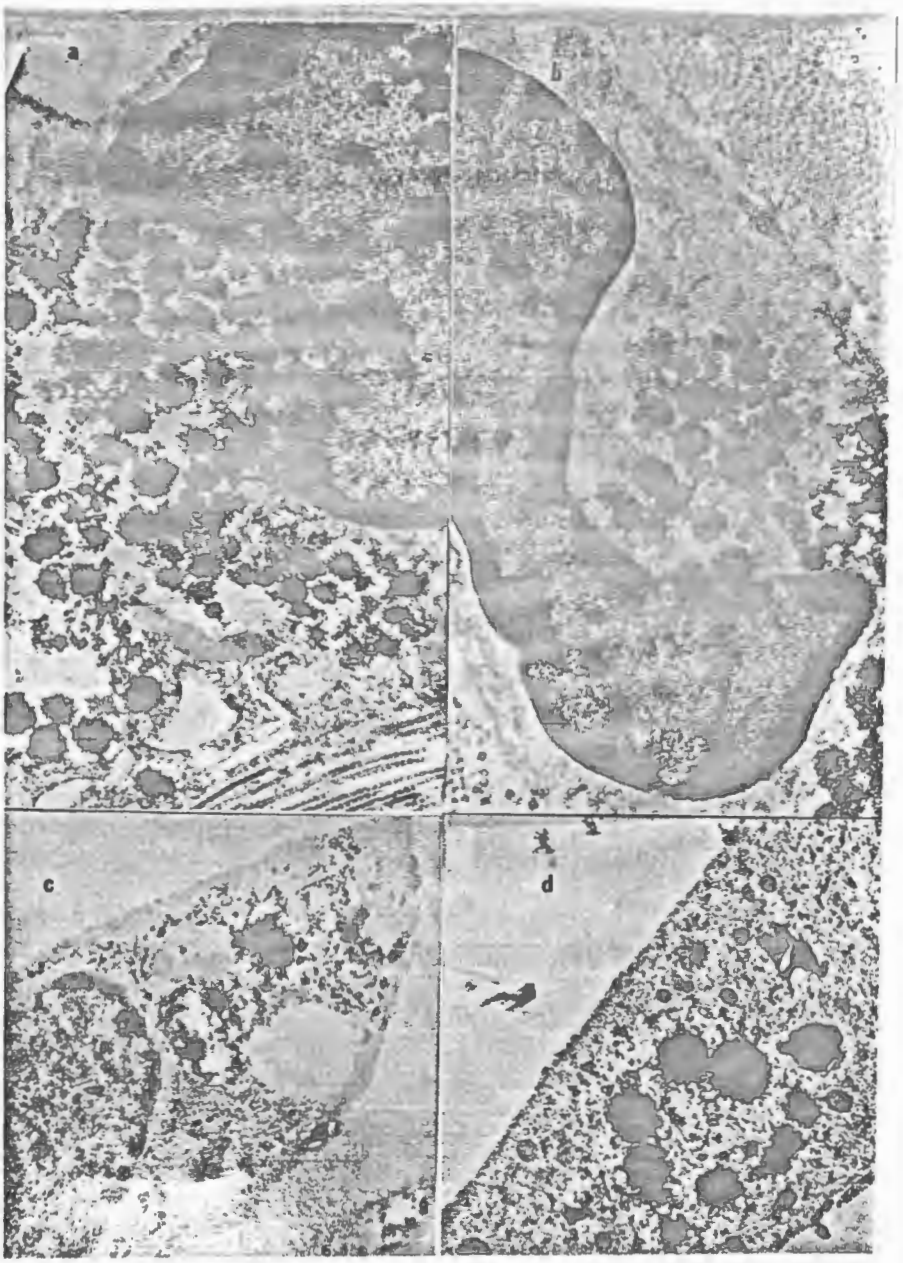


Fig. 3. In vivo and in vitro aurosomes. Synovial tissue (a and b) and synovial cells (c and d) were not osmium stained. (a) In the lower right-hand corner is collagen; in the upper right-hand corner is the nucleus. The many electron dense areas to the left of the nucleus are gold aggregates in a lysosome. (b) Aurosome to the right of the nucleus. (c) and (d) Electron dense gold aggregates in the lysosomes of synovial cells in culture.

The patient donor of this section had been receiving gold therapy for 2 years and had discontinued gold therapy for several months before synovectomy. The aurosome structures are nearly identical in both the sections of synovial tissue and in the gold induced aurosome in the cultured synovial cell.

b. Genesis of the aurosome in culture.

Fig. 4 is a proposed sequence in the development of an aurosome. First the gold is precipitated with proteins to form an electron dense aggregate outside the cell. The gold aggregates are phagocytosed or pinocytosed. The protenous core is broken down (possibly by enzymes) and plate-like structures and small round electron spheres or rods in cross-sections become numerous. Eventually the proposed protenous core is dissolved leaving just aggregates of lamellae structures.

c. Nuclear change.

The exposure of cultured synovial cells to GST (125 uM) results in a nuclear change. Chromatin clumping near but not attached to the nuclear envelope occurred in some nuclei (Fig. 5). This was not found as consistently as aurosome formation. An anti-malarial drug quinaqrine can cause similar nuclear changes (LaDuc et al., manuscript in preparation). Anti-malarial drugs are also second-line drugs used in RA.

Fig. 4. Genesis of aurosomes. Cells were not osmium stained. (a) An electron dense aggregate is pinocytosed. (b) Many small particles break off the electron dense material in a lysosome. (c) Lamellae structures form in the lysosome. (d) The protein core is dissolved.

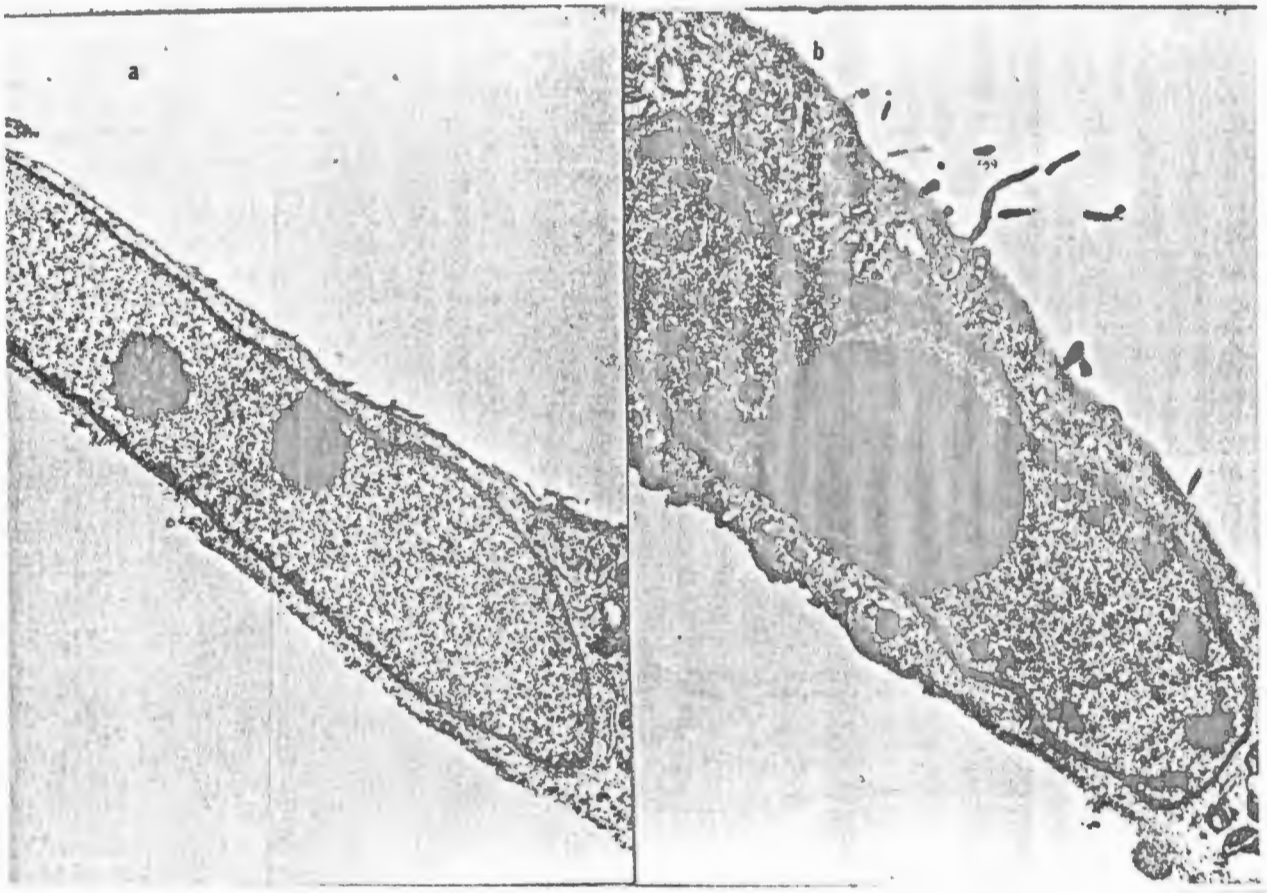


Fig. 5. GST induced nucleus change. The cells were stained with osmium. (a) A control synovial cell nucleus with two nucleoli. (b) A nucleus in a synovial cell that was exposed to 125 uM GST for one week. One nucleoli is in the nucleus with many electron dense areas (clumped chromatin) near the nuclear membrane.

B. Biochemistry

1. Proliferation.

Because GST has been shown to prevent the progression of the pannus (Sigler et al., 1974) in RA, the effect of GST on the proliferation of synovial cell cultures was studied. Clinically, the effect of gold is persistent but not permanent, therefore, the reversibility of GST effects on synovial cells was also investigated.

a. Cell number and size.

Fig. 6 demonstrates the effect of two GST concentrations on the synovial cell growth curve. Cells were plated on day zero with 10,000 cells and on day one 25uM or 250uM GST were added to the medium. The normal doubling time of these cells is approximately 5 days. There was no significant difference in cell number after five days of GST exposure. After 10 days there was a suppression in cell number with both concentrations. After an initial doubling of the culture growing with the 250uM GST, there was a continuous decrease in the number of cells. When the 250uM GST was removed from the medium after 10 days of exposure, there was no change in cell number after 6 days. With the 25uM GST containing cultures the cell number increased but at a slower rate than the controls.

GST did not cause a significant decrease in cell viability (Table 1) but did significantly decrease cell number. This can be explained on the basis of cell death which causes

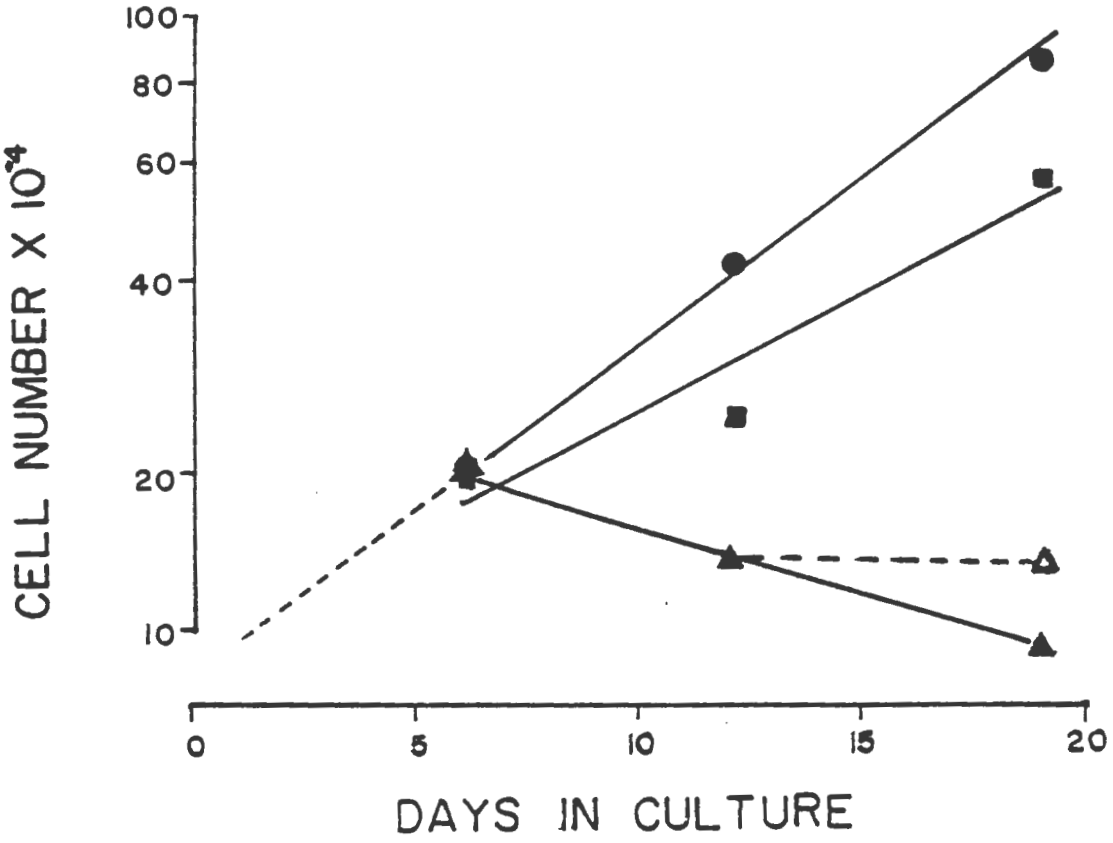


Fig. 6. Effect of gold sodium thiomalate (GST) on cell growth curve. Cells (10^5) were plated in 25cm^2 flask on day zero. The medium was changed on day 1 with the addition of GST to the gold treated flask. GST was added after medium changes on days 6 and 12. Each point represents the mean of three flasks. Cells were counted using a hemocytometer. (●) Control, (■) 25uM GST, (▲)250uM GST and (△) GST was not added after medium change.

