STROMAL ALTERATIONS DURING CUTANEOUS CARCINOGENESIS

by

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In Memory of my Mother

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I. GENERAL INTRODUCTION

Although an all inclusive definition of cancer poses difficulties, it must admit a defect in those processes directing and controlling differentiation and growth. In the skin, as in other tissues, normal differentiation and growth are directly dependent upon an interaction between epithelium and mesenchyme. In these tissues, alterations of the normal epitheliomesenchymal interaction occur both in the cancers themselves and in the processes leading to their formation. As will be seen, these alterations are a constant feature of carcinogenesis independent of organ, species or carcinogen.

Epitheliomesenchymal interactions depend on both physical and humoral factors and are necessary for normal differentiation and growth. These interactions are regionally specific, the specificity for growth generally residing in the epithelium and for differentiation in the mesenchyme.

In order to understand the rationale behind the studies to be presented later, research indicating that the connective tissue underlying epithelium becomes altered during carcinogenesis will first be reviewed. Additionally, data will be presented which indicate that such a stromal response can influence the neoplastic response of its overlying epithelium. Research on these stromal modifications falls into three categories: (a) histological, (b) histochemical, and (c) biochemical, and will be discussed in that order.

A.   Histological and Histochemical Changes

Alterations of collagen and elastin in stroma associated with
cancerous and pre-cancerous epithelia are a common finding.
Bonney (15) in 1908 first reported the changes taking place in con-
nective tissue during carcinogenesis. He noted that "In the area of
primary carcinoma there has always occurred a complete disappear-
ance of yellow elastic tissue, and it is in this de-elasticized area
that the first epithelial down-growths occur." He also described a
constant loss of connective tissue and hyaline changes in the collagen.

Orr (119a) treated the skin of mice with methylcholanthrene
for three weeks and observed that the collagen fibers in a narrow
subepithelial zone became thinner and lost their bi-refringence. As
carcinogenesis proceeded, the alteration in the collagen fibers
gradually spread until the whole dermis appeared rarified. Con-
comitantly, the elastic fibers underwent an initial disappearance,
then reappeared in above normal amounts. This new elastin appeared
coarser and sometimes granular. Orr concluded that the neoplastic
change in the epithelium which occurred after the onset of these
dermal changes "may be secondary to nutritional alterations depend-
ent on the state of the connective tissue."

Herovici (66) studied the connective tissue reaction in tumors
of the uterine cervix. Precollagen fibrils followed the contour of

localized tumors and the cells were mostly of the lymphocytic or plasmocytic series, whereas with invasive tumors, precollagen fibrils were completely disorganized and the cells were predominantly fibroblasts.

O'Shevskaya (118) implanted pieces of cellophane of varying sizes subcutaneously. After six months they were surrounded by a dense collagen capsule. Tumors arose only in cases where prolonged proliferation of fibroblasts was accompanied by abnormally extensive collagen formation. The extent of the connective tissue reaction varied directly with the size of the piece of cellophane. During experimental cancro-cirrhosis induced by p-dimethylamino-azo-benzene (butter yellow), Bartoloni-Saint Omer (8) observed histologically both a proliferation of elastic tissue and "elastotic" degeneration of collagen in the liver.

Mehregan (103) in a study of 530 cases of basal cell epithelioma showed five different patterns of change of elastic tissue associated with the tumors. In the most common pattern, newly-formed fibers surrounded the epithelial components. Other patterns consisted of clumps, sheaths and networks of new or degenerate elastic tissue both without and within the epithelial nests.

Alterations in the mucopolysaccharide compartment of connective tissue associated with precancerous and cancerous epithelia have also been observed. Prodi and Maltoni (133a) painted rabbit

skin with the carcinogen, dimethylbenzanthracene (DMBA) and the promoting substances, Croton oil and Tween 60. Within twenty-four hours both toluidine blue and alcian blue stains revealed a "highly chromotropic substance" in the superficial layers of the dermis. As treatment continued, this substance advanced to the deepest dermal layers and became concentrated near the sites of epithelial proliferations, but after eighty days of treatment, it had largely or completely disappeared. No chromotropic substance appeared with either promotor regardless of their ability to elicit a hyperplastic response. The intensity of the metachromasia of this substance was proportional to the degree of hyperplasia. Although the chromotropic substance was sensitive to hyaluronidase, its nature cannot be deduced since both hyaluronate and chondroitin sulfate are attacked by this enzyme.

Tighe (163) studied the histochemistry of mucopolysaccharides (MPS) with toluidine blue, alcian blue and Hale's dialyzed iron in combination with testicular hyaluronidase in myxomatous, fibrous and fatty breast tumors. Although the reaction of the mucin varied with tumor type, Tighe thought that either chondroitin sulfate A or C was the principal mucopolysaccharide in the majority of these con-nective tissue tumors on the basis of strong metachromasy, Hale positivity, alcian blue positivity, PAS negativity and hyaluronidase

sensitivity. In this study, liposarcomas appeared to contain hyal-
uronate. No correlation was found between the type of polysacchar-
ide, the degree of malignancy or the tendency to recurrence in any
of the tumors.

Majewski et al (96) investigated the ground substance of the
dermis in skin cancer in a series of eighty-two human patients. In
basal cell epithelioma they found an increased activity of acidic
mucopolysaccharide (AMP) around the neoplastic infiltrations,
accompanied by a proliferation of fibroblasts and mast cells. In
keratinizing and non-keratinizing squamous cell carcinomas both the
AMP and connective tissue cells varied in reactivity. These authors
suggested that in cancer of the skin the cellular reaction represents
the main defense of the connective tissue to neoplastic infiltration.
An increased production of AMP accompanied the increased pro-
liferation of fibroblasts and mast cells according to Gasic et al (42).
They studied solid tumors with different metastatic behavior in mice
and found that the quantity of AMP in the intercellular spaces as
revealed by Hale staining of primary tumors correlated negatively
with the frequency of metastases. However, when the same tumors
were incubated with pepsin before Hale staining, the reactivity cor-
related with metastatic behavior. According to the authors, proteins
partially or completely mask the acidic character of AMP, blocking
the staining of these substances; thus, the pepsin "unmasked" the

AMP. The question arises whether the differences in metastatic behavior could depend more on the amount of protein combined with acidic groups of the intercellular AMP than on quantitative or qualitative differences in AMP content. In a related study, Steigleder et al (158) found a relation between proteolytic activity and the visibility of AMP in the dermis associated with skin tumors. The proteolytic activity, however, permitted no conclusions regarding the degree of malignancy with the possible exception of squamous cell carcinoma.

Poole and Williams (131) in a histological and histochemical study of the effect of an invasive malignant rat tumor on xiphisternal cartilage in vitro found that when the tumor was implanted next to the xiphisternum, the cartilage immediately adjacent to the tumor lost metachromasy with toluidine blue. This returned to normal with increasing distance from the tumor mass. Chondrolysis was observed in the immediate vicinity of the tumor. Xiphisternal cartilage is of the hyaline variety and is thus rich in chondroitin sulfate.

A histochemical characterization of the glycosaminoglycans of salivary gland tumors was performed by Quintarelli and Robinson (124d) using a sequential extraction technique developed by Scott, Dorling and Quintarelli (152b). The method is based on the reversal of AMP-alcian blue complexes upon the addition of increasing concen-

trations of magnesium chloride to the dye solutions. This technique resembles the selective precipitation of polysaccharides with the polycation cetylpyridinium chloride and their sequential solubilization with increasing salt concentrations. Alcian blue thus stains with increasing selectivity as the critical electrolyte concentration for each specific AMP is successively reached. Quintarelli and Robinson showed by this technique that the mesenchymal portions of this tumor contained HA and Ch-4-and/or Ch-6-SO_4 and that both the epithelial and the mesenchymal portion of the tumor reacted histochemically in the same manner as their corresponding tissues of origin.

An altered stromal cellularity has also been found frequently associated with epithelial neoplasia. Moore, Stevenson and Schoenberg (109) in an investigation of the connective tissue in human basal cell carcinoma, squamous cell carcinoma, seborrheic keratosis and verruca vulgaris noted the above changes, as well as an increase in the number and immaturity of the fibroblasts. They suggested, however, that the intensity of the connective tissue reaction was not a function of the aggressiveness of the tumor although the non-malignant lesions of seborrheic keratosis and verruca vulgaris showed none.

The benign connective tissue tumor of man, the histiocytoma, consisting of a proliferation of fibroblasts and histiocytes within a

fibrotic and metachromatic stroma, is frequently associated with downward proliferation of the overlying epidermis (150). Occasionally this proliferating epidermis can become malignant (19).

In several studies a multiplicity of changes have been noted to progress during the course of carcinogenesis. While these cannot as yet be considered a connective tissue "syndrome," it should be noted that they commonly occur in the presence of cancerous and pre-cancerous epithelia under the influence of a variety of physical and chemical carcinogenic stimuli. Dobson and Griffin (29b), investigating the histochemistry of dermal alterations during 2-amino-anthracene carcinogenesis in the rat, described an increase in Mowry's colloidal iron-positive material considered to be indicative of an increase in acid mucopolysaccharides. They also noticed a concomitant decrease in Luxol fast blue reactivity. Luxol fast blue is thought to stain mature collagen (56). Other findings included a fragmentation and clumping of the basement membrane, fragmentation and rarefaction (i.e., thinning and loss) of dermal collagen, increase of elastin in the dermal papillae, marked vascular dilatation, and an increase in mast cells and fibrocytic-type cells which "were larger than normal and contained irregular hyperchromatic nuclei." Most of these changes progressed until cancer appeared.