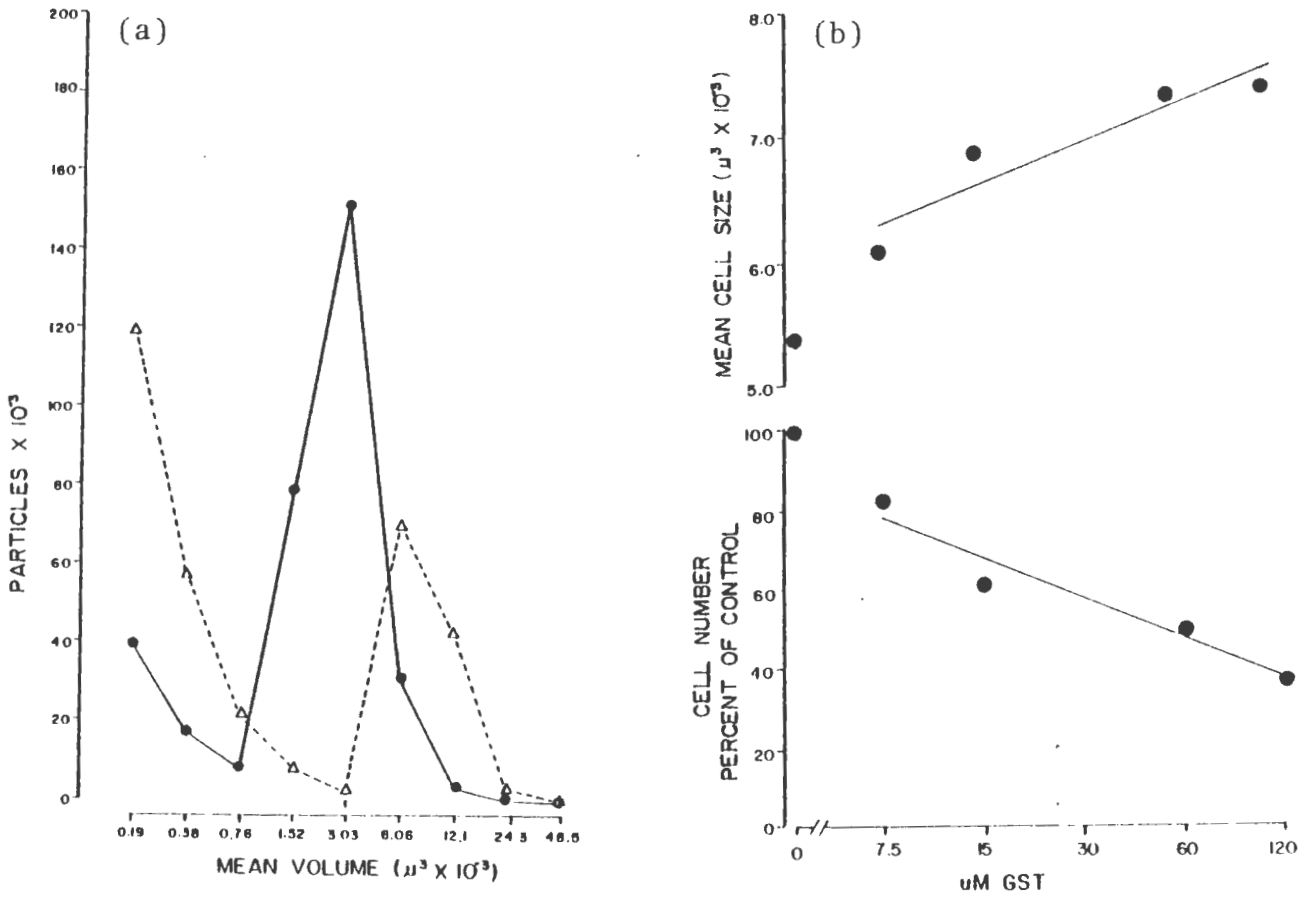


Fig. 7. Effect of gold sodium thiomalate (GST) on cell number and size. (a) Coulter counter distribution of trypsin released cell layer (●) control and (Δ) 250uM GST for 10 days. (b) Concentration dependent decrease in cell number and increase in cell size. Cells were exposed to GST for 13 days.

the cells to detach from the flask. The gold treated cells appeared larger than the nongold exposed cells in the hemocytometer. In order to determine if gold caused a change in cell size, treated cells were counted using a Coulter counter. Fig. 7a shows the size and number of particles counted in the channels of the Coulter counter. In the presence of GST, there was a shift to larger particles and a decrease in the number of particles. No clumping was observed at the counting orifice. The increase in small particles may be due to the presence of gold protein aggregates. This increase in cell size and a decrease in cell number was concentration dependent between 7.5-125uM GST (Fig. 7b). These concentrations are equivalent to the gold concentrations observed clinicly in serum and synovial tissue of patients, respectively (Gerber, 1972; Grahame et al., 1974 and Gottlieb et al., 1972).

In order to determine if the increase in cell size was related to the decrease in proliferation, synovial cells were incubated with latex beads (0.5u) and/or GST for 14 days (Table 2). The latex beads increased the cell size but did not decrease [^{3}H]thymidine ([^{3}H]TdR) incorporation. GST and GST plus latex beads decreased [^{3}H]TdR incorporation. A mixture of latex beads and GST caused a decrease of cell size when compared to the cell size of cells exposed to latex beads alone. Ugai et al. (1979) reported GST inhibits phagocytosis. This would account for the decrease in cell volume with the mixture of latex beads and GST.

b. DNA content and rate of synthesis.

In order to further characterize the antiproliferative effect of GST on synovial cells, the dose (time and

TABLE 2

EFFECT OF LATEX BEADS AND GOLD SODIUM THIOMALATE (GST) ON PROLIFERATION AND CELL SIZE

Latex[a] Beads (dilution)[b]	GST (uM)[a] 0		30		60	
	$[^3H]$TdR[c]	Cell size[d]	$[^3H]$TdR	Cell size	$[^3H]$Tdr	Cell size
	13.31±1.2[e]	5.97±0.13	2.0	8.00[f]	---	---[g]
1:10	11.6	9.17	1.6	8.70	0.8	7.18
1:20	15.9	9.20	2.0	8.20	1.0	7.27

[a]Cells were exposed to GST and latex beads for 14 days.

[b]Latex beads (0.5u) were diluted in sterile water and then a 1% (v/v) was added to the culture medium.

[c] $[^3H]$thymidine ($[^3H]$TdR) was the CPM X 10^{-3} incorporated into TCA precipitable material.

[d]Cell size was the mean volume, $\mu^3 X10^{-3}$ as determined by Coulter counter.

[e]Control, mean ± S.E. for three flasks.

[f]Duplicate determination from one flask.

[g]Culture lost.

concentration) dependent effect of GST on DNA synthesis and accumulation in culture synovial cells were measured. Fig. 8a shows the effect of a high (100uM) and low (10uM) drug concentration on a DNA growth curve. The high and low drug concentration inhibited the DNA growth curve in a similar manner as found with the cell growth curve (Fig. 6). Removal of GST from the medium resulted in only a partial recovery in DNA accumulation at the high drug (100uM GST) concentration and a complete recovery at the low (10uM GST) concentration. After one month without 100uM GST in the medium, the culture did not grow to confluency. Fig. 8b shows the concentration dependent inhibition of GST on DNA content of flasks after 5, 15 and 20 days. The curves shift to the left with increasing exposure time again demonstrating that dose is time and concentration dependent.

To confirm the ability of synovial cell proliferation to resume normal division rate after removal of 10uM GST from the medium, but not 100uM GST, the dose dependent effect of GST treatment on [^{3}H]TdR was investigated. The inhibition of [^{3}H]TdR was concentration dependent (Fig. 9b). Fig. 9a shows that the removal of 10uM GST resulted in a return to the control levels of DNA synthesis. The removal of the high (100uM) drug concentration illustrates a partial recovery, but the continued downward trend indicates incomplete recovery. The inhibition of DNA synthesis preceded the inhibition of DNA accumulation (Figs. 8a, 9a) which preceded synthesis (Fig. 10).