Dobson and Pinto (29c) investigated the effects of the carcinogens, 2-aminoanthracene (AA), 3-methylcholanthrene (MC) and 9, 10-dimethylbenzanthracene (DMBA) alone and in combination with the promoter, dodecylbenzene, on the skin of the Rhesus monkey. Although AA and MC alone produced little change, DMBA alone and all carcinogens in combination with DDB produced extensive alterations in the dermal connective tissue equivalent qualitatively and quantitatively to those observed in rodents.

In mice irradiated with ultraviolet light, Epstein and Epstein (31) noted an accumulation of acid mucopolysaccharides, a loss of mature collagen, a proliferation of mast cells and fibroblasts and a thickening and dissolution of the basement membrane preceding epidermal neoplasia. In sun-damaged human skin, Sams and Smith (146a) reported a marked increase in the dermal content of elastin. In this regard, according to Urbach, Davies and Forbes (165b), 86.5% of human squamous cell carcinomas occur in sun-exposed areas.

In human basal cell epitheliomas Sams, Smith and Finlayson (146b) noted that dissolution of normal collagen and elastic fibers, proliferation of argyrophilic fibers, an increased amount of acid mucopolysaccharide, mainly hyaluronate, and an increased number of fibroblasts and mast cells occurred in response to tumor invasion.

In 1962, Van den Hoof (166) in a sequential study of the skin
of mice treated with methylcholanthrene found an initial increase
in argentophilia and periodic acid-Schiff (PAS) staining of the con-
nective tissue followed by a rarefaction of the fibrous structure and
breakdown of the basement membrane. In the final phase, he ob-
served collagen proliferation, increased argentophilia, and increased
PAS staining. The PAS reaction occurs with neutral polysaccharides,
sialic acid and mucoproteins but not with glycosaminoglycans (152a).

B.   Electron Microscopical Changes

In an investigation of fibrous-long-spacing (FLS) collagen in
tumors of the central nervous system, Ramsey (136) was able to
demonstrate altered periodicity of the collagen. A random arrange-
ment of collagen fibrils with crystallite deposited irregularly along
them was found to be present in beryllium-induced osteogenic sar-
coma (124). Collagen in human Schwann cell tumors both in vivo
and in vitro appeared similar to the FLS form of reconstituted
collagen. Tarin (162) in an investigation of experimentally-induced
carcinogenesis in mouse skin showed an initial increase in dermal
ground substance and decrease in the diameter of the collagen fibers
with thickening of the basement membrane and loss of basement
attachment devices; the latter was accompanied by separation of
the epidermal cells from the basement membrane. Subsequently,

large amounts of fragmented material similar in consistency to the basement membrane and lying in the superficial dermis coalesced, forming organized structures which resembled a reduplication of the basement membrane. Although the organization of collagen was found to be disturbed throughout the dermis, its periodicity remained intact. The number of mast cells, fibroblasts and plasma cells was increased. In squamous cell carcinomas, the basement membrane completely disappeared and epithelial cells lay in direct contact with connective tissue. At this naked junction, the dermal surfaces of tumor cells extended pseudopods into the connective tissue; these ballooned, constricted at the necks and became free vesicles which burst with the consequent dissolution of collagen and severe disruption of the superficial connective tissue. Gaps in the basement membrane appeared long before any tumor was seen. Although these changes were also found associated with papillomas, they were not nearly as marked.

The fine structure of the basement membrane in epidermal tumors experimentally induced in mice with DMBA and croton oil has been studied by Frei (38). In several cases the basement membrane was found to have g aps through which epidermal pseudopods protruded. Frei concluded that the neoplastic epidermal tissue grows faster than a basement membrane can be formed beneath it. This, however, seems unlikely in the light of information to be presented in the general discussion.

Several studies have indicated that these connective tissue changes might play an important inductive role in epithelial carcinogenesis. Vasiliev (168a), studying the dermal connective tissue of rats treated with 7,12-DMBA, reported that all doses of DMBA inhibited connective tissue proliferation and depressed cell multiplication and reactive proliferation of connective tissue. Higher doses produced drastic degenerative changes in the ground substance, atypical collagen formation and focal proliferations of atypical fibroblasts. The intensity of these changes correlated with the carcinogenicity of the various dosages of DMBA.

Mizukami (107) investigated the role of the mesenchymal system in the growth and infiltration of cancer. The injection of cortisone or trypan blue, which block the function of the mesenchymal system, promoted cancer production by methylcholanthrene or 3,4-benzpyrene. When he transplanted Ehrlich's cancer intramuscularly, the local injection of hyaluronidase intensified the infiltration of the cancer. On the other hand, "nekrohormone," a substance obtained by ligation of the splenic artery, which "activates the vitality of the mesenchymal system" clinically and experimentally suppressed cancer development.

In order to determine whether the primary induction occurs in the dermis or in the epidermis, Billingham, Orr and Woodhouse

(12a) cross-transplanted epithelia between carcinogen-treated skin

and untreated skin. The untreated epidermis grafted onto a treated

dermis became cancerous while a treated epidermis grafted onto an

untreated dermis developed no tumors. Although they concluded that

the neoplastic determinant lay in the dermis, they admitted that the

observed tumors could have arisen from residual follicular epithel-

ium.

Although it is difficult to make any general conclusions from

such heterogenous data, certain unifying points emerge.

First, all reports indicate that there is an alteration of the

connective tissue associated with carcinogenesis or the presence

of a cancer in adjacent epithelium.

Second, these connective tissue changes precede the onset

of neoplasia in the epithelium. It cannot be stated whether the ob-

served changes are the direct result of the carcinogen on the con-

nective tissue, or the indirect result of an influence exerted by a

carcinogen-altered epithelium. Because of the time course of their

appearance, however, it can be said that the connective tissue changes

are not produced by the cancerous epithelium, even though they can

persist in the presence of, and thus be found in association with such

an epithelium.

Third, certain manifestations of the stromal response are

constant and predictable. The most evident of these is the collagen

response. In the dermis of pre-cancerous skin, the collagen first undergoes rarefaction (i.e., thinning) and fragmentation in the superficial layers which gradually spreads to involve the entire dermis. The collagen in carcinogen-treated skin, based on its altered histochemical affinities, differs from the normal mature form of untreated skin. Increases in elastin also appear to be a constant feature of pre-cancerous connective tissue. The ground substance undergoes histochemical changes, the nature of which remains relatively unexplored. Some have considered the change to be quantitative, others qualitative, while still others have considered the histochemical changes to be due to an unmasking of the AMP which is independent of qualitative or quantitative change. The discrepancies on this point lie, however, in the interpretations of the histochemical findings rather than in the findings themselves. Additionally, a relatively common feature of pre-malignant connective tissue is an increased cellularity, manifested by a mild mastocytosis and fibrocytosis. Fibroblasts generally appear less mature than those in normal connective tissue. Finally, the basement membrane underlying a pre-malignant epithelium is always clumped and fragmented. The basement membrane appears to be replaced at regular intervals in the course of growth of a tumor. Pierce (125) has shown that tumor cells are capable of producing their own basement membrane as they invade.

The fourth point which emerges clearly is the universality of these changes. They are predictable and reproducibly observed in the dermal connective tissue of rats, rabbits, guinea pigs, mice, hamsters and monkeys. Though reports are less frequent in other tissues, it would appear that the changes are not tissue-specific. The response is the same, regardless of the carcinogen, provided the stimulus is carcinogenic to the species tested. The pre-neoplastic changes seem to be independent of the eventual tumor type, but once the tumor has been established, a difference in connective tissue response coincides generally with degree of malignancy. A relatively non-malignant tumor will elicit an increased fibrotic response with the ultimate formation of a collagen pouch surrounding the tumor from which the tumor can be dislodged en masse. Conversely, a malignant tumor will cause a rarefaction and dissolution of the surrounding stroma.

Although there are certain features which characterize the connective tissue response to a carcinogenic stimulus, a carcinogen-treated dermis generally resembles immature connective tissue (43), connective tissue during wound healing (45), and chronically sun-damaged skin (146a). Thus, although these histological and histochemical alterations of the stroma are specific, they are not unique. However, the one critical feature which is shared by all epithelia that

abut such connective tissue, a feature which lends significance to these findings, is a tendency in each case toward rapid proliferation and infiltration of the epithelium into that tissue.

Histological, electron microscopical and histochemical observations, however valid, are not in themselves, always convincing, since it is nearly impossible to obtain unanimity of interpretation. The obvious approach to this problem would be an attempt to confirm these connective tissue changes biochemically and to express results in terms less liable to subjective interpretation. Some approaches in this direction have been made.

C. Biochemical Changes in Collagen

Ma in 1940 (93) was the first to analyze connective tissue changes during cutaneous carcinogenesis. With Lowry's method for the determination of collagen and elastin, Ma found that fibrous proteins in the skin of methylcholanthrene-treated female Swiss mice first slightly increased and then decreased. The chemical fluctuations in elastic tissue content corresponded to his histological findings. The number of elastic fibers increased progressively for thirty days after initiation of painting accompanied by thickening and irregularity of individual fibers. This was followed by a decrease in the number of fibers and loss of epithelial anchorages and terminal swellings, eventuating in the localization of all elastic

tissue to fibrous islands in a dermis otherwise free of elastic fibers.
However, since the Lowry technique involves separatory and assay
procedures which are crude and unreliable by present standards, the
data of Ma are questionable. Current procedures for the isolation
and quantification of collagen will be discussed in experimental
section II.

Grabowska (48) measured the collagen content of normal con-
nective tissue, of connective tissue surrounding a tumor and of
growing rat sarcoma by the technique of Neuman and Logan and
found that the collagen content in neoplastic tissue, as percent dry
weight, depended on the weight of the tumor. An initial decrease in
the proportion of collagen with increasing tumor weight was followed
by a stabilization of the relationship as the tumor grew. The collagen
content of normal tissue exceeded that of the tissue surrounding
the tumor. The author concluded that the surrounding stroma reacts
to the tumor either by decreased synthesis or by increased catabolsim
of collagen. Nomura measured the content of collagen in prostatic
hypertrophy and prostatic carcinoma (116). The average collagen con-
tents as percentage dry weight were as follows: normal prostate 6.3%;
prostatic hypertrophy 9.8%; glandular carcinoma 8.6%; fibro-gland-
ular carcinoma 10.4%; mixed carcinoma 10.9%; microfollicular
hyperplasia 9.8%; occult carcinoma 11.3%; scirrhous type carcinoma
13.3%.

Fels and Greco (33) determined changes in dermal hydroxy-proline (a specific marker of collagen comprising about one seventh of the total amino acid residues) during carcinogenesis induced by methylcholanthrene in benzene. A marked progressive decrease in hydroxyproline occurred in both the experimental and the benzene-painted control animals. After cessation of painting, the hydroxy-proline increased in the controls, but continued to decrease in the carcinogen-treated animals. In transplanted tumors, Gullino (55) showed that histologically similar tumors produced by the same carcinogen contained markedly different amounts of collagen. Anderson (3) has assayed the collagen content of a human chondro-sarcoma; in comparison to normal cartilage, the collagen was reduced. Chondroitin sulfate was markedly reduced in the actively growing part of the tumor. These studies are of limited value due to the unavail-ability of an appropriate control. A decreased concentration of col-lagen in a mass of rapidly dividing tumor cells can be expected. If the percentage of collagen in a tumor is increased in comparison to normal tissues, then an increase in the fibrous support of the tumor should be suspected. Danishefsky, Oppenheimer, Heritier-Wilkins, and Willhite (25b) recently investigated changes in the connective tissue pocket surrounding subcutaneously-imbedded plastic. The soluble collagen was higher in the early stages and subsequently de-

creased, whereas the insoluble collagen increased constantly in comparison to glass-imbedded controls. The differences between imbedded glass and plastic, however, were slight and suggest that all changes could be explained by simple aging of the connective tissue since, during aging, there is normally seen a decrease of soluble collagen and an increase of insoluble collagen.

Shelton (154a, b) examined the effect of tumor cells on preexisting collagen and on collagen production by normal cells grown in diffusion chambers. When mouse and rat tumors were combined with normal mouse peritoneal cells and normal connective tissue, the hydroxyproline content was reduced. Rat hepatoma placed in a similar chamber and surrounded by tumor tissue showed a decreased DNA content. The author concluded that the observed inhibition of collagen formation was due to an inhibition of the proliferation of collagen-producing cells rather than an inhibition of their metabolism. The tumors did not have the capacity to destroy pre-existing collagen. Gilfillan (44) has recently studied the effects of heteroploid, diploid, and primary kidney cells from several species on elastin. Established human heteroploid cell lines and virus-transformed tumorigenic hamster cells actively phagocytized and degraded intracellular elastin, while human diploid fibroblasts did not. Epithelial-like cells from primary kidney cultures showed less active phagocytosis and somewhat less active elastolysis than heteroploid cells.

Hamer and Marchant (58) measured extractable and non-extractable collagen hydroxyproline, tyrosine, hexosamine and reducing sugar in normal mouse skin and the skin of mice painted with 20-methylcholanthrene (MC) to establish a chemical basis for the histological changes they observed in connective tissue. Since no pronounced chemical changes were detected, they concluded that physical alterations in the collagen, rather than changes in chemical composition, were responsible for its altered histologic appearance.

Green, Todaro and Goldberg (50) investigated collagen synthesis in established fibroblast cell lines transformed by oncogenic viruses. Under conditions which allowed maximum rate of collagen formation, transformed fibroblasts produced less collagen per unit of protein synthesized than the parent line. A second differentiated function, hyaluronate synthesis, was reduced in one line transformed by virus. The fibroblasts transformed by oncogenic viruses also have an enhanced metabolic autonomy, as their collagen synthesis was less dependent on exogenously supplied ascorbate than the parent line.

Several indirect indications of abnormal collagen metabolism associated with cancer have been established. Bonadonna, Merlino, Meyers and Sonenberg (14) showed an increase in urinary hydroxyproline in patients with various cancers. Platt, Doolittle and Hartshorn (130) investigated urinary hydroxyproline excretion in

a series of patients with metastatic carcinoma of bone, carcinoma

in soft tissue, some with metastases to other soft tissues, a group

of patients with various diagnoses not known to involve collagen, and

normal adults. In ascending order, the mean hydroxyproline excre-

tion per 24 hours in this series was as follows: controls, patients

with varied diagnoses, patients with carcinoma of soft tissues,

patients with metastatic carcinoma of bone and a five year survival,

patients with same disease but less than five year survival. Accord-

ing to Prockop and Kivirikko (132b), such increased hydroxyproline

excretion can derive from (1) an increased rate of collagen synthesis

with collagen excretion remaining normal, (2) a decreased rate of

aggregation, that is the conversion of soluble collagen to the more

insoluble forms, (3) an increased rate of degradation of insoluble

collagen to more soluble forms, or (4) an increased rate of degrada-

tion of various forms of collagen to free and peptide hydroxyproline.

McCormick (99) asked whether cancer could be a collagen

disease secondary to a nutritional deficiency. He cites Eickhorn

as having found a pronounced deficiency of vitamin C in patients with

cancer. Bodansky (13) found a marked reduction in the concentration

of ascorbic acid in plasma and white blood cells of patients with

cancer as compared with the values in patients with non-cancerous

chronic diseases. Russel, Ortega and Wynne (143) found that an

ascorbic acid deficiency decreased the latent period for the onset of neoplasia in methylcholanthrene-treated guinea pigs. Ascorbic acid (vitamin C) is necessary for the hydroxylation of proline to hydroxyproline, an essential component of collagen. Since hydroxyproline itself cannot be incorporated directly into the peptide precursors of collagen, but rather must derive from the ascorbate-requiring hydroxylation of the proline already present in these peptides, the absence of ascorbate could cause either a complete block to collagen synthesis or production of a defective collagen molecule. A paucity of new collagen or an abundance of defective collagen could lower connective tissue resistance to tumor invasion, or could loosen the attachments of cells which have a normal propensity to migrate and divide when uninhibited by close contact with each other. However, it is unlikely that cancer would result merely from the influence of an ascrobic acid deficiency on collagen since cancer is not normally associated with the collagen changes in scorbutics.

The potential contribution of ascorbate fluctuations in carcinogenesis encourages examination of the influence of various other collagen defects on cancer and carcinogenesis. Lathyrism induced by β-aminoproprionitrile (βAPN) had no effect on either the frequency of takes or the rate of growth of transplanted Walker 256 carcinoma in the rat (100). On the other hand, Madden and Carpas (94) showed

that Walker tumor 256 implanted into the flank initially grew larger

and remained larger in weanling rats made lathyritic with βAPN than

in pair-fed tumor-implanted, non-lathyritic controls. Similarly,

Schweppe, Baserga, Harris and Jungmann (151) found that lathy-

rism accelerated the induction and growth of mammary tumors and

reduced the tensile strength of the collagen but did not alter metastatic

spread.

D.   Biochemical Changes in Mucopolysaccharides

Chemical alterations in the mucopolysaccharide (glycosamino-

glycan) compartment of connective tissue undergoing carcinogenic

treatment or associated with cancer have been investigated in an

attempt to clarify the observed histochemical changes. Kabat, Kaiser

and Sikorski (79) were the first to demonstrate the presence of hyal-

uronic acid (HA) in virus-induced Rous chicken tumor. HA has also

been extracted from Rous and Fujinami myxosarcomas (122), from

the interstitial fluid of pleural and peritoneal mesotheliomas (105a),

in one case of pleural effusion due to adenocarcinoma, in human

liposarcoma (163), and in one case of "giant" human carcinoma (25).

Physicochemical characterization of tumor HA by infra-red spec-

troscopy (120), determination of electrophoretic mobility and

sedimentation characteristics, and viscosity reduction with testicular

hyaluronidase (172) agreed closely with data from mammalian hyal-

uronic acid. Recently, Kirchheiner (84b) fractionated AMP in ascorbic acid-deficient carageenan granulomas by column chromatography on ECTEOLA cellulose. Three fractions were obtained-- an HA-like, a $ChSO_4$-like and a residual fraction. Higher concentrations of both the HA-like fraction and the $ChSO_4$-like fraction characterized the scorbutic granulomas.

Grossfeld (54) studied the production of hyaluronic acid by fibroblasts from explants of Walker tumor 256 and the subsequent production of hyaluronidase by the tumor cells. After subcutaneous transplantation in rats, Walker tumor 256 grew slowly for seven days. At about day seven, however, the growth of the tumor accelerated enormously. To determine the relation between tumor growth and the presence of spreading factors or mucolytic enzymes, the author cultured both the early and late phases of this tumor. Explants of the young tumor contained a distinctive cell type different from the carcinoma cells of the older tumor. This cell was considered to be a fibroblast, and in culture it formed a mucinous clot in the supernatant which was removed by hyaluronidase. Extracts of large infiltrative Walker tumors depolymerized the HA produced by these fibroblasts in culture. Gossfeld interpreted the course of growth of Walker tumor transplant as an initial production by the fibroblasts of hyaluronic acid which blocks the spread of the tumor; the small

tumor then begins to produce hyaluronidase which digests hyaluronic acid and allows infiltrative growth. However, it is possible that other mucopolysaccharides are involved as well, since it is now known that certain of the chondroitin sulfates are also sensitive to hyaluronidase digestion.