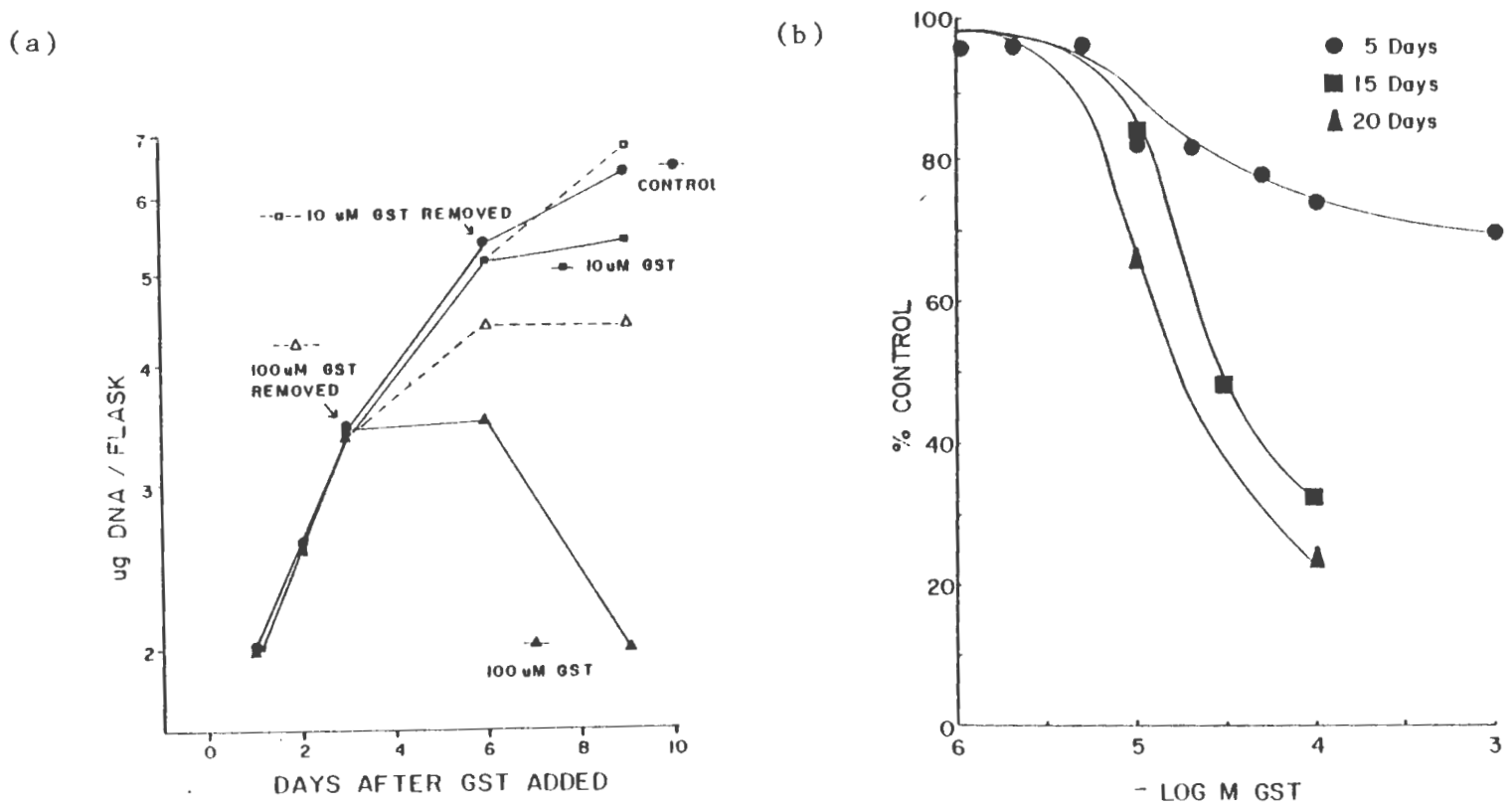
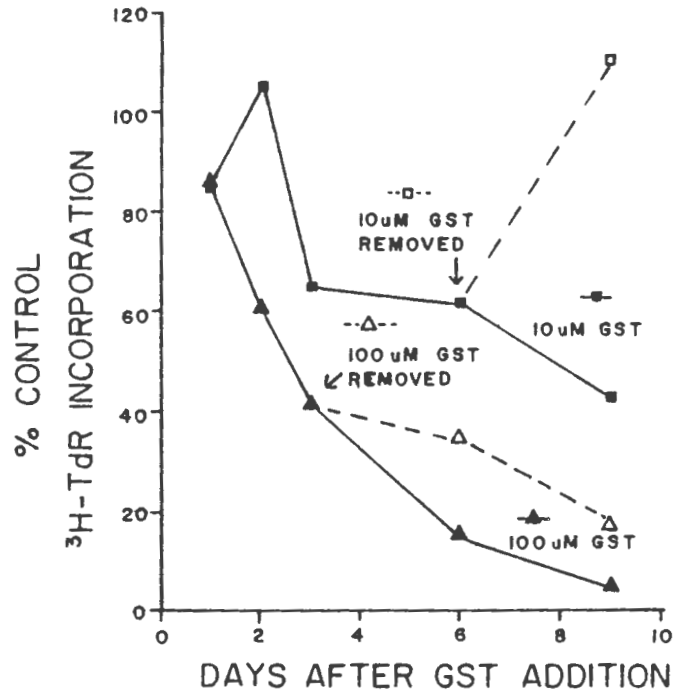


Fig. 8. Dose dependent effect of gold sodium thiomalate (GST) on DNA accumulation. (a) Effect of GST on DNA growth curve. One day after subculturing the medium was changed and GST added to the ($25cm^2$) flasks. Medium was changed after 3 and 6 days of exposures. (●) control, (■) 10uM GST, (▲) 100uM GST, (□)10 UM GST and(△) 100uM (GST was not added to medium after medium change. (b) Concentration and time effect on DNA accumulation. Each point is a single determination from one flask. Each curve is a separate experiment from different cell lines. (●) 5 day exposure, (■) 15 day exposure, (▲) 20 day exposure.

(a)



(b)

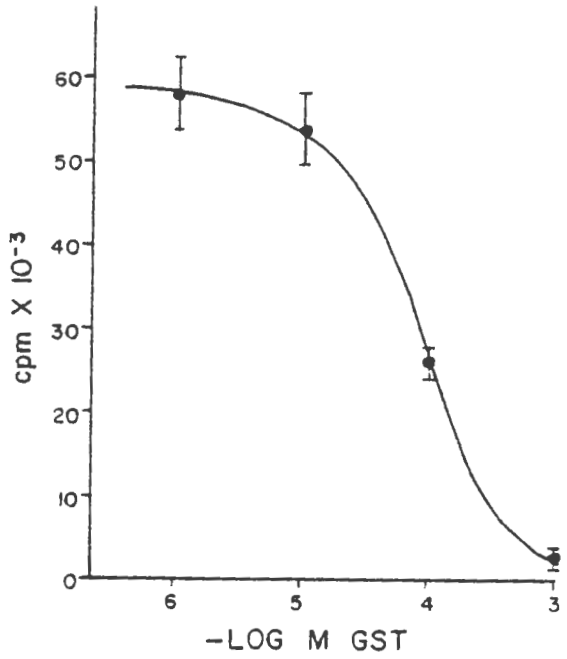


Fig. 9. Dose dependent effect of gold sodium thiomalate (GST) on DNA synthesis. (a) Time course for [^{3}H]thymidine ([^{3}H]TdR) incorporation into cell layer TCA precipitable material. Each point is the mean of duplicate flasks percentage of duplicate control flasks for days of exposure. Protocol and DNA content in Fig. 8a. (b) Concentration effect of GST on [^{3}H]TdR incorporation into DNA. Each point is the mean $\pm$ S.E. for three flasks of cells exposed to GST for 5 days.

2. Protein synthesis.

Fig. 10a shows the relative time course for inhibition of protein synthesis. At 100uM GST the 50% inhibition for DNA synthesis and protein synthesis was 25 and 9 days, respectively. The delay of onset of significant inhibition was also longer for protein synthesis (after 5 days) than DNA synthesis (after 2 days).

a. Noncollagen protein and collagen synthesis.

Fig. 10b shows the concentration dependent inhibition of collagen and noncollagen protein synthesis. Because a limited number of drug concentrations over a narrow range were used to characterize the inhibition curve a probit transfermation was used to examine the correlation ($r > 0.95$) between drug effect and concentration. Collagen synthesis was inhibited at each concentration more than noncollagen synthesis (Fig. 10). Fig. 11c shows that there is a concentration dependent inhibition of the percentage of collagen synthesized with exposure to increasing GST concentrations.

Both proliferation and protein synthesis were inhibited by GST, but the inhibition of proliferation preceded the inhibition of protein synthesis. The dose dependent effect of GST on collagen per cell and per DNA was biphasic (Fig. 11) with first an increase in the amount of protein synthesized per cell up to 30uM and a decrease to below control levels at higher doses. Using 100uM GST, a time dependent increase in protein per cell was found but this increase was not found with protein per unit of DNA (Fig. 11a). However, an increase

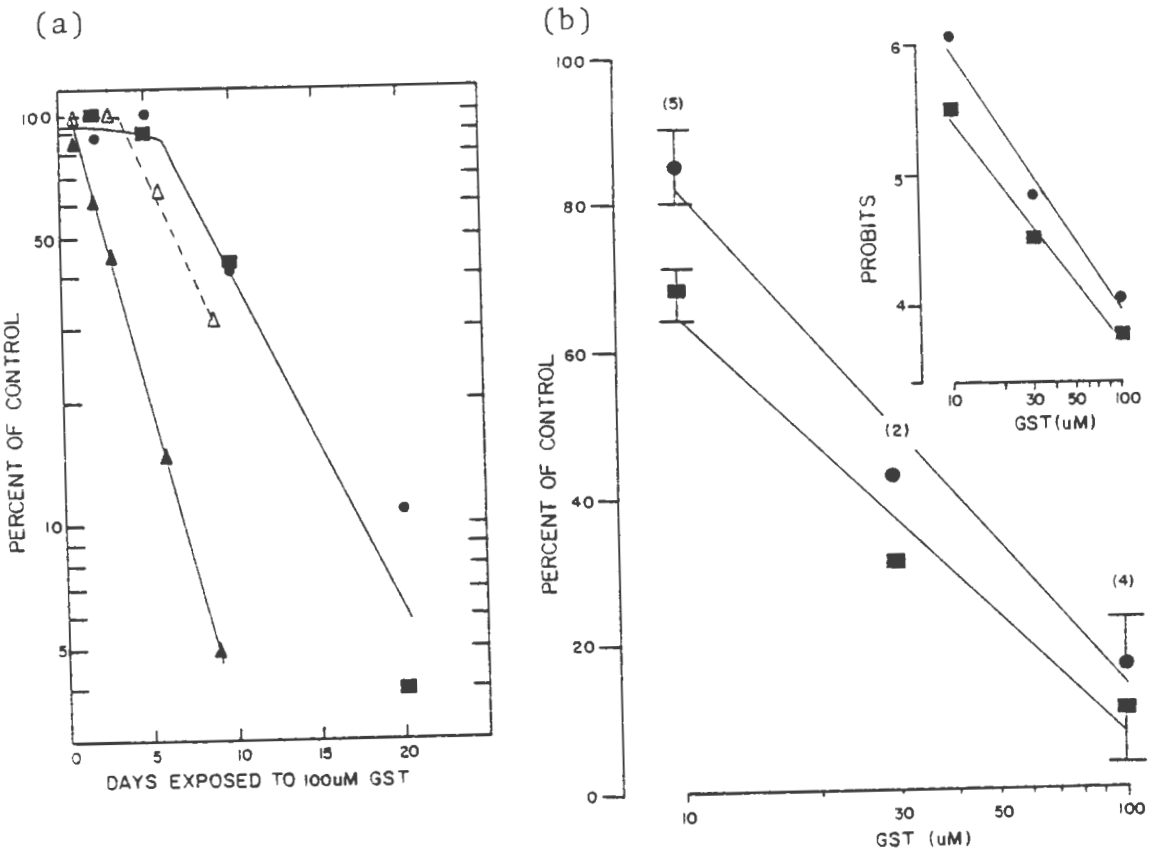


Fig. 10. Dose dependent effect of gold sodium thiomalate (GST) on collagen and non-collagen protein synthesis. (a) The relative time course for inhibition of (▲) DNA synthesis, (△) DNA content, (●) non-collagen protein synthesis and (■) collagen synthesis by 100uM GST. (b) Concentration dependent inhibition of (●) non-collagen protein and (■) collagen synthesis after 20 days exposure to GST. Insert probit transformation. Each point represents the mean ± S.E. (number of flasks) from two experiments.