Prodi and Laschi (133b) examined mucopolysaccharide (MPS) metabolism in the dermis of rabbits after treatment of the skin with irritating compounds. Rabbits, injected intravenously with $10\mu c$ glucose^{14} C/Kg body weight, were painted on one side of the dorsum with croton oil. The contralateral unpainted side was used as a control. The treated site shows an accelerated synthesis of dermal MPS evident within three hours after the first painting. The greatest increase was in the synthesis of hyaluronic acid indicated by a greater incorporation of label into glucosamine. The authors concluded that the inflammatory stimulus promoted an accelerated synthesis of MPS in fibroblasts which was directed chiefly toward the production of hyaluronic acid; they also state that carcinogenic polycyclic hydrocarbons do the same.

Various findings regarding the chondroitin sulfates during carcinogenesis have been reported. In fibrosarcomas induced in rats by polystyrene films, $Ch-4-SO_4$ together with heparin and another, as yet unidentified, glycosamine-containing polysaccharide were demonstrated chromatographically by Danishefsky, Oppenheimer, Willhite,

Stout and Fishman (26a). They also described an increased hexo-samine concentration and sulfate uptake reaching peaks at two to four months and decreasing slowly therafter. The MPS activity remained higher than normal, however, even after six months. Adams (2) demonstrated enzyme systems in human osteochondro-sarcoma which activate and transfer sulfate to chondroitin sulfate. The enzyme activity, similar to that found in embryonic cartilages, exceeded that of adult costal cartilage. The bulk of the glycosamin-oglycan isolated from the tumor was chondroitin-4-sulfate and possibly a small amount of chondroitin-6-sulfate.

Keratan sulfate has been shown by Bentley and Kircheim (10) using the histochemical technique of Scott, Dorling and Quintarelli (152b) to be the only AMP specifically localized to prostatic and bladder cancers.

Boström, Friberg, Larsson and Nilsonne (16) studied the in vitro incorporation of S^{35}-sulfate into samples of two surgically-removed chondrosarcomas of long bone using samples of adjacent joint cartilage as controls. By means of biochemical and radio-autographic techniques, they were able to show (1) a 1.5 to 15 fold increase in sulfate incorporation in tumor tissue, (2) that keratan sulfate and chondroitin sulfate were the main constituents of the tumor tissue, (3) that the synthesis of keratan sulfate was greatly

accelerated in relation to that of the chondroitin sulfate (s), and (4) that the greater cellular incorporation of S^{35} in the tumor appeared to account for the discrepancies in isotope uptake.

The incorporation of $^{35}SO_4$ into heparin by a microsomal fraction from mast cell tumors was studied by Silbert (155). Sulfation occurred with the same fraction involved in AMP polymerization, thus suggesting a close proximity of these activities within the mast cell.

Large quantities of heparin have been found in mast cell tumors from dogs (117), cattle, cats and mice (140). There appears to be a marked heterogeneity of heparin from these tumors, particularly in the amount of sulfation (141).

Sasaki (148) studied the enzymes involved in AMP synthesis in the DMBA-treated abdominal skin of mice undergoing treatment with various hormones known to influence AMP metabolism. The three enzymes, UDPG-DH, L-glut.-F6P-transamidase and L-glut.- G6P-transamidase, which synthesize the monosaccharide units, UDPGA and Gm-6-P known to be important intermediates in AMP synthesis, were found to be higher in the DMBA-treated than in the normal skin. Cortisone acetate, testosterone and DOCA decreased the levels of these enzymes. Estradiol caused an increase in UDPG- DH in both DMBA and normal skin.

Several attempts have been made to clarify the role of MPS in growth regulation. Ozello, Lasfargues and Murray (121), studying the behavior of "a purely epithelial strain" of mammary carcinoma cells in tissue culture, noted growth-promoting activity of certain MPS added to the culture medium. The cells depended on the presence of hyaluronic acid and chondroitin sulfate to grow and spread, and even for survival. Tryptic digestion products of these MPS of low molecular weight had very little growth-promoting effect. Morrison, Murata, Quilligan, Schjeide and Freeman (111) investigated the effect of various MPS on Hela cell growth as measured by cytocrit determinations, cell counts, cell volumes and dry weights. The MPS test substances included an extract from calf aorta, which the authors claimed is rich in MPS, chondroitin sulfate A (CSA), and chondroitin sulfate C (CSC). All test groups and the controls were also treated with a mild growth stimulant, human lipemic serum (HLS). CSA and aorta extract most effectively increased cell volume, whereas, CSA produced the most significant change when growth was measured in terms of total dry weight. The aorta extracts and HLS alone increased cell numbers most effectively. While such attempts to ascribe a role for specific MPS in the regulation of growth are theoretically sound, practically, they are difficult or impossible to interpret due to the large number of

manipulations exerting uncontrolled and thus, indeterminant influences on growth.

Takeuchi recently investigated the effect of intra-peritoneal and subcutaneous injections of chondroitin sulfate on the growth of Ehrlich Ascites tumor (161a) and the influence exerted by other interstitial components on the effects of these injections (161b). Previous studies by this author established that AMP promoted the growth of the tumor when a subcutaneous injection of an AMP solution was immediately followed by tumor inoculation into the same site, and that the tumor growth tended to be accelerated as the concentration of chondroitin sulfate in the tumor inoculation site was increased. In these studies, the stimulation to tumor growth and the increase in the urinary concentration of $ChSO_4$ were both greater in those animals injected intraperitoneally with the $ChSO_4$ solution than in those receiving a subcutaneous injection. The stimulatory effects of chondroitin sulfate on tumor growth was markedly inhibited by egg albumin, calf serum and muramidase, but only weakly inhibited by a collagen preparation, L-hydroxyproline and fibrinogen. Takeuchi believes chondroitin sulfate protects the surface of tumor cells and promotes the exchange of their metabolites through its polyanionic function. He regards the inhibitory effects of the substances tested as being due to the decrease of the negative electric charge of chondroitin sulfate.

Finally, Lippman (90b), in an extensive investigation of the influence exerted by various glycosaminoglycans and glycoproteins on mouse L-cell and ascites tumor cell growth both in vitro and in vivo, showed that sulfated glycosaminoglycans inhibited cell division but that protein-polysaccharide complexes from a variety of sources produced a marked stimulation of cell division. L-cells inhibited by polysaccharide treatment in vitro produced large transplantable malignant tumors when injected into mice. Histochemically the polymers were seen to be adsorbed to the cell surface. The author proposes that this influences DNA synthesis by controlling nucleotide pool utilization.

Rich and Meyers (138) investigated the urinary excretion of MPS in patients with malignant neoplastic disease and showed a significant increase of AMP in these patients as compared to normal controls. The AMP isolated from several patients with cancer or leukemia was found to have a composition similar to that of chondroitin sulfate. However, such a finding could merely reflect the commonly observed breakdown of tissues adjacent to a tumor and could be independent of the neoplastic process.

Although considerable knowledge of stromal alterations during carcinogenesis exists, it has been recorded from such diverse models that a constructive conclusion from the various data is difficult. To obtain a more thorough understanding of this area, it seemed desirable

to use a single animal-carcinogen system and attempt to define the

epithelio-mesenchymal response first on a histological and histo-

chemical basis and later on a biochemical basis. It would be desir-

able to explore, first, those collagen alterations characterized by

morphological changes and by changes in tinctorial affinities. Such

a study should include the entire course of carcinogenesis and

should address itself to possible defects in collagen synthesis, cross-

linking or breakdown. The histochemically observed alterations in

the ground substance should also be explored at the biochemical

level. Finally, as techniques in the area of connective tissue

chemistry develop, the dermal mucoproteins and elastin should

similarly be considered.

II.   EXPERIMENTAL SECTION

A.   Histology and Histochemistry of Stromal Alterations During

Cutaneous Carcinogenesis.

1.   Introduction

The histological changes in dermal connective tissue assoc-

iated with cancerous and precancerous epithelia would appear quite

pertinent to the carcinogenic process since the interaction of an

epithelium with its mesenchyme can be a critical determinant in its

movement, growth and differentiation. Since carcinoma of the skin

represents an abnormal growth and differentiation of epithelium, a

re-evaluation of those factors involved in normal epithelio-mesenchymal

interaction would be of potential value in understanding neoplastic

activity. Additionally, a precise definition of those stromal altera-

tions which are consistently and universally found in diverse models

of carcinogenesis and which could result in the loss of differentiation

and control over growth of associated epithelia would be desirable.

Once armed with this information we should be in a much better position

to evaluate the actual pertinence of these dermal alterations to carcin-

ogenesis. Therefore, the present studies represent an attempt to

define at a histological and histochemical level those stromal alter-

ations which are consistently found in different systems of cutaneous

carcinogenesis, which precede the onset of carcinoma, and which

could possibly play a key role in the evocation of a neoplastic response in the overlying epidermis.