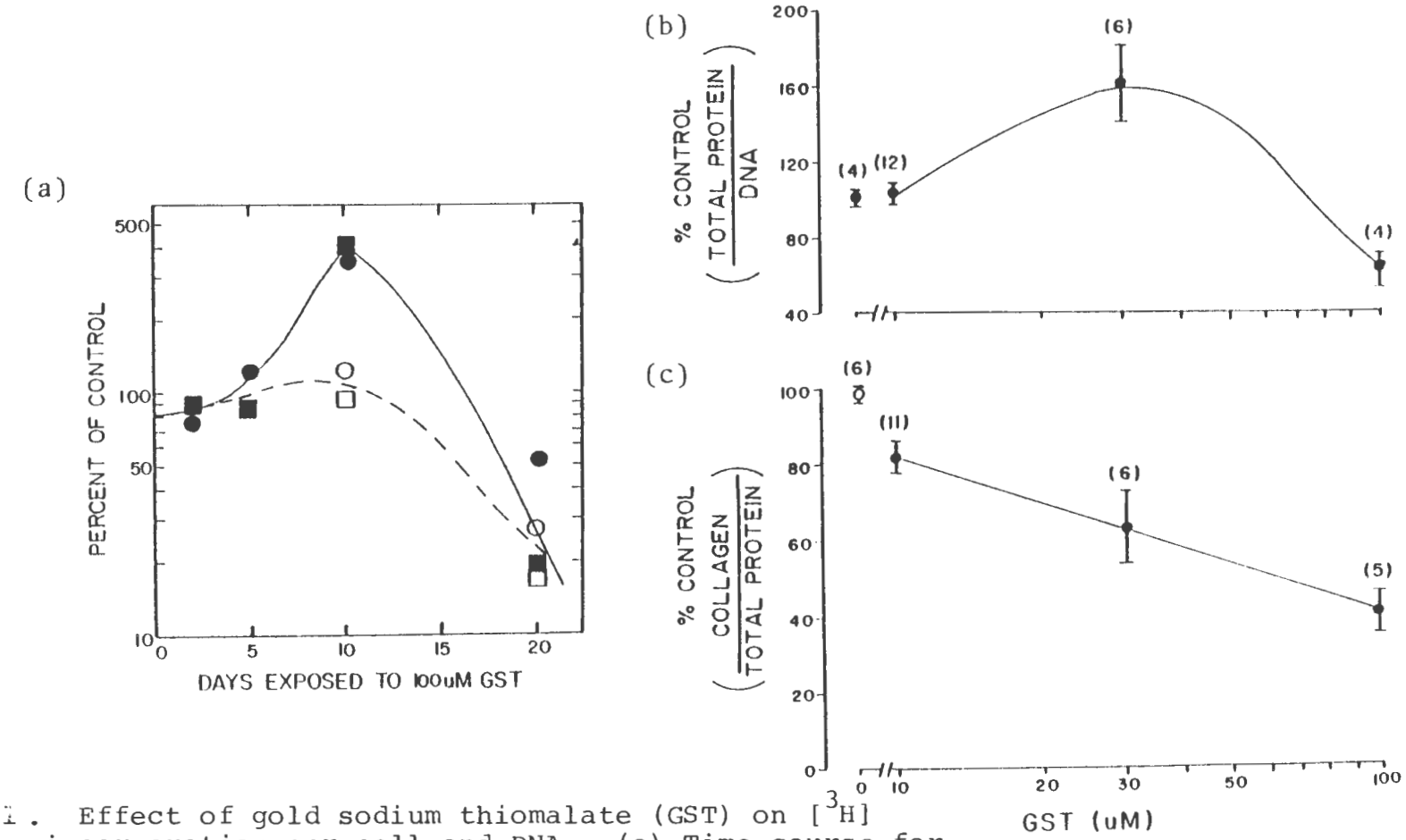


Fig. 11. Effect of gold sodium thiomalate (GST) on [^{3}H] proline incorporation per cell and DNA. (a) Time course for the rate of (■) collagen and (●) non-collagen protein synthesis per cell and (□,○) per DNA. (b) Concentration dependent effect on the medium CPM total protein per DNA. Each point is the mean ± S.E. (number of cultures) from two to three experiments for a 15-20 day exposure. (c) Concentration dependent effect on the % collagen synthesized as measured by the collagenase digestible protein assay.

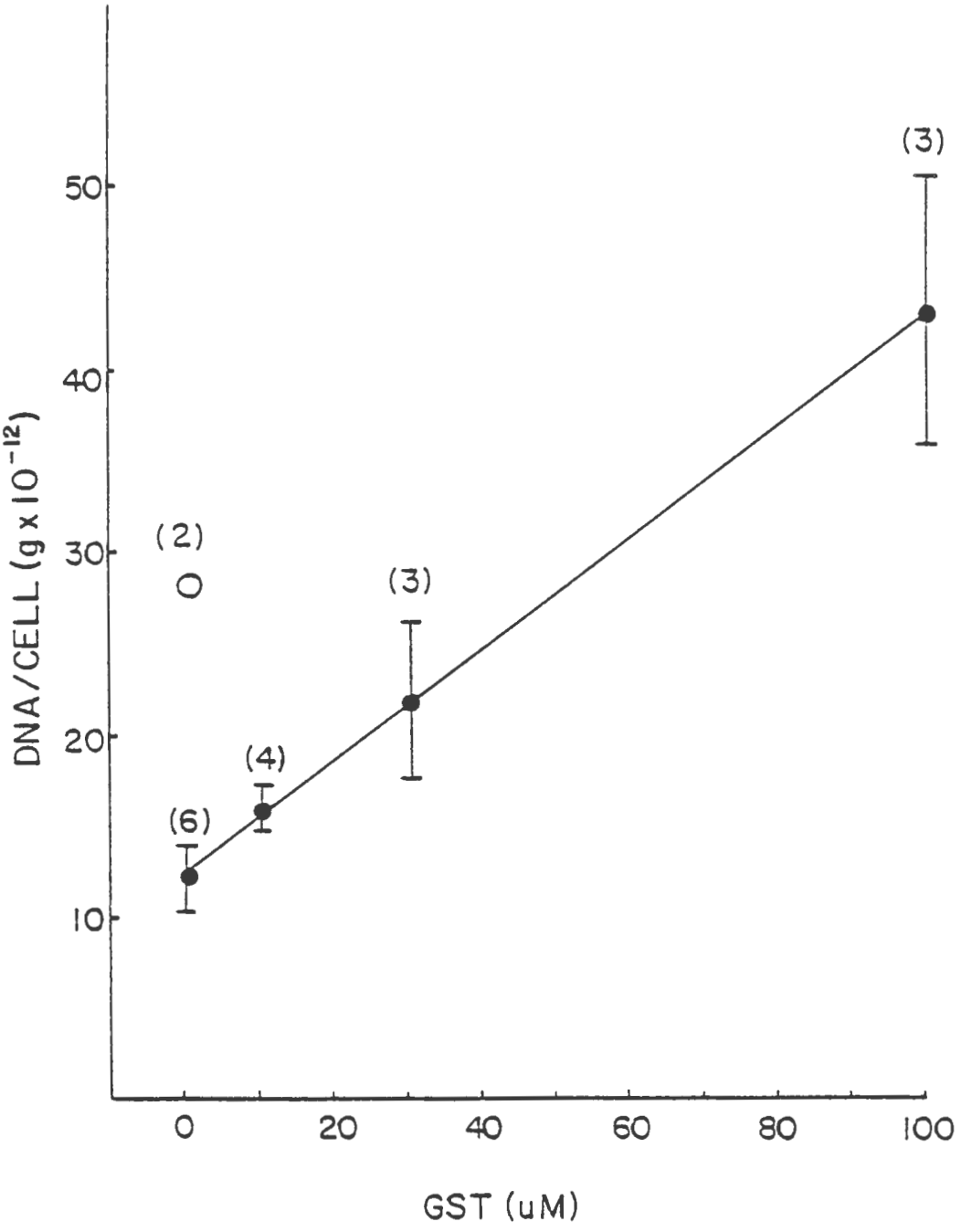


Fig. 12. Effect of gold sodium thiomalate (GST) on DNA content per cell. The DNA content was measured with ethidium bromide and cell number was determined using a hemocytometer. Each point (●) is the mean + S.E. (number of flasks) from two to three experiments; (Ō) mean of two flasks grown for five days in serum free medium.

in protein synthesized per DNA content was found at 30uM GST after 15-20 days of exposures (Fig. 11). This discrepancy is explained on the basis of the higher DNA content per cell with increasing GST concentrations (Fig. 12). The increased ethidium bromide-DNA fluorescence is not due to GST in the sample because the addition of GST to cell sonicates did not increase fluorescence in this assay. When cell proliferation was arrested by removal of the fetal calf serum (FCS) from the medium, an increase in DNA content per cell was also found. Thus, increases in cell size and DNA content per cell are most likely consequences of proliferation inhibition (Greenberg et al., 1977 and Cristofolo and Kritchevsky, 1969). Because the anti-proliferative effect of GST is dose dependent for both cell number and DNA, both were used to express the cellularity of the culture though cell number is the more conservative estimate of the two. Therefore, the increase in collagen and noncollagen protein synthesis is more dramatic per cell number than per microgram DNA.

b. Collagen types.

i. Electrophoresis. - Fig. 13 is a photograph of a SDS-polyacrylamide gel containing purified types I and III calf skin collagen standards. Beneath the gel is a densiometric scan of the Coomassie blue stained proteins. With Coomassie blue, reduced α1(III) migrates a little slower than α1(I) using the Neville (1971) gel system. With MDPF labelled chains the reduced α1(III) and α1(I) migrate together (Goldberg and Fuller, 1978). Because BAPN is used with

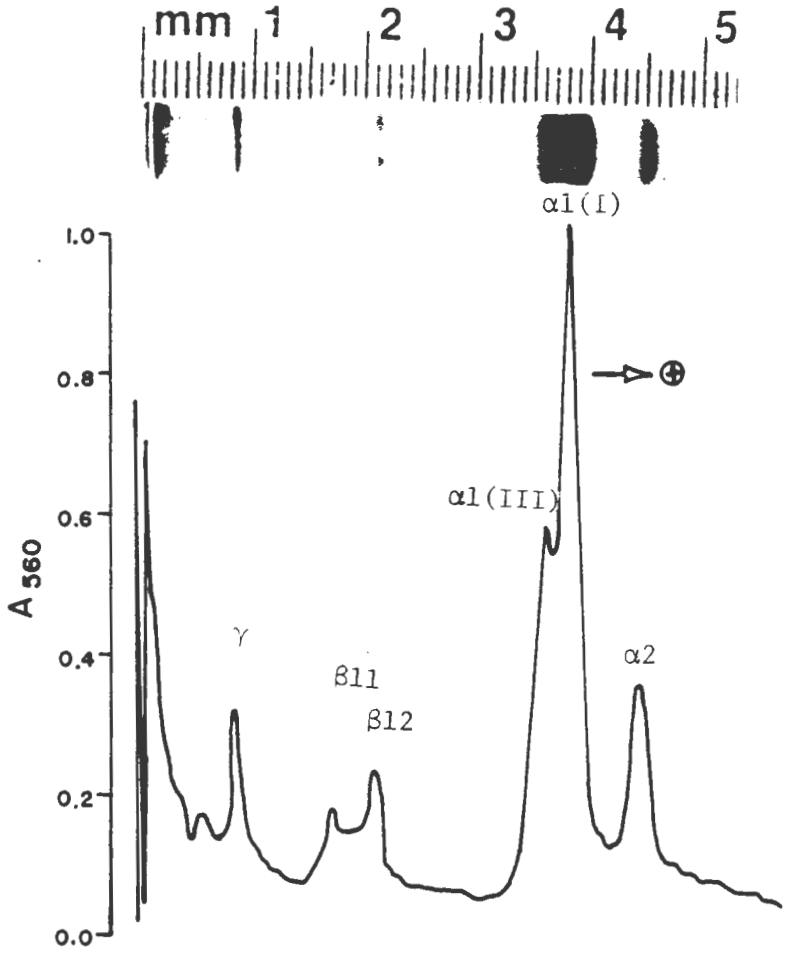


Fig. 13. Photograph and scan of standard type I and III collagen in a SDS-polyacrylamide gel.

the cell culture system the dimers, β 11 and β12, are not found in the medium extracts of the cell culture system. Type III collagen is held together as a trimer, γ , and possibly higher molecular weight forms by disulfide bonds. The reduction of the disulfide bonds removes the γ peak and causes a concomitant increase in the α1 peaks in extracted synovial cell medium collagen (Fig. 14). The peaks remaining at the top of the gel are the interfaces of the stacking and running gels. The fluorescence observed between the α and γ peaks did not contain radioactivity and was not found consistently.