2.   Material and Methods

Primate Carcinogenesis: Twelve preadolescent male Rhesus monkeys were divided into three groups of four animals. Each group was treated with a different carcinogen. These were 0.5% 20-methyl-cholanthrene (K & K Laboratories, Inc.), 1.2% 2-amino anthracene or anthramine (Aldrich Chemical Co., Inc.) and 1% 9,10 dimethylbenz-anthracene (K & K Laboratories, Inc.) all in acetone. The carcinogens were freshly prepared before use and were applied topically twice weekly on three areas about four cm. in diameter on the upper, mid, and lower back. After three months, the lower site was also painted with a promoter, dodecylbenzene ("Alkane 56" - Standard Oil Co.) after each application of the carcinogen. Biopsy specimens were obtained from the painted areas every three months and stained by the following procedures: hematoxylin and eosin (H & E), Luxol fast blue-periodic acid Schiff (LFB-PAS) (56), Mowry's colloidal iron (MCI -PAS) (113) and silver-orcein-aniline blue (SOAB) (74). The tissues were fixed in Helley's formaldehyde (9:1). At the end of three years of treatment the painting was stopped and the animals were observed, biopsies being taken only when the appearance of the skin warranted it.

Rabbit Carcinogenesis: Twenty-four young male New Zealand white rabbits were divided into three groups of eight animals. An 8 x 8 cm. area on the interscapular region of the first group was painted twice weekly with 1% solution of 9,10-dimethylbenzanthracene in acetone. The lumbar region of the back was left untreated as a control site. The second group was painted in the interscapular area with the promoter, dodecylbenzene. The third group was unpainted. After three weeks when tumors began to appear, all animals were killed by the intracardiac injection of pento barbital sodium (Nembutal), biopsy specimens were taken from both the painted and unpainted sites and the remaining tissue from each site was used for biochemical studies as described later. The histological stains employed were H & E, LFB-PAS, MCI-PAS, and SOAB, and the tissues were fixed in Helley's formadehyde (9:1).

Rat Carcinogenesis: One hundred fifty weanling male rats were divided into two groups. The first group was painted over the entire left dorsum (an area approximately 7 x 12 cm.) twice weekly with a 1.2% (w/v) solution of 2-amino anthracene in acetone. Every two weeks for 14 weeks, 10 animals from the first group were killed with an intracardiac injection of Nembutal. Biopsy specimens were removed from painted and contralateral unpainted sites, fixed in Helley's formaldehyde (9:1) and stained with H & E, LFB-PAS, MCI-

PAS and SOAB stains. The remainder of the tissues from the treated and contralateral untreated areas were used for collagen analyses to be described later. The second group served as unpainted controls, six of which were killed every four weeks and the tissues processed as above.

Tumor Histochemistry: The animals remaining from the latter experiment were allowed to survive without further painting until they developed large tumors. At this point they were killed and the tumors removed and fixed in formal-alcohol to precipitate mucopolysaccharides in situ. Fresh tissue from large tumors in rabbit skin treated with DMBA for 7 weeks were similarly fixed. Both sets of tumors were stained by the technique of Scott, Dorling and Quintarelli for the identification of acid mucopolysaccharides. (The technique, as discussed in detail in Section II of the Appendix, involves the differential staining of mucopolysaccharides by alcian blue in solutions of $MgCl_2$ of varying concentrations. Further resolution of the chondroitin sulfates (A, B and C) and keratan sulfate can be achieved by hyaluronidase digestion to which CS-A and C are sensitive and CS-B and KS are resistant. The interpretation of the alcian blue-$MgCl_2$ reaction was made by comparing the critical electrolyte concentration at which staining was found to be suppressed with that in model tissues known to contain predominantly or exclusively single mucopolysaccharide species (152b).

3.   Results

In all of the animal-carcinogen systems studied, the response
of the dermis was similar, consisting of an inflammatory reaction
initially which, in some systems, (i.e., rat-anthramine, monkey-
anthramine, monkey-methylcholanthrene), subsided quickly, but in
others (i.e., rabbit-DMBA, monkey-DMBA-DDB), persisted.   The
epithelium typically became acanthotic with foci of hydropic degener-
ation in the stratum basale.

Stromal changes were consistently found in all the animal-
carcinogen systems. Since the rat-anthramine system has been
studied most extensively, it will serve to illustrate the response in
all systems. Figures I - XXI, taken from the rat carcinogenesis study,
illustrate the following changes:

a. An alteration of the normal tinctorial response of the dermis
to Mowry's colloidal iron (MCI) stain. MCI stains acid mucopolysac-
charides (AMP) but is non-specific. In this technique, the sections
are treated with a colloidal solution of ferric hydroxide which is ad-
sorbed primarily by the polyanionic AMP. The iron bound in the
colloid is then identified with the Prussian Blue reaction. The chrom-
ogen represents a precipitate of ferric ferrocyanide and appears blue
or blue-green. Unpainted skin shown in Figure I contains little or
no blue material. This most likely results either from leaching of

the AMP during processing, or "masking" (i.e., blocking of the reactive sites) of the AMP. After about six weeks of painting, foci of bright blue staining appear either scattered throughout the dermis as in Figure II or, more commonly, localized to the dermo-epidermal junction (Figures III and IV). In the latter case, the point of reaction is generally adjacent to a hair follicle, probably reflecting the route of penetration of the carcinogen through the pilosebaceous apparatus. Less commonly, a generalized rather than a focal response is seen (Figure V). In the contralateral unpainted site of the animal at this time, (Figure VI), none of the blue MCI(+) material is seen.

    b. A concomitant decrease in Luxol fast blue reactivity occurs in the same area. Luxol fast blue is alleged to stain mature collagen (56), although this is open to some question (22). Luxol fast blue (LFB) is a light, heat, acid, and alkali-fast, alcohol-soluble amine salt of sulfonated copper phthalocyanine (123). Figure VII shows the typical intense blue staining of collagen with Luxol fast blue before painting. As painting continues, the blue reactivity is lost, first from the superficial dermis (Figure VIII), and eventually throughout the entire dermis (Figure Ix). At all times blue staining persists in the contralateral unpainted site (Figure X).

    c. Fragmentation, fraying and rarefaction of dermal col-lagen begins at the apex of the dermal papillae and gradually spreads

downward. Dermal collagen, which normally has a regular, relatively homogenous appearance, is arranged in criss-crossing bundles parallel to the surface. Figure XI shows unpainted rat dermis stained with SOAB. After six weeks of painting there is rarefaction and fraying of the collagen (Figure XII, same magnification). The epidermis at this time is acanthotic. Basement membrane changes, which will be discussed later in more detail, are also seen. After eight weeks of painting, the fragmentation and rarefaction are maximal, Figures XIII and XIV). Normal fibers of homogenous appearance as seen in Figure XI are not present.

d. The painting results in fragmentation and clumping of the basement membrane, best seen with SOAB staining, (Figures XII, XIII, and XIV). The epithelium descends through gaps in the basement membrane and, as it proceeds, a series of new basement membranes are laid down ahead of it, each of which is successively breached.

e. Elastin which stains orange with the orcein component of the SOAB stain, increases in the dermal papillae. Although not easily seen in photographs, numerous orcein-positive fibrils of elastin can be detected in the upper dermis (Figure XX) in the immediate subepithelial zone.

f. A small increase in fibroblasts and mast cells in the dermis occurs, (Figures III, IV, XIII, XIV, XVIII and XIX represent this

finding). Although vascular dilatation and increased toruosity of dermal vessels have been noted, these findings are not consistent.

A consistent feature of these studies and one demonstrable only by histological means, is the confinement of all dermal changes to a fairly well circumscribed area. Figures XV, XVI and XVII represent the same locus stained with MCI, LFB, and SOAB respectively, from the painted area on the back of a rat that had been treated with anthramine for fourteen weeks. Figure XV shows the typical MCI positivity underlying an area of mild acanthosis and hydropic degeneration of epithelial cells. The dermo-epidermal junction is irregular and a cellular infiltrate is present in the area of dermal change. The MCI positive material seen in the dermis also appears in the intercellular spaces of the germinative cells of the overlying epithelium. Figure XVI shows the same site (turned 180°). The tissue, stained with LFB, shows a complete loss of blue reactivity in the dermal collagen underlying the junction between follicular and surface epithelium. Figure XVII is an SOAB-stained section of the same material. The fragmentation and clumping of the basement membrane are readily seen, as are the increased dermal cellularity and elastosis. Figures XVIII-XXI are similarly arranged to show changes confined to the same area. Figure XIX outlines the area well because of its MCI positivity. As in the former series, this locus is adjacent to a hair follicle and

is also associated with mild acanthosis. Figure XXI shows the LFB
negativity and the increased dermal cellularity limited to the area.
Again, the overlying basal cells show early hydropic degeneration.
Figure XX shows the same area stained with SOAB. The orcein-
positive material (i.e., elastin), is quite prominent in the immediate
subepithelial zone of the dermis. Figure XVIII is an H & E-stained
section of the same locus demonstrating the rarefaction and fraying
of the dermal collagen which was obscured by the orcein positivity in
the SOAB-stained section.

Identification of the tumor mucopolysaccharide was made by
comparison of the critical electrolyte concentration (CEC) at which
Alcian blue (AB) staining was abolished in the tumor tissue with those
CEC's established by Scott et al (152b) for MPS species in model
tissues. These authors found the following:

Species	$MgCl_2$ concentration above which Alcianophilia is lost
Poorly charged mucins	0.3M
COOH-containing MPS	0.3M
Chondroitin sufate (in hyaline cartilage)	0.7M
Heparin (in mast cells)	0.9M
Keratosulfate (in cornea)	1.0M

In the present study, the majority of alcianophilia of the con-
nective tissue around the tumor was abolished at 0.2M $MgCl_2$. Ac-
cording to Quintarelli and Dellovo (134c) this material represents a

poorly charged mucin. However, between a $MgCl_2$ concentration of 0.4M and 0.8M a second and complete loss of staining occurred. It was found that human umbilical cord, which was fixed and stained in the same manner as the tumor tissue, also lost its staining at this concentration. Since umbilical cord contains predominantly hyaluronic acid, if the studies of Scott et al are applicable here, the loss of tumor staining between 0.4M and 0.8M $MgCl_2$ represents both hyaluronate and chondroitin sulfate.

Sections which had been extracted with 0.4M $MgCl_2$ were then digested with hyaluronidase. After this treatment, approximately 2/3 of the alcianophilia was lost. As mentioned, hyaluronidase digests chondroitin sulfate-A (CS-A), chondroitin sulfate-C (CS-C) and hyaluronic acid (HA) while chondroitin sulfate-B (CS-B) and keratan sulfate (KS) are resistant to this treatment. The skin contains goodly quantities of HA, and condroitin sulfate, but no KS (10). Thus, it is likely that the tumor stroma contains predominantly HA, CS-A, CS-B and CS-C. Histopathological examination of these tumors indicated that they were squamous cell carcinomas and basal cell epitheliomas in the rat and exclusively squamous cell tumors in the rabbit.

4.   Discussion

The significance of these histochemical findings is speculative

at this point. It can be said, however, that there is a definite and consistent response of the dermis to carcinogenic treatment, that this response is generally associated with epithelial acanthosis, and that it precedes a frank epithelial neoplasia, although a causative relationship cannot be inferred.

These histologic studies permit a broad definition of the dermal response during carcinogenesis. This response involves both the fibrous and mucoid components of the connective tissue. The epidermal basement membrane invariably breaks down. Locally increased dermal cellularity is a common feature. These dermal alterations always precede carcinomatous invasion by the epidermis, and occur consistently and predictably in all carcinogen-animal systems.

Although the histochemical changes are somewhat more specific, they allow only a tentative identification of the dermal components involved in the alterations. The loss of staining with LFB is thought to represent collagen alteration. However, this reaction is not unique to carcinogen-treated skin, as it has been observed in the abnormal collagen of a variety of dermal diseases (56). A recent review of collagen staining by Constantine and Mowry (22) discusses the selectivity of the various methods presently available, including the Luxol fast blue stain and concludes that none of the currently used collagen stains is truly selective for collagen. While Mowry's colloidal iron

probably stains mucopolysaccharides, its specificity is questionable. It is quite possible that the "Mowry's positive material" indicates an epithelial mucin or a product of enzymatic digestion (152a), or any one of a number of tissue or blood group substances that could bind the ferric hydroxide. The orceinophilia seen with SOAB stain appears to indicate and to be specific for mild elastosis, but here also, the orcein could be staining collagen in which the polar groups have in some way been masked, as has been shown to occur by Fullmer and Lillie (40). The staining of tumors with alcian blue in various concentrations of $MgCl_2$ perhaps represents the most specific of the histochemical techniques used, but even here the comparison of the staining of one tissue with that of another very different tissue involves some precarious assumptions. Nevertheless, the natural occurrence in tumors of chondroitin sulfate, the specific mucopolysaccharide found to stimulate tumor growth artificially (161a) has exciting implications. These will be discussed in more detail in the general discussion.

Thus, although the histology and histochemistry have served to focus our attention on specific changes in the dermis during carcinogenesis, and have provided insight into which components of the dermis are most markedly affected during that process, they, in themselves, are not particularly specific.

5. Summary

Dermal changes in response to topically applied carcinogens were studied histologically and histochemically in rats using 2-anthramine, rabbits using 9,10-dimethylbenzanthracene, and monkeys using anthramine, dimethylbenzanthracene and methylcholanthrene alone and in combination with the promoter, dodecylbenzene. Regardless of animal species or carcinogen, the dermal response was consistent and preceded any evident epidermal neoplasia. It consisted of alterations in all dermal components. These alterations were as follows: (1) a rarefaction, fragmentation and fraying of the collagen fibers and a loss of their normal affinity for Luxol fast blue. (2) increased Mowry's colloidal iron positivity of the acid mucopolysaccharides, (3) increased orceinophilia of the elastic tissue in the superficial dermis, (4) a mild mastocytosis and fibrocytosis, and (5) a variable dilatation and increased tortuosity of the dermal vasculature. These changes were limited initially to small loci throughout the superficial dermis, but as carcinogenesis proceeded they spread to involve the whole of the dermis. Extraction of alcian blue-mucopolysaccharide complexes with increasing concentrations of $MgCl_2$ for the specific identification of the mucopolysaccharide species in cancerous rabbit and rat skin indicated the presence of chondroitin sulfate A, B and C and hyaluronic acid.

The specificity and utility of the findings are discussed. Their pertinence to carcinogenesis in particular and to tissue interaction in general will be considered in the General Discussion.

Figure I: Normal rat skin stained with Mowry's Colloidal Iron-
Periodic Acid Schiff (MCI-PAS) x 100. No MCI positive blue
material is seen.

Figure II: Rat skin painted for eight weeks with 2-aminoanthracene.
Foci of MCI Positivity can be found throughout the dermis. Mild
thickening of the epidermis is seen. MCI-PAS x 160.
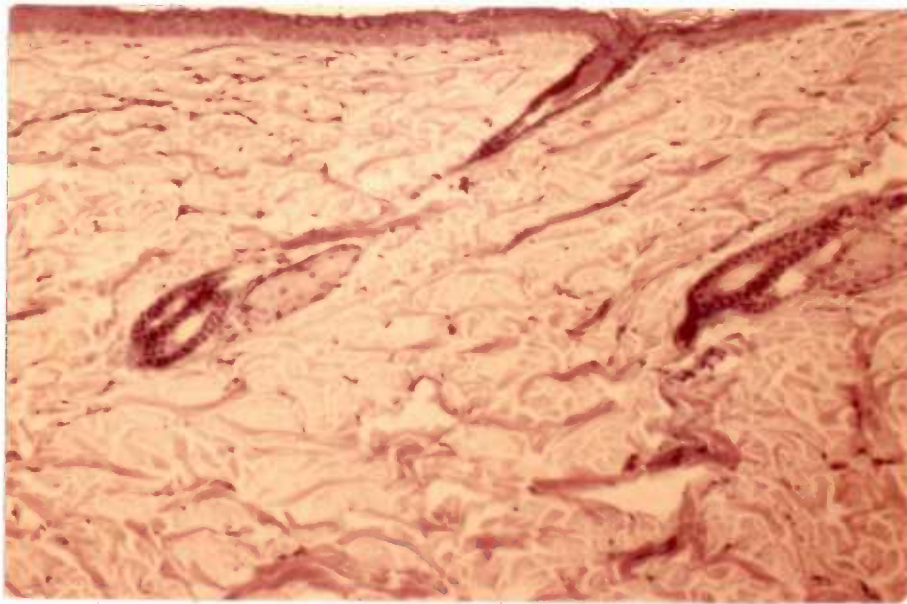
figure I
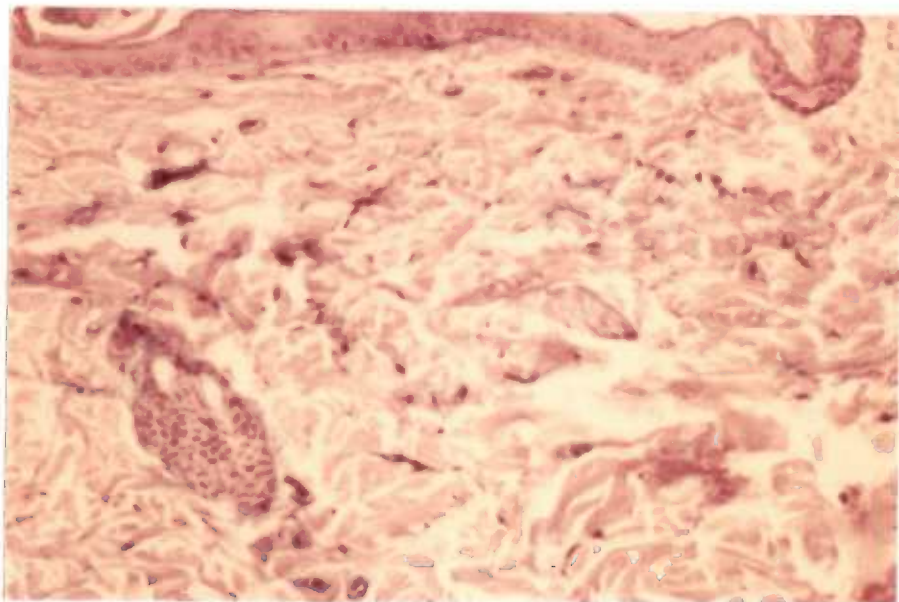
figure II

Figure III: MCI positivity in the dermis adjacent to a hair follicle.
Rat skin painted for eight weeks with 2-aminoanthracene. MCI-PAS
x 160.