The fluorescence is the amount accumulated for 5 days; the radioactivity reflects the rate of synthesis for the last 2 days. After 15 days of exposure to GST (7.5-30uM) a dose dependent increase in collagens synthesized per cell was found using the MDPF-SDS-PAGE separation procedure (Fig. 16). This increase in collagen was predominantly type I collagen. The increase in collagen content, by fluorescence, and the rate of synthesis by the incorporation of radioactivity, were similar, indicating that GST was not effecting [^{14}C] proline transport. Because the number of cells and percent of collagen both decrease at high GST concentrations, not enough collagen was extracted to quantitate collagen types at concentrations above 30uM GST.

ii. Chromatography. - Fig. 16 is the CMC chromatograph obtained from synovial cell medium. The arrows represent the peaks of purified types I and III calf skin

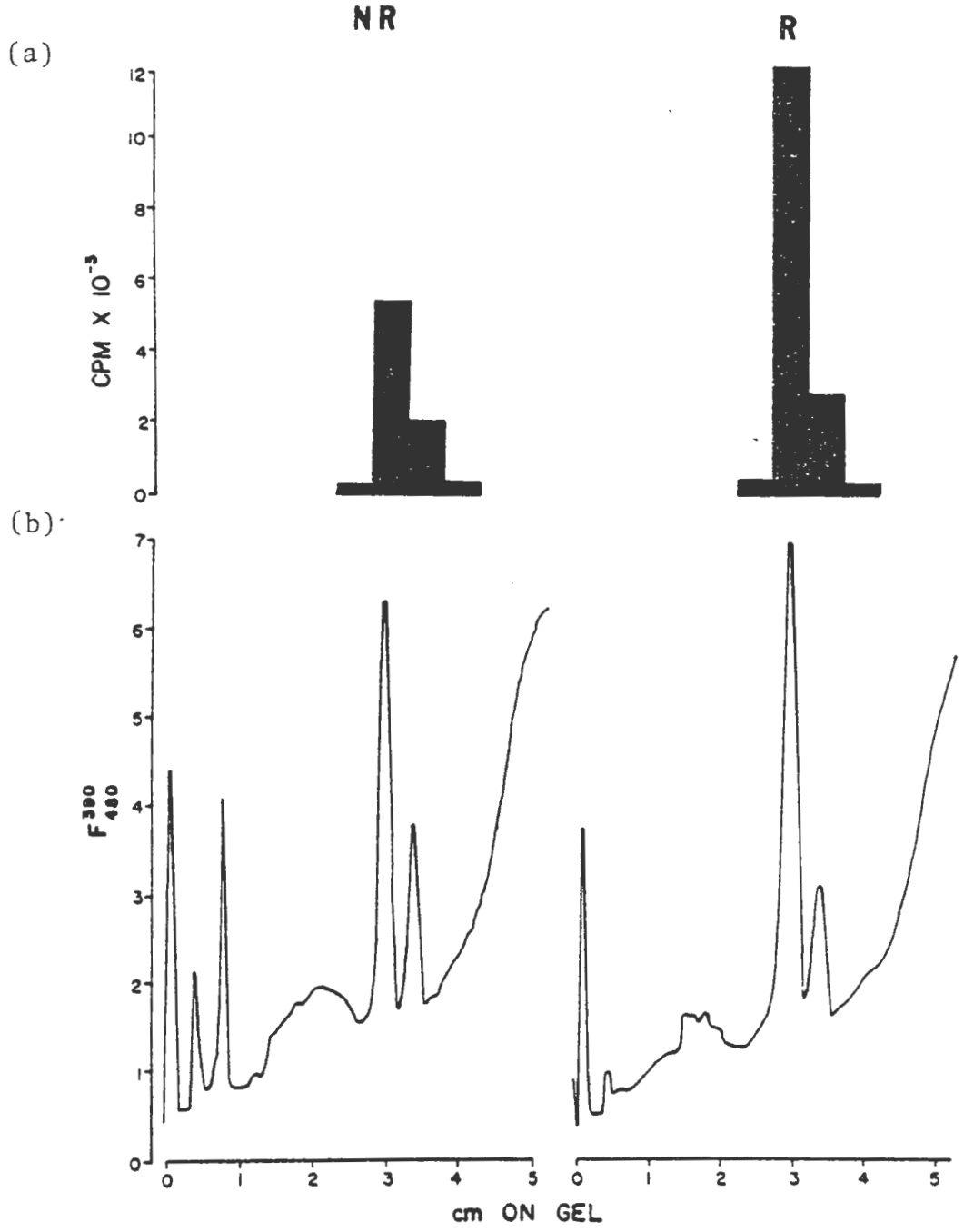


Fig. 14. Synovial cell medium collagen, non-reduced (NR) and reduced (R) after MDPF SDS-AGE. (a) CPM in 5mm gel slices; (b) fluorescent gel scans.

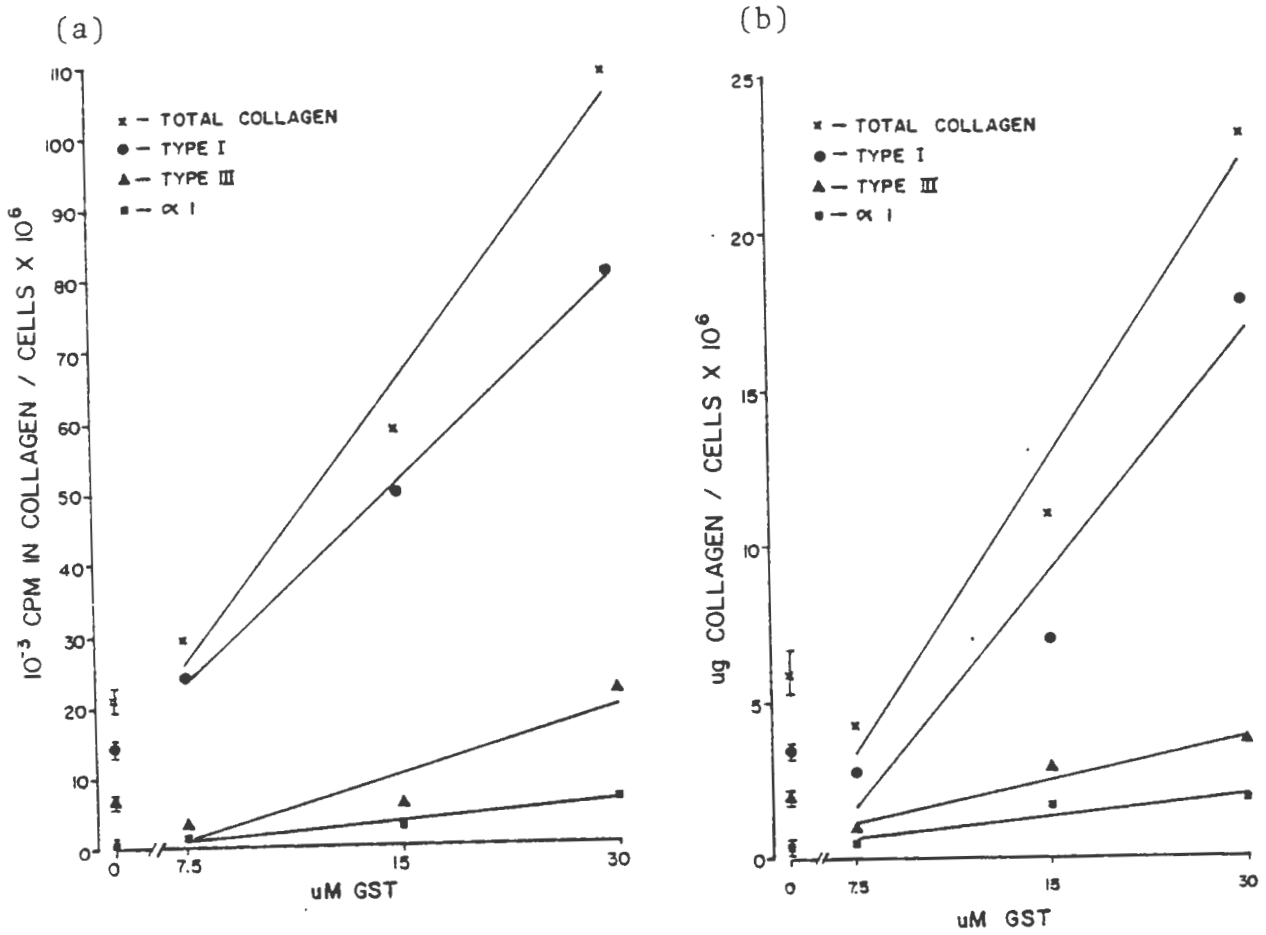


Fig. 15. Effect of GST on total collagen and collagen types per cell after separation by MDPF-SDS-PAGE. (a) [^{14}C]proline (CPM) in 5mm gel slices; (b) MDPF-protein fluorescence area beneath the curve (RFIXmm). (X) total collagen (●) type I, (▲) type III, (■) αl other.

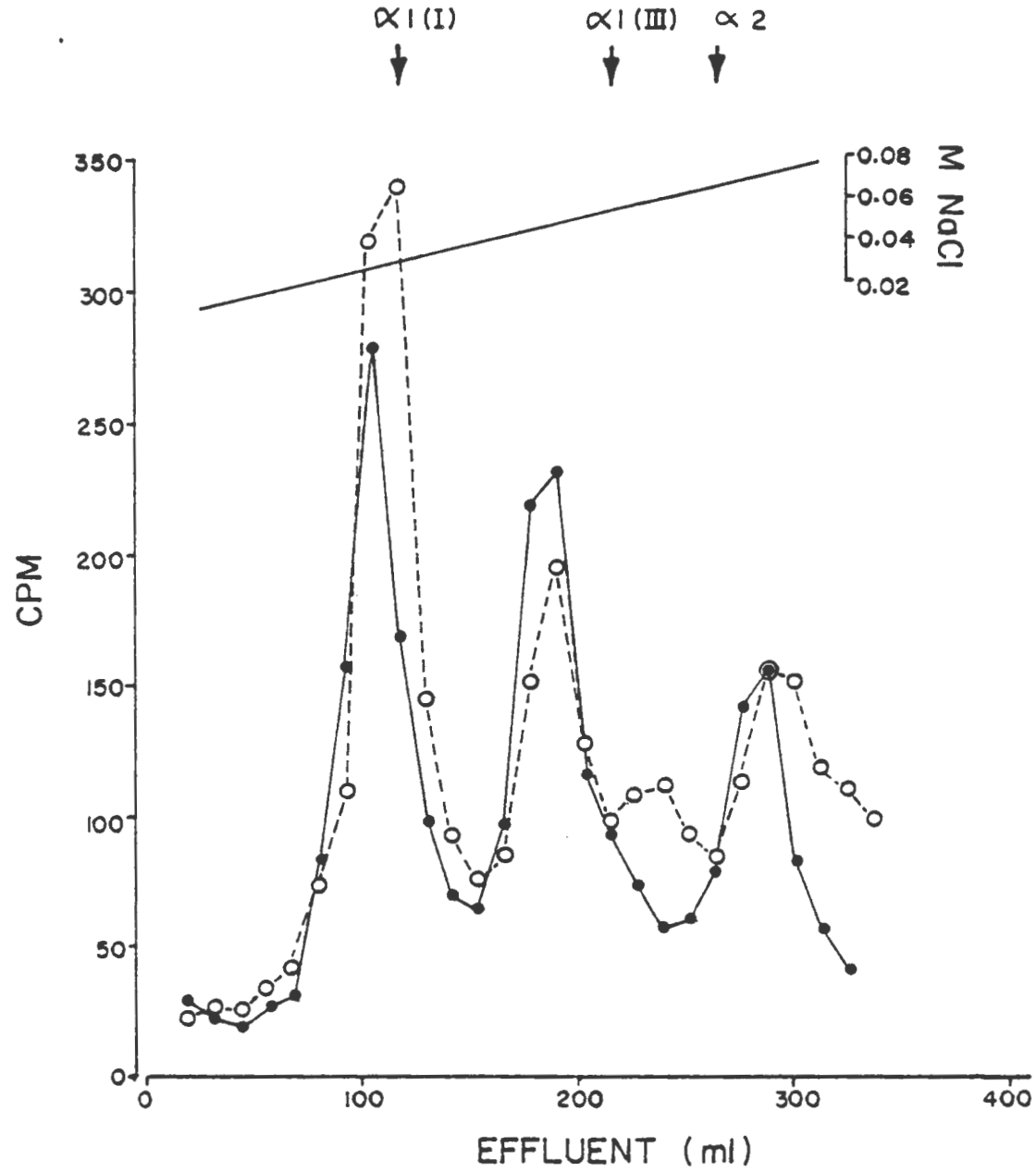


Fig. 16. Carboxy methyl cellulose chromatography (CMC) of medium collagen extracts from control and GST exposed synovial cells. (●) control and (○) 7.5 uM GST for 15 days.

α chains used as standards. The medium collagen was obtained from two pooled cultures exposed to 7.5uM GST for 15 days or from two pooled control flasks. The small peak between α1(III) and α2 was not seen with the 15uM GST exposed cultures. The effect of GST on collagen types as quantitated using fluorescence or radioactivity after separation by SDS-PAGE is compared to the results obtained when the radioactive samples were separated by CMC chromatography (Table 3). SDS-PAGE separates the collagens by molecular weight with type III collagen being the increase in the α1 position after reduction. CMC separates by ionic charge with α1(III) being more positive than α1(I) and less positive than the α2 chain of type I collagen. GST (7.5-30uM) significantly ($P < 0.05$) decreased the percent type III collagen synthesized by about 50%. This decrease was not found to be dose dependent. The estimation of the portion of type III was greater when separated by CMC than SDS-PAGE but the decrease in percent type III collagen is still evident by both separation techniques. (Parrott et al., 1980) using rheumatoid arthritis, osteoarthritic and normal synovial cell lines found the estimation of percent type III collagen was greater by CMC than SDS-PAGE. These estimates obtained with the two procedures were significantly correlated ($P < 0.01$).