Figure IV: MCI positivity underlying acanthotic epithelium adjacent
to a hair follicle. Rat skin treated for eight weeks with 2-anthra-
mine. MCI-PAS x 160.
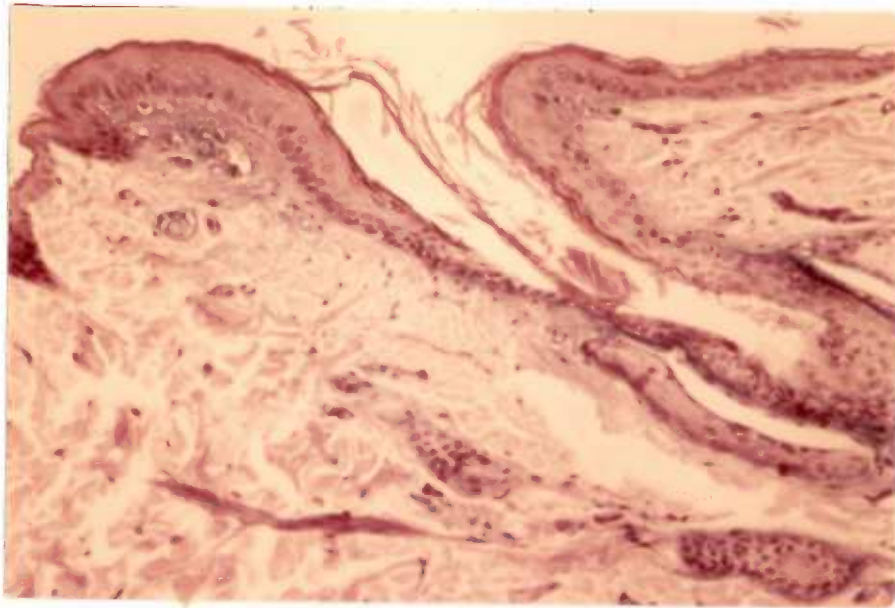
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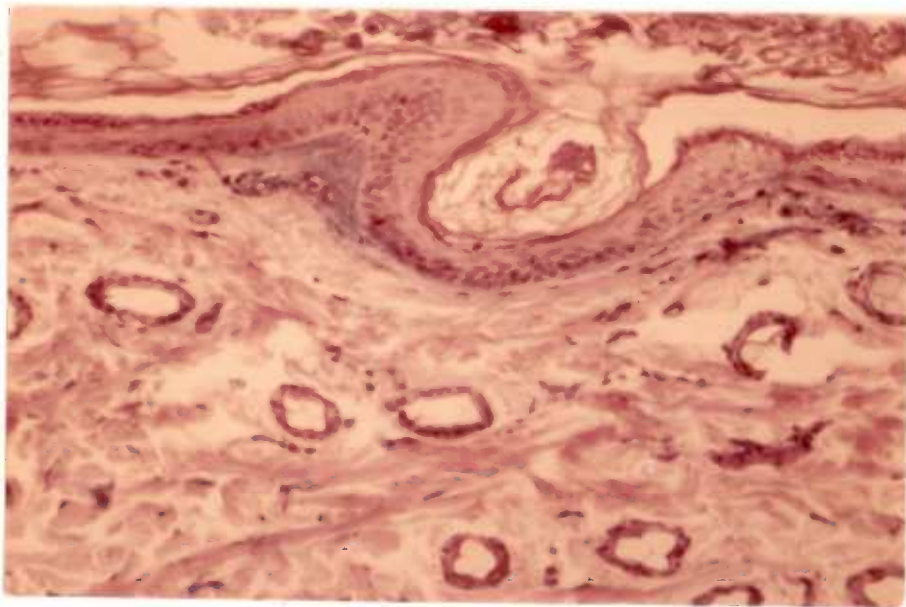
figure IV

Figure V: Less-commonly observed generalized MCI positivity in anthramine-treated rat skin. MCI-PAS x 100.

Figure VI: Contralateral unpainted back skin of a rat painted for eight weeks with anthramine. No MCI positive material is seen in the dermis, and the epidermis is of normal thickness. MCI-PAS x 100.
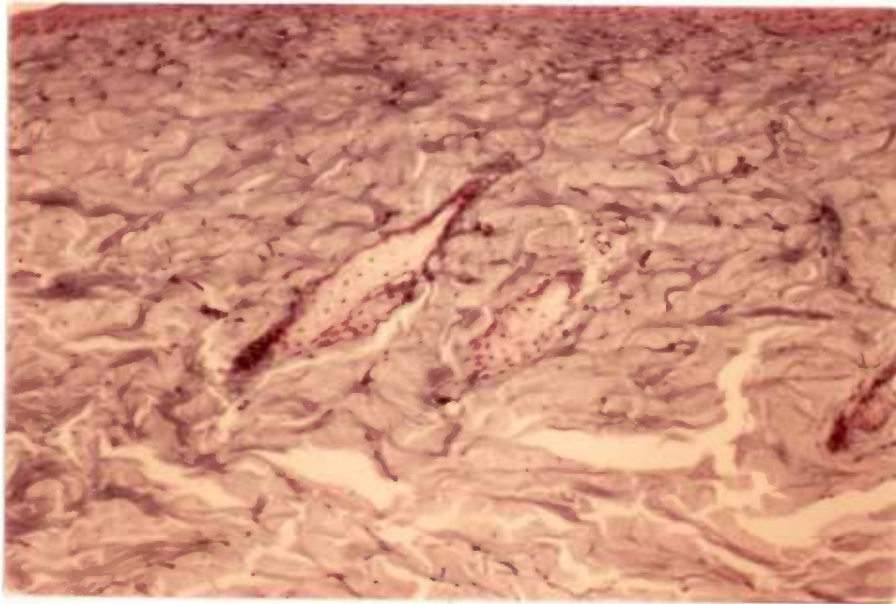
figure V
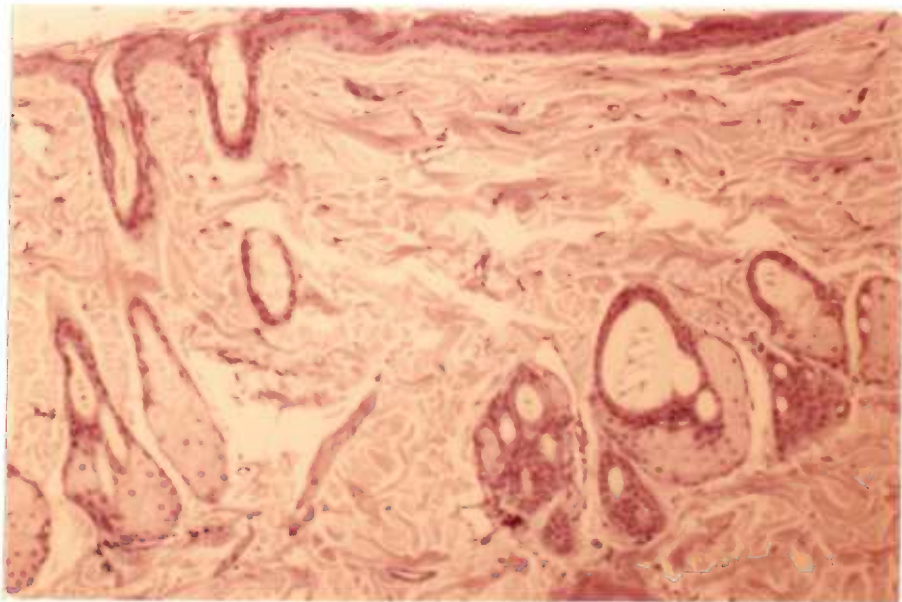
figure V I

Figure VII. Normal rat skin stained with Luxol Fast Blue-PAS.

Mature collagen stains blue. LFB-PAS x 100.

Figure VIII. Rat skin painted for six weeks with anthramine.

Epidermis slightly thickened. Loss of LFB staining in superficial

dermis. LFB-PAS x 100.
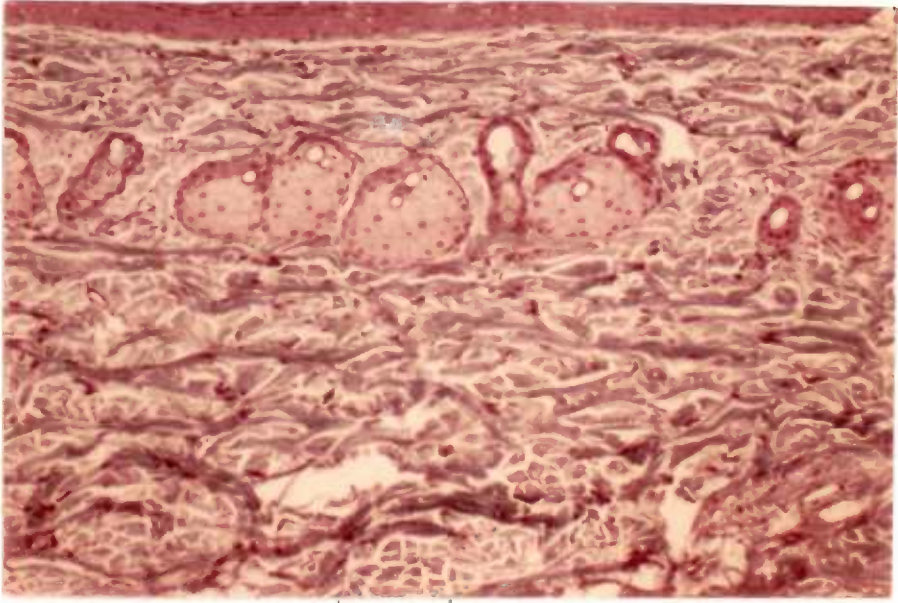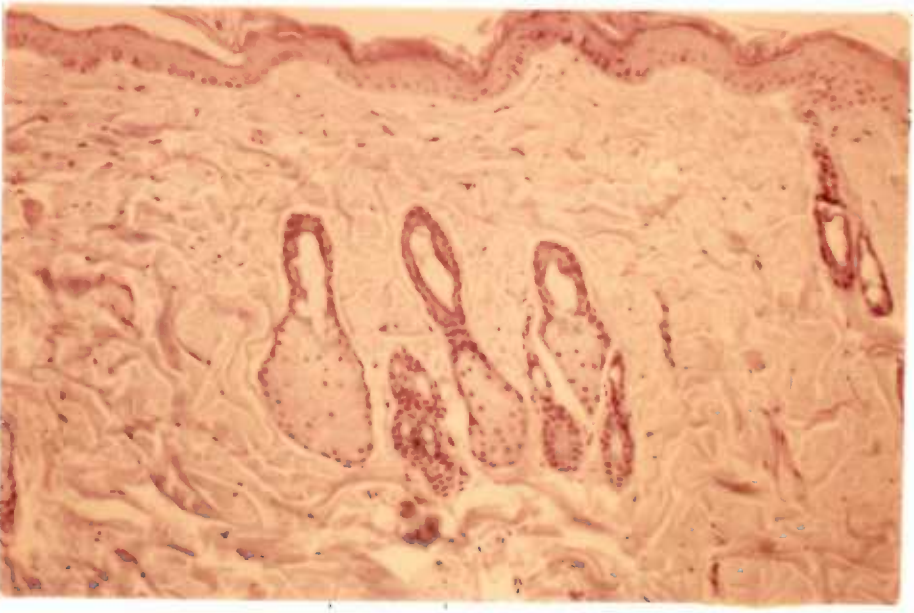
figure VII

figure VIII

Figure IX: Rat skin painted for eight weeks with 2-anthramine. LFB affinity has been lost from the deep as well as the superficial dermis. LFB-PAS x 100.