3. Reversibility.

In order to further characterize the reversibility of the effect produced by GST, the gold content of cell

TABLE 3

EFFECT OF GOLD SODIUM THIOMALATE(GST)ON THE PERCENT OF TYPE III COLLAGEN SYNTHESIZED BY SYNOVIAL CELLS

GST[a] uM	Electrophoresis(MDPF-SDS-PAGE) CPM[b]	F_{480}^{390}	Chromatography(CMC) CPM
0	32±6[c]	31±3	39[d]
7.5	13±1[e]	10±3[e]	26
15	9±3[e]	8±3[e]	21
30	13±3[e]	20±2[e]	--[f]

[a]Cells were preincubated with GST for 15 days. Fresh medium without GST but continuing BAPN and ascorbate was then added; the medium collagen was harvested 5 days later.

[b][^{14}C]proline was added 2 days before the medium was harvested.

[c]Mean ± S.E. from triplicate flasks.

[d]Single determination from two pooled flasks.

[e]$P < 0.05$.

[f]Insufficient collagen for quantitation.

layer sonicates was determined by atomic absorption spectroscopy. Fig. 17 shows that GST caused a dose dependent accumulation of gold associated with the trypsin released and sonicated cell layers. These cell layers were washed once with PBS and once with 0.1% trypsin PBS pH 7.3, thus, the gold detected with the cell layer is in and possibly on the surface of cells. The deposition of gold in the cell layers has a lag time of a day and is inversely related to the inhibition of DNA synthesis (Fig. 17a). The relationship of medium concentration of GST to gold in the cell layer is log linear (Fig. 17b). When the cell layer volume was estimated (by Coulter counter) there was approximately a 1000 times greater concentration in or on the cells than in the medium. Table 4 shows that the removal of gold from the medium causes a decrease in gold associated with the cell layer but that complete removal is not achieved after 14 days. Table 5 shows the recovery of some biochemical parameters after the removal of GST from the medium. The recovery of the biochemical parameters was not complete but neither was the removal of GST from the cell layers. The increase in gold content per unit of DNA from experiments 1-3 was linearly related to the decrease in the percent of control of DNA content (slope = -1.6, intercept = 79, $r = 0.64$, $p < 0.01$ and $n = 17$ flasks); total protein synthesized (slope = -1.3, intercept 72, $r = 0.61$ and $n = 17$); percent collagen (slope = -0.93, intercept = 76, $r = 0.57$, $p < 0.01$ and $n = 17$). The

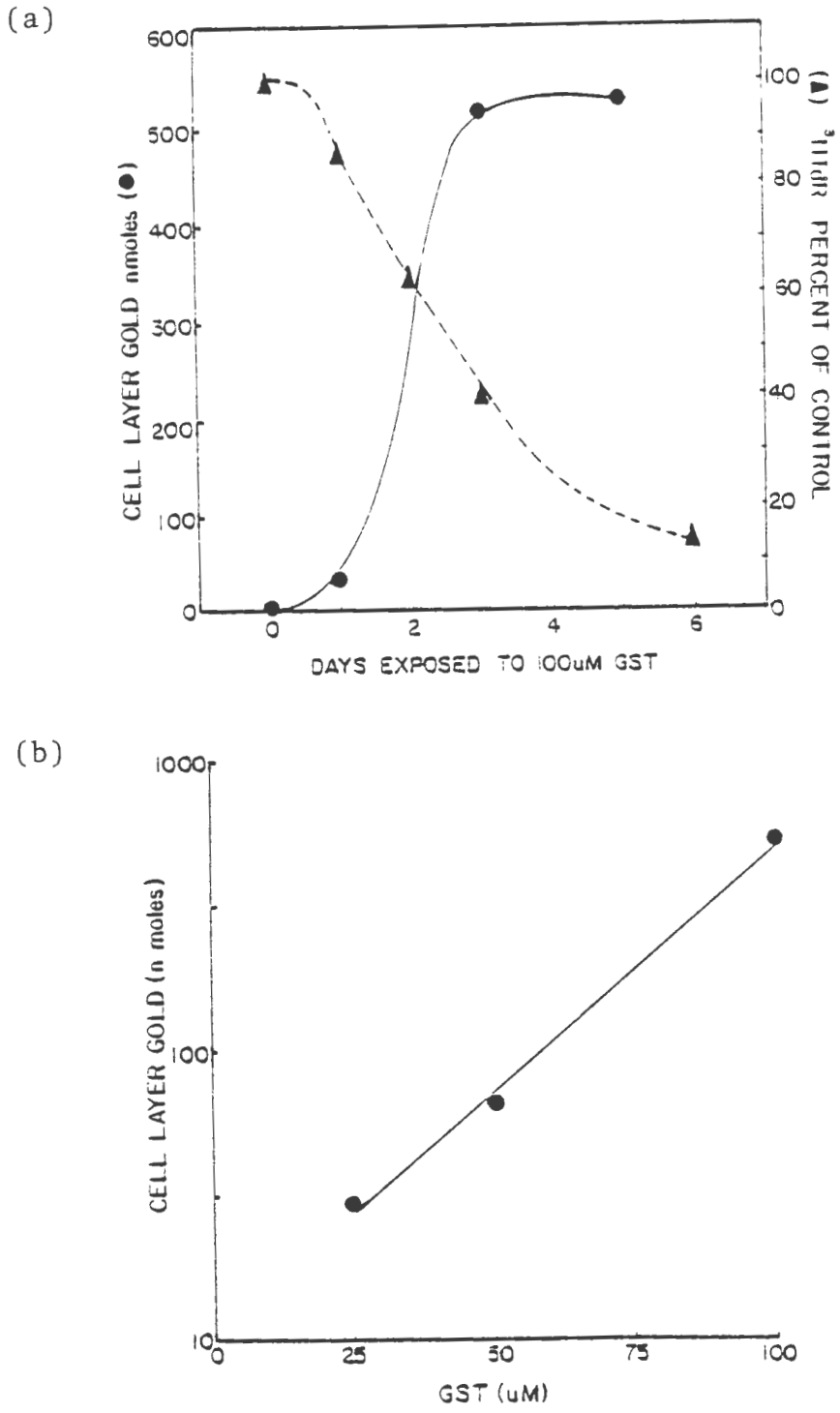


Fig. 17. Dose dependent accumulation of gold with the cell layer after exposure of synovial cells to gold sodium thiomalate containing medium. Gold was determined in cell layer sonicates by atomic absorption. (a) Time course for gold deposition (●) in or on the cells; (▲) inhibition of DNA synthesis; (b) concentration dependent accumulation of gold associated with the cell layer after 5 days of exposure. Each point is a duplicate determination from one ($25cm^2$) flask.

TABLE 4

QUANTITATION OF GOLD ASSOCIATED WITH SYNOVIAL AND SKIN CELL LAYERS AFTER EXPOSURES TO GOLD SODIUM THIOMALATE (GST) CONTAINING MEDIUM

Cell Line		GST (uM)	Days with GST in Medium	Days without GST in Medium		[a] Moles gold / g DNA X10^{12}	[b] Molecules gold / Cell X10^{-9}
RA-23[c]	(2)[d]	10/10	17	0	EXP 1	2.0	17
	(2)	10/0	9	8		1.2(0.9-1.5)[e]	8(7-9)
	(2)	30/30	17	0		40 (35-44)	400(180-610)
	(2)	30/0	9	8		18 (11-26)	10 (10-11)
					EXP 2		
RA-15	(2+3)	10/10	10(2)[c]+19(3)	0		0.45(0.43-0.46)	---[f]
	(2+3)	100/0	10(2)	14(3)		12.0	---
					EXP 3		
RA-16	(2)	10C[g]	14	0		0.8	---
	(3)	10L	20	0		0.8	---
	(2)	100C	10	4		38.6	---
	(3)	100L	12	8		4.7	---
					EXP 4		
PY-O[h]	(2)	30C	5	0		10.3	44
	(3)	30L	5	0		44.1	470

[a] Gold was determined by atomic absorption and DNA by the ethidium bromide assay.

[b] Cells were counted using a hemocytometer. [c] Rheumatoid synovial cells.

[d] Passage number (2) or (3). [e] Mean (range). [f] Cell number not determined.

[g] GST added to medium during confluency (C) or log phase (L). [h] Skin cells.

TABLE 5

RECOVERY OF BIOCHEMICAL PARAMETERS AFTER GOLD SODIUM THIOMALATE (GST) EXPOSED SYNOVIAL CELLS ARE GROWN IN MEDIUM WITHOUT GST

GST treatment (uM)	Gold[a] (m moles)	DNA[b] (ug)	Total Protein[c] ($CPMX10^{-6}$)	Collagen[c] (%)	Collagen Type III[d] (%)
		EXP 1[e]			
0/0	0	9.1[f] (10.2-8.0)	0.38 (0.36-0.39)	12.6 (12.4-12.8)	18 (16-20)
10/10	18.3	9.1	0.32	8.7	8
10/0	8.7 (8.7-8.6)	7.6 (5.9-9.2)	0.31 (0.31-0.31)	9.6 (9.5-9.7)	13 (11-15)
30/ 30	149 (104-193)	3.7 (3.0-4.4)	0.13 (0.13-0.13)	6.9 (6.8-7.0)	7
30/0	47 (43-51)	3.0 (2.0-4.0)	0.21 (0.17-0.24)	6.8 (4.2-9.4)	9 (4-14)
		EXP 2[e]			
0/0	0	37.1 (32.6-41.6)	4.0 (3.4-4.6)	9.8 (9.6-9.9)	18 (14-21)
10/10	15.0	33.5 (32.3-34.7)	3.2 (3.0-3.5)	8.2 (7.5-8.9)	4
0/10	14.3	39 (37.9-40.0)	3.5 (3.1-3.9)	9.9 (8.4-11.4)	5 (3-6)
100/0	44.3	3.7 (3.0-4.3)	0.3 (0.3-0.4)	3.9 (3.4-4.3)	---[g]

[a] Gold was quantitated using atomic absorption.

[b] DNA content was determined by the ethidium bromide assay.

[c] Total protein and % collagen were determined by the collagenase digestible assay.

[d] Collagen type III % was determined radiometrically after separation by electrophoresis.

[e] Exposure time for Exp. 1 and 2 are the same as that on Table 4.

[f] Mean (range) for two flasks.

[g] Insufficient collagen for quantitation.

percent type III collagen was not linearly related to gold content per unit DNA or to gold content of flasks ($r = 0.12$). The correlation coefficients for gold content versus biochemical parameters are not as high as GST medium concentrations versus biochemical parameter ($r > 0.90$).

4. Effect of GST on Cells during log phase and confluence.

In order to determine the effect of confluency and log phase on cells, second passage confluent cells (75 cm^2 flask) were subcultured. After waiting one day for the subcultured cells to attach, the medium of third passage log phase cells and second passage confluent cells were exposed to GST. Table 6 shows the effect of GST on gold content, proliferation, total protein, percent collagen and percent type III collagen. The skin fibroblast doubling time was approximately two days, therefore the GST exposure time was less than the synovial cells. With the synovial cells the percent type III collagen produced was increased during log phase growth whereas with the skin fibroblasts the percent type III collagen produced was decreased. With both cell lines in confluency or log phase the percent type III collagen decreased due to GST exposures. GST did not decrease the DNA content (more than 10%) during confluency, but depressed both when exposed during log phase growth. In synovial cells, GST induced a decrease in the total amount of protein synthesized and a decrease in the percent collagen

produced during log phase growth.

5. Anti-rheumatic drugs and effects on GST.

The effect of hydroxycloroquine, dexamethasone, penicillamine and gold sodium thiomalate (GST) on the percentage of type III collagen produced by synovial cells after 10 days of exposure was measured in order to determine if other anti-rheumatic drugs had GST-like effects on synovial cells. Again, GST decreased the percentage of type III collagen by 50%; while the other anti-rheumatic drugs did not decrease the percent type III collagen synthesized.

TABLE 6

EFFECT OF GOLD SODIUM THIOMALATE (GST) ON BIOCHEMICAL PARAMETERS OF SYNOVIAL AND SKIN CELLS DURING LOG PHASE AND CONFLUENCY

GST[a] (uM)	Gold[b] (m moles)	DNA[c] (ug)	Total[e] Protein ($CPMx10^{-6}$)	Collagen[c] (%)	Collagen[f] Type III (%)
a) Synovial cells during second and third passage (Exp. 3).					
			Confluency		
0	0	19.4[g]	2.2	2.0	13
10	22	21.5	2.0	1.6	8
100	725	18.8	1.7	1.1	--
			Log Phase		
0	0	14.7[h]	1.3	4.0	23
10	(0-0)	(13.3-16.1)			
	10	12	0.9	2.6	9
	(9-11)	(10.6-13.3)	(0.8-1.0)	(2.2-3.0)	(7-11)
100	38	5.9	0.5	2.5	--
	(38-39)	(3.6-8.2)	(0.2-0.8)	(1.8-3.2)	
b) Skin fibroblast during second and third passage (Exp. 4).					
			Confluency		
0	0	23.6	1.3	3.4	31
30	221	21.4	1.6	3.5	4
			Log Phase		
0	0	8.4	0.85	6.4	17
30	234	5.3	0.70	3.8	3

[a] Exposure time for Exp. 3 and 4 are the same as in Table 4.

[b] Gold associated with cell layer sonicates from (75 cm^2) flasks was quantitated by atomic absorption.

[c] DNA content was determined by ethidium bromide fluorescent assay.

[d] Cells were counted using a hemocytometer.

[e] Medium total protein and % collagen was determined by the collagenase digestible assay.

[f] Percent type III collagen was determined radiometrically after separation by electrophoresis.

[g] Single determinations from one flask. [h] Mean (range) of two flasks.

TABLE 7

EFFECT OF ANTI-RHEUMATIC DRUGS ON PERCENT TYPE III COLLAGEN SYNTHESIZED BY CULTURED SYNOVIAL CELLS

Drug (uM)	Type III[b] Collagen (%)	DNA[c] (ug)
Control	13 ± 2[d]	35 ± 1
Hydroxycloroquine (10)	19 ± 5	31 ± 1
Dexamethasone (10)	12 ± 1	27 ± 4
Control	17 ± 5	14 ± 3
Penicillamine (20)	22 ± 2	22 ± 5
Gold Sodium Thiomalate (10)	7 ± 5	18 ± 4

[a]Cells were preincubated with the drug for 10 days, followed by 3 days with drug, [^{3}H]-proline, BAPN and ascorbate before medium was harvested.

[b]Type III collagen % has determined radiometricly after separation by electrophoresis.

[c]Cell layer sonicates were analyzed by the ethidium bromide DNA assay.

[d]Mean ± SE for three flasks.

V. DISCUSSION

The efficacy of chrysotherapy (gold therapy) in inducing the remission of rheumatoid arthritis has been confirmed by several controlled clinical studies (Sigler et al., 1974, Research Subcommittee of the Empire Rheumatism Council, 1960, 1961 and Cooperative Clinics Committee of the American Rheumatism Association, 1973). The present study demonstrates that, in cultures of human synovial cells, gold sodium thiomalate (GST), at pharmacologically relevant concentrations, has a direct effect on cells derived from the synovial tissues of patients with rheumatoid arthritis. Serum gold concentrations of 1 to 3 ug/ml have been reported in rheumatoid arthritic patients during chrysotherapy. This is equivalent to 5-15 uM GST. Synovial tissue gold concentrations are approximately 20-30 ug/g, which are equivalent to 100-150 uM GST. Thus, the concentrations used to treat cells in culture in these experiments were intermediate between the serum and tissue concentrations observed during chrysotherapy (Gerber et al., 1972; Mascarehas et al., 1972; Jessop and Johns, 1973; Rubinstein and Dietz, 1973 and Gottlieb et al., 1972; Vernon-Roberts et al., 1976). Lipsky and Ziff (1972) have shown that the gold moiety of GST or gold chloride inhibits lymphocyte proliferation (thiomalic acid was without effect). None of the GST effects reported here were observed when sodium thiomalate was added to the medium. Therefore

in accord with Lipsky and Ziff (1977), the gold moiety is responsible for the observed changes in synovial cell morphology, proliferation and collagen synthesis.

No one experimental model has all the characteristics of human RA. The model used in this investigation to study the effects of GST on the proliferative lesion of RA, was the synovial cell explant cell culture model from human rheumatoid synovial tissue. The advantages of this model are: the cells used in culture are the cells most likely responsible for the proliferative lesion in RA, experiments can be conducted in a convenient and controlled manner, and biochemical effects due to GST can be detected more rapidly (days) in culture than clinical benefit in patients' joints (months). The limitations are: the cells selected are the more rapidly dividing cells, the immunological and inflammatory cells and factors are not present, and, after subculturing, an extracellular matrix is reformed by the synovial cells. These limitations are advantageous in this investigation because the focus of this study is on the direct effect of GST on the proliferating synovial cells and the extracellular matrix formed by these cells.

This model does not deal with the complex interactions of the immunological, inflammatory and synovial cells. Conversely, the cell population generated by this culture technique is heterogeneous (Marsh et al., 1974) so the biochemical effects measured could be due to a selectivity for certain

cell populations. The interpretation of drug induced changes in synovial cell metabolism in the flask to the RA joint has the limitations of relating an *in vitro* to an *in vivo* condition.

Protein bound drugs in the serum are usually inactive and the amount of free drug is usually correlated with pharmacological activity. Albumin bound gold and gold denatured proteins (Gerber, 1964) may be phagocytosed by gold neutrophils and monocytes. This may be another reason in addition to increased blood flow, that an inflamed synovium accumulates more gold than a nonrheumatoid joint (Grahme *et al.*, 1974). The accumulation of gold in kidney, liver and the reticuloendothelial system (Gottlieb *et al.*) may be due to the phagocytosis and filtration of gold aggregated and denatured proteins. Thiomalate is more rapidly eliminated than the gold moiety of GST (Jellum *et al.*, 1980). Hence, the distribution of gold in the body may be more dependent upon the gold-protein aggregates than upon the free parent compound GST.

The pharmacodynamic receptor(s) for gold has (have) not been identified. The fact that gold can form stable coordination complexes with a variety of ligands (sulfur > carbon and nitrogen) (Harvey, 1976) would imply an immense number of possible binding sites. There are several billion molecules of gold bound per cell (Table 4). Therefore, much of the binding, for example albumin, is not related to the mechanism of action.

GST causes a morphological change in cells both in culture and in tissues. Exposure of synovial cells to GST containing medium causes dose dependent vacuole formation (Fig. 1) as observed by light microscopy, which are gold containing membrane bound vesicles by EM (Fig. 2) and resembles the aurosomes in patients' tissues (Fig. 4). In the case of synovial tissues observed after administration of GST to RA patients, the predominant cell type in which aurosomes occur is a phagocytic macrophage cell type (Fig. 4a & b), whereas in the explant culture system, the fibroblast like cells contained the aurosomes (Figs. 2 and 3). Ghadially (1979) and Norton et al. (1968)have previously reported that aurosomes occur in the phagocytic cells of the synovium after in vivo exposure to GST. The cultured fibroblast like synovial cells used here also have phagocytic properties, as demonstrated by the increase in cell size after exposure to latex particles (Table 2) or following exposure to GST (Fig. 7a). In the synovium the cells more specific for phagocytosis would be expected to take up more gold protein aggregates. The kidney, liver and adrenals have higher gold concentrations than the synovium (Gottlieb et al., 1972). Therefore, the highest concentration of gold in a cell type or in a tissue does not identify the cell or the tissue as the target for the therapeutic agent.

The macrophage may be involved in both the mechanism of action of gold and in the processing of gold for distribution.

In the macrophage, the aurosomes could inhibit the degradative enzymes (Persillin and Ziff, 1966) or inhibit phagocytosis (Ugai et al., 1979). Fig. 3 shows a proposed maturation of the aurosome. The gold precipitants or aggregates enter the cell via phago- or pinnocytosis. The protein moiety of the aggregates in vacuoles may be dissolved by proteases, thereby leaving low molecular weight soluble gold complexes that may interact within either the phagocytic cell itself or be passed directly to another cell type (possibly a type B synoviocyte) and where biochemical changes result (Goldberg et al., 1980). Also, a dead phagocyte may release gold into the extra cellular matrix where binding to the collagen occurs (Adam et al., 1965 and 1968) reforming protein aggregates which may then be phagocytized by an adjacent type B sunoviocyte. GST has been shown to interfere with the proliferation of lymphocytes by interfering with the macrophage function (Lipsky and Ziff, 1977). Macrophages process antigens for presentation to lymphocytes for antibody and lymphokine production. The possibility exists that macrophages assist in the clinical benefit of gold by processing gold aggregates and presenting them to a target cell (the proliferating type B synoviocyte), in this way preventing the growth of synovial cells of the enlarging pannus.

The hypothesis that processed gold-protein aggregates are digested in the lysosomes and may interact with cytoplasmic proteins or other cellular components is supported

by the gold distribution study of Lawson et al. (1977). In the lysosomes they found a wide range of molecular weight species, whereas in the cytoplasm several distinct peaks of bound gold were found. This finding would be expected if high molecular weight gold-protein aggregates were in the process of being digested. The low molecular weight gold or free gold would leave the lysosome and bind to certain proteins with a high affinity for gold. Van De Stadt and Abbo-Tilstra (1980) found that at low gold concentrations (<1 ug/ml) gold preferentially binds to proteins other than albumin. Thus, it is feasible that the lysosomes are processing the high molecular weight aggregates by degrading the protein moiety and releasing soluble complexes that could bind to cytoplasm "macromolecules."

Heavy metals in general have an antiproliferative effect, but this is the first time the mechanism of action of gold in rheumatoid arthritis has been linked to the antiproliferative effect on hyperplastic synovial cells. The onset of the antiproliferative effect of GST takes several days. No significant difference in cell number was found until after 5 days of GST exposure. No significant difference in DNA content was found until after 4 days of GST exposure, while a change in DNA synthesis was seen after 2 days. The onset delay of inhibition of DNA synthesis is correlated with the accumulation of gold in the cell layer (Fig. 7). This would indicate that gold needs to be internalized

(Fig. 3) prior to the inhibition of proliferation or the occurrence of aurosome formation. The delay of onset (which is observed clinically since several months of gold therapy is necessary before clinical benefits are seen) argues for the presence of an internal receptor which provides a less rapid onset than a cell surface receptor (Passow et al., 1959). The growth curve for both cell number (Fig. 6) and DNA (Fig. 8a) shows continued cell growth for precisely one doubling before inhibition by GST. The [^{3}H] thymidine inhibition preceded the DNA content decrease (Fig. 10) which implies inhibition before or during S phase of the cell cycle. The DNA content per cell increases with GST implying inhibition after S phase but before M phase (possibly G_2 phase). To determine where in the cell cycle gold inhibits, a cloned cell line that can be syncronized would be necessary. To date, no clones have been developed from the synovium. The inhibition of cell proliferation may be related to the nuclear change in Fig. 5. Penney et al. (1976) found ^{195}Au in nuclear subcellular fractions though this could have been due to lysosomal contamination. Ghadially (1979) reported accumulation of bismuth and lead in the nucleus of cells but not gold. There has been no report of gold in the nucleus by X-ray probe analysis. In the present study, an X-ray probe analysis of one electron dense area in the nucleus failed to detect gold. This would indicate the majority of the electron dense area as chromatin clumps rather than gold

but it is also possible there was not enough gold to detect by X-ray analysis. Gold may be bound to DNA, RNA or proteins in the nucleus but the amount found in the nucleus is far less than the gold in the aurosomes. The data presented here suggest that aurosomes participate in the transport and processing of gold and the primary site of action is the nucleus.

The decrease in protein synthesis per flask (Fig. 10) like proliferation, is an effect common to most heavy metals and could be just heavy metal toxicity. However, the change in protein synthesis per DNA was byphasic with the transition for the concentration dependent decrease the same as the threshold concentration for aurosome formation. GST caused a concentration dependent decrease in the percentage of collagen to total protein synthesized by synovial cells (Fig. 12). Peniacillamine has also been shown to decrease the percent of collagen to total protein synthesized by skin fibroblasts in culture (Shorn et al., 1979), but the concentration of peniacillamine used in culture for this study was far higher than those obtained clinically.

The influence of cell crowding on collagen and protein synthesis has been studied in a number of laboratories. Mann (1971) and Steinberg (1978) both found a decrease in total protein synthesis and an increase in the percentage of collagen synthesized by 3T6 mouse fibroblasts from log phase to confluency. Steinberg (1978) found an increase rate of collagen synthesis in 3T6 during confluency; Priest and Davis (1969) using 3T6 and rat PR105 cell lines and Abe

et al. (1979) using guinea pig skin fibroblasts found a decrease rate of collagen synthesis from log phase to confluency. Both human synovial cells and human skin fibroblast produce less protein per unit of DNA during confluency and an even greater decrease in collagen protein synthesis was found during confluency (Table 6). Gold depresses the percentage of collagen synthesized by synovial cells in both log phase and confluency and by skin cells in log phase only.

The increase in collagen synthesized per cell as detected by the collagenase digestable assay (Fig. 12 and Table 6) was also found when the medium collagens were separated on SDS-polyacrylamide gels (Fig. 15). The increase in collagen was found to be predominantly type I collagen with the dose dependent increase for type III collagen at a lower slope than type I collagen (Fig. 15). The percentage of type III collagen to total collagen was found to be lower ($p < 0.05$) in gold exposed (7.5-30 uM) cultures than untreated controls when separated by both SDS-gel electrophoresis and carboxymethyl cellulose chromatography (Table 3).

Hance and Crystal (1978) reported that guinea pig lung fibroblasts produce the same ratio of III/I collagens with passages and cell density. Parrott et al. (1980) found that osteoarthritis, normal and rheumatoid arthritis derived synovial cells produce a similar ratio of III/I with passage. With the osteoarthritis and normal synovial cells a downward trend with passage was found, and with rheumatoid

arthritis synovial cells an increase trend was found (but not statistically significant). Abe et al. (1970) reported an increased percent type III collagen synthesis during confluency for human and guinea pig skin fibroblasts. This observation was confirmed in Table 6 for human skin fibroblast but the opposite was true for synovial cells. The percent of type III collagen synthesized was decreased by GST in both skin fibroblasts and synovial cells in both log phase and confluency. Also, in an experiment in which cell number was unchanged due to the low GST concentration (7.5uM) a decrease in type III collagen was also found. Therefore, the change in the percent synthesis of type III collagen is not due to cell density changes.

The quantitation of medium collagen, by the fluorescent method used here, represents the accumulation of [^{14}C]proline into collagen α chains in the synovial cell medium over a 5 day period. The measurement of total accumulation circumvents interpretation problems with regard to the influence of pool size changes and amino acid transport deficiencies on label incorporation which may be altered by gold (Finkelstein et al., 1977). The radioactive values represent the incorporation of [^{14}C]proline into collagen α chains during a 2 day incubation and subsequent separation of the radioactive collagen chains by both SDS-polyacrylamide gel electrophoresis and carboxymethyl cellulose chromatography. The exposure of human synovial cells to

GST caused a decrease in the percentage of type III collagen synthesized (as detected by both separation techniques) and also in the percentage of type III collagen which accumulates in the medium (based on the quantitation of media collagen by fluorescent gel scanning techniques). In view of this, and the premise that the production of both type I and type III collagen is dependent on the same precursor pools, indicates that the direct effect of gold on amino acid transport or pool specific activity is not responsible for the changes in collagen synthesis reported here.

The biochemical mechanism by which gold compounds alter proliferation and collagen synthesis in synovial cells can only be postulated from these experiments. The cell population generated by this culture technique is heterogeneous (Marsh et al., 1974, Smith, 1971, Castor, 1970) so that the dose-dependent effect of GST on proliferation and, therefore, also on collagen synthesis could be due to a selectivity for certain cell populations. The alteration in collagen types observed here could be due to direct stimulation of the synthesis of type I collagen, but we cannot rule out the possible inhibition of intracellular degradation (Bienkwski et al., 1978). Granulocyte collagenase has been shown to preferentially degrade type I as compared to type III collagen (Horowitz et al., 1977). Therefore, intracellular inhibition of a similar enzyme may result in the secretion of increased amounts of type I collagen.

A pannus can be considered a form of granulation tissue. Gay et al. (1978) demonstrated by immunofluorescence that type III collagen is the early collagen laid down in granulation tissue followed by type I collagen. In contrast, the predominant collagen type in normal scar is type I collagen (Bailey et al., 1973, 1975). A pannus responds to inflamation by generating granulation tissue, and further maturation results in the formation of scar tissue. Thus, it is conceivable that the alteration of the composition of the synovial matrix is related to or dictates the functional nature of the synovium in rheumatoid arthritis. Recent preliminary experiments by Dayer (personal communication) indicate that a blood mononuclear cell factor may stimulate proline incorporation into collagen, with the preferential stimulation of a protein tentatively identified as type III procollagen. Parrott (manuscript in preparation) found an increaase in the percentage of type III collagen synthesized by cultured synovial cells after mononuclear cell factor stimulation.

The data reported here establish that GST has a direct effect on synovial cell proliferation, and on the amount and nature (genetic composition) of the extracellular matrix they produce. This shift in synthesis or accumulation of type I collagen over type III collagen may represent a switch from a proliferative granulation tissue to end stage scar tissue. This forced maturation may in fact be responsible for aborting the progressive proliferative component of the

rheumatoid arthritis lesion, and may represent one of the important mechanisms responsible for the therapeutic efficacy of gold therapy in the treatment of rheumatoid arthritis.

Ugai et al. (1979) reported in vitro aurosome formation in monocytes at doses similar to those used here to form aurosomes. However, biochemical changes in cultured synovial cells were found below the dose for vacuole (i.e., aurosome) formation. GST-induced vacuoles appeared to be irreversible (present after subculturing and for a month without GST in the medium) and biochemical recovery and elimination of gold (Figs. 6, 8 and 9; Tables 4 and 5) was only partial at doses that would cause vacuole formation. Patients withdrawn from gold therapy usually have a relapse or reactivation of RA after several months (Freyberg, 1972). Gold does decrease in the synovial tissue after patients are withdrawn from chrysotherapy but is still present years later, as measured by atomic absorption spectrometry (Grahme et al., 1974) and as found by EM (Fig. 4) and X-ray probe analysis (Ghadially et al., 1978). Vernon Roberts et al. (1976) using neutron activation analyzed the localization of GST in the synovium. During chrysotherapy gold was abundant in synovial lining cells. Twenty-three years after stopping chrysotherapy gold was found in the subsynovial connective tissue.

The presence of aurosomes or the presence of gold in tissues does not imply a level of pharmacological activity compatible with clinical benefit. The presence of gold in the cell layer after removal of GST from the medium for 4-14

days (Table 4) was significantly correlated (r =0.57) with biochemical changes (Table 5) but not to the degree observed when GST was present in the medium (r > 0.9) (Fig. 7-12). Thus, the gold-induced suppression in the percentage of type III collagen synthesized and cell proliferation return after removal of GST from the medium at low (10 uM) but not high (100uM) GST.

The GST induced morphological change of the synovial cells in culture has a direct correlation to the morphological change in patients' synovial cells due to chrysotherapy. This lends credence to the experimental model as one in which there is a similar processing of gold in the cell cultures as in the rheumatoid joint. The biochemical effects of inhibition of proliferation and shift in collagen metabolism to the formation of a matrix not conducive for expansion may be a mechanism by which gold causes remission of RA. The means by which these biochemical alterations occur can only be speculated.

Steroids are known to bind to a cytoplasm receptor. The drug-receptor is modified to an "active complex"and enters the nucleus. Gold is internalized by cells, highly protein bound, effects DNA replication and gene expression. It is unlikely that the sequestered gold in the aurosomes can have these effects on the nucleus. Thus, the processing of gold in the aurosomes may be a means of forming an "active complex" similar to the "active complex" with steroids.

Another means by which these biochemical effects of gold on synovial cells may occur is by blocking or denaturing necessary factors for cell proliferation and metabolism.

Lastly, the biochemical effects of gold have striking similarities to the biochemical changes due to aging. Gold decreases the solubility of the collagen matrix by increasing cross-linkages of collagen (Adam et al., 1965 and 1968). With age, there is an increase in collagen crosslinkages (Bailey and Shimokomaki, 1971; Davison and Patel, 1975). Gold decreases the relative percent of collagen synthesized by cells in culture. With age, there is a decrease in the relative percentage of collagen synthesized in animal tissues and cells in culture (Newman and Lagner, 1975; Paz and Gallop, 1975). An age dependent shift from a type III collagen predominance to a type I collagen predominance was found from fetal to adult skin (Epstein, 1974). GST causes a dose-dependent increase in type I collagen. Nontransformed cells in culture have an aging process in which proliferation slows down and stops after a finite number of doublings. An increase in cell size and DNA content per cell has also been found by aging cells in culture (Greenberg et al., 1977; Pec, 1970; Cristofalo et al., 1969). GST has an antiproliferative effect in which there is an increase in cell size and an increase in DNA content per cell. Therefore, a quicker maturation of the pannus granulation tissue is a means by which expansion of the hyperplastic synovial cells into other connective tissue components of the joint can be inhibited, and may be an explanation of the efficacy of gold in rheumatoid arthritis.

VI. CONCLUSION

Gold sodium thiomalate (GST) causes biochemical and morphological changes in cultured synovial cells at pharmacologically relevant concentrations (10-100uM) which may be related to the efficacy of gold treatments to rheumatoid arthritis patients.

1. GST causes an analogous morphological change, aurosomes, in cultured human synovial cells as in the synovial tissue of patients. Hence, processing of gold *in vivo* and *in vitro* are similar.

2. Clinically, GST prevents the hyperplasia of the synovial cells in joints of patients with RA and inhibits cell division in cultured human synovial cells. The inhibition is reversible at low gold concentration (10uM) but not at high gold concentration (100uM) in culture.

3. GST alters the synthesis of extracellular matrix proteins. The shift in the synovial cell synthesis from collagen to noncollagen protein and from a type III collagen (new, repair) to a type I collagen (end stage, scar) would cause the formation of an unfavorable matrix scaffold for pannus expansion. The recovery of the cell to synthesize type III collagen was found when GST (10uM) was removed from the medium.

Therefore, a mechanism of action of gold in rheumatoid

arthritis can be a direct effect on synovial cells. The gold induced alterations in extracellular matrix formation and the gold induced anti-proliferation of the synovial cells may work synergistically to halt the pannus. The clinical benefits obtained from gold therapy, in a complex disase like rheumatoid arthritis, lie in a specturm of mechanisms working on a variety of receptors and cells in concert. The direct effect of GST on synovial cells could be instrumental in preventing irreversible joint damage.

VII. Appendix

A. Cell lines used in experiments.

Cell Line	Patient Initials	Sex	Age (yrs)	Date of Biopsy	Area of Biopsy	Diagnosis
RA-17S	A.T.	M	69	9/15/78	L.hip synovium	RA
RA-14S	A.P.	F	60	9/20/78	L.hip synovium	RA
RA-18S	C.P.	F	26	9/17/78	L.wrist synovium	RA
RA-19S	A.P.	F	60	9/22/78	L.hip synovium	RA
RA-16S	C.M.	F	53	9/13/78	R.knee synovium	RA
RA-21S	L.N.	F	54	8/30/78	R.hip synovium	RA
RA-13S	E.H.	M	66	11/28/77	R.knee synovium	RA
RA-15S	L.N.	F	54	8/30/78	R.knee synovium	RA
RA-23	J.R.	F	-	9/5/79	L.knee synovium	RA
RA-15	J.F.	M	62	8/29/79	L.hip synovium	RA
RA-16	D.B.	F	66	10/2/79	R.knee synovium	RA
RA-26	F.B.	F	74	12/12/79	R.knee synovium	RA
RA-25	G.H.	F	-	12/11/79	R.knee synovium	RA
RA-27	A.B.	F	71	1/15/80	R.knee synovium	RA
RA-30	G.D.	F	53	1/30/80	knee synovium	RA
NS-17	G.C.	M	31	9/4/79	R.knee synovium	Normal
OA-16	V.S.	F	59	8/28/79	R.hip synovium	OA
PY-0	-	F	90	12/10/79	skin	Normal

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