Figure X: Unpainted back skin from a rat painted for ten weeks with anthramine. In contrast to the painted side, this contralateral site retains its affinity for LFB, LFB-PAS x 100.
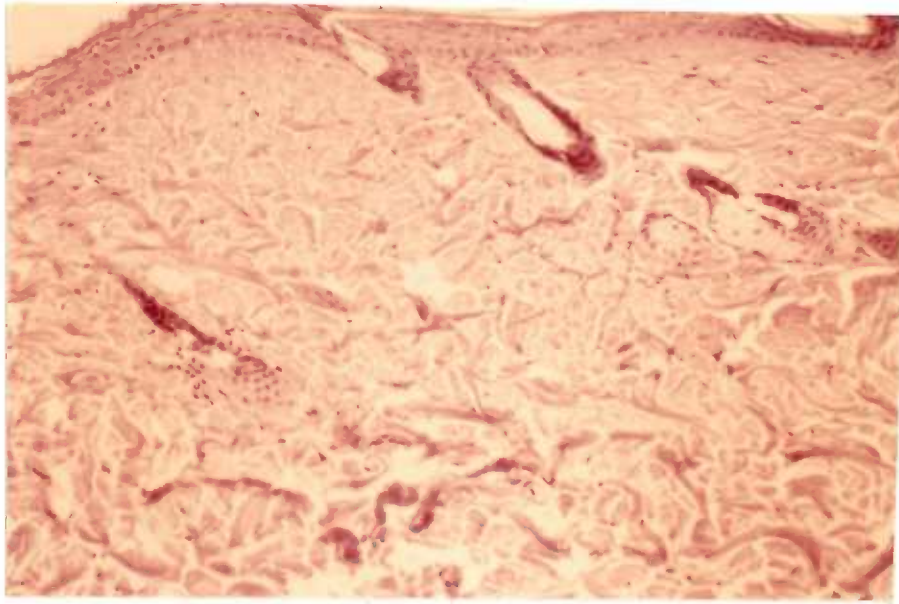
figure IX
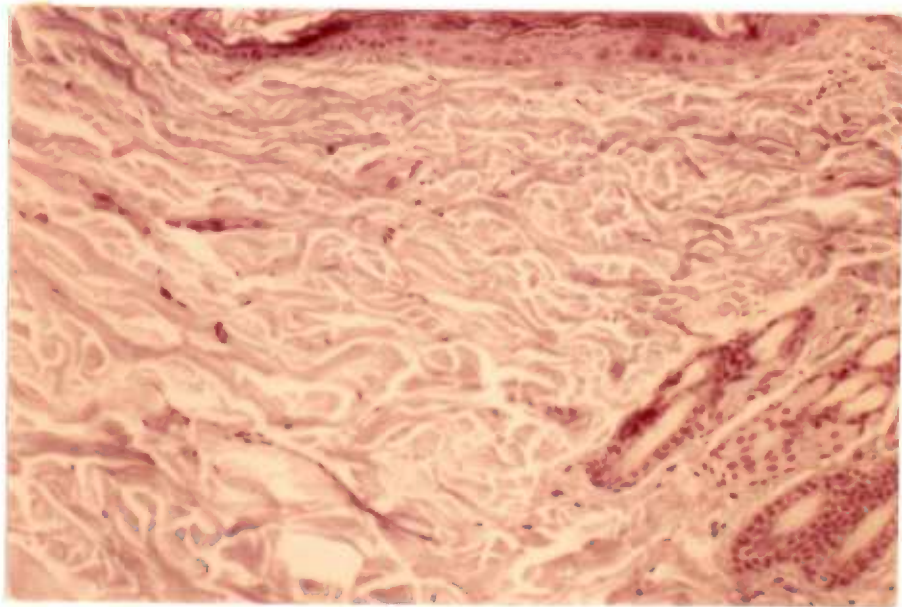
figure X

Figure XI: Normal rat skin stained with silver Orcein Aniline Blue (SOAB). Note heavy, regular fibers criss-crossing parallel to the surface. SOAB x 160.

Figure XII: Rat skin treated for six weeks with anthramine. Note epithelial irregularity and thickening. Superficial dermal collagen is frayed, and the basement membrane appears to be missing in some areas, thickened in others. SOAB x 160.

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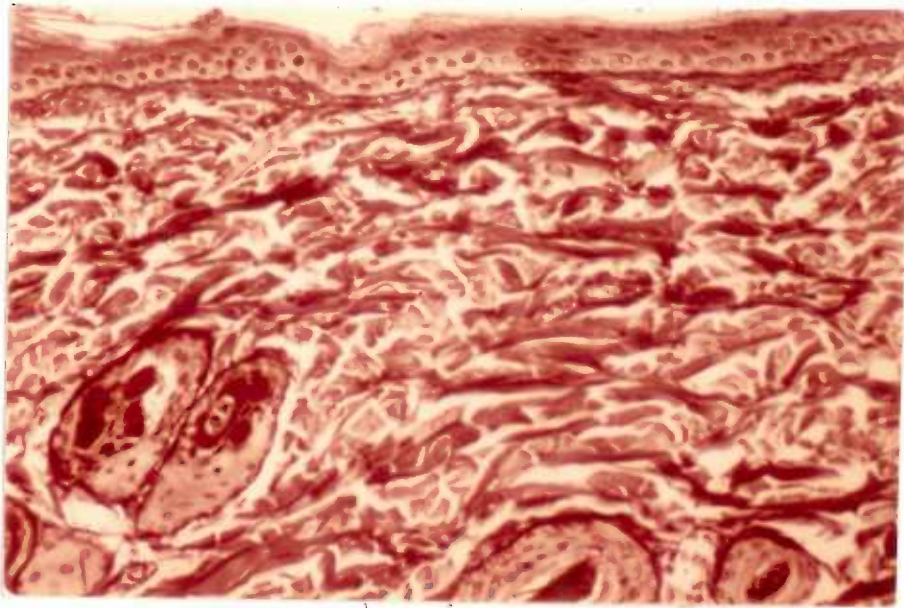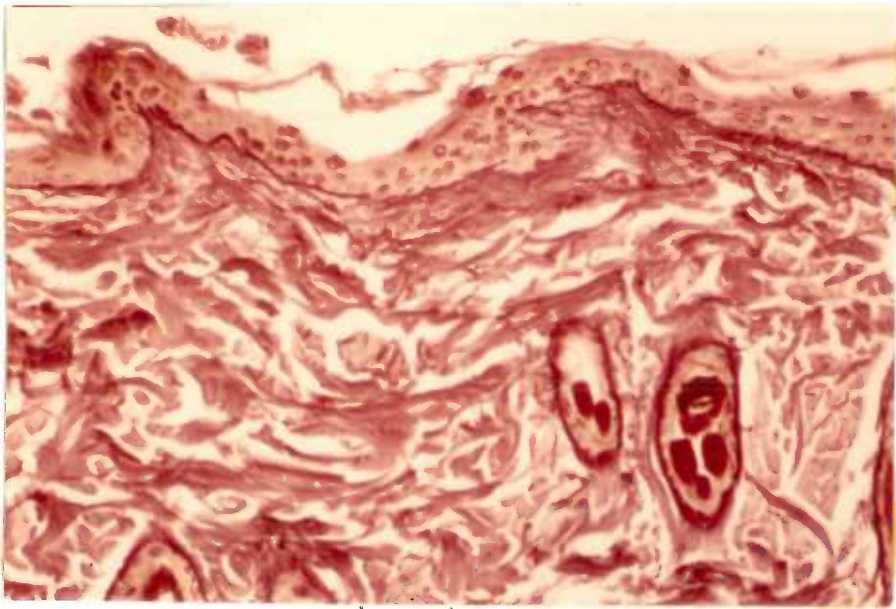
figure XI

figure XII

FIGURE XIII: Rat skin treated for eight weeks with anthramine. The epithelium is acanthotic and shows hydropic degeneration in the stratum basale. The superficial dermis shows increased cellularity and irregularity and fragmentation of the fibrous components. The basement membrane appears to be thickened, fragmented, and in some areas, missing. SOAB. x 400.

FIGURE XIV: Rat skin painted for eight weeks again showing fragmentation of dermal collagen in the superficial dermis associated with disruption of the basement membrane. SOAB. x 512.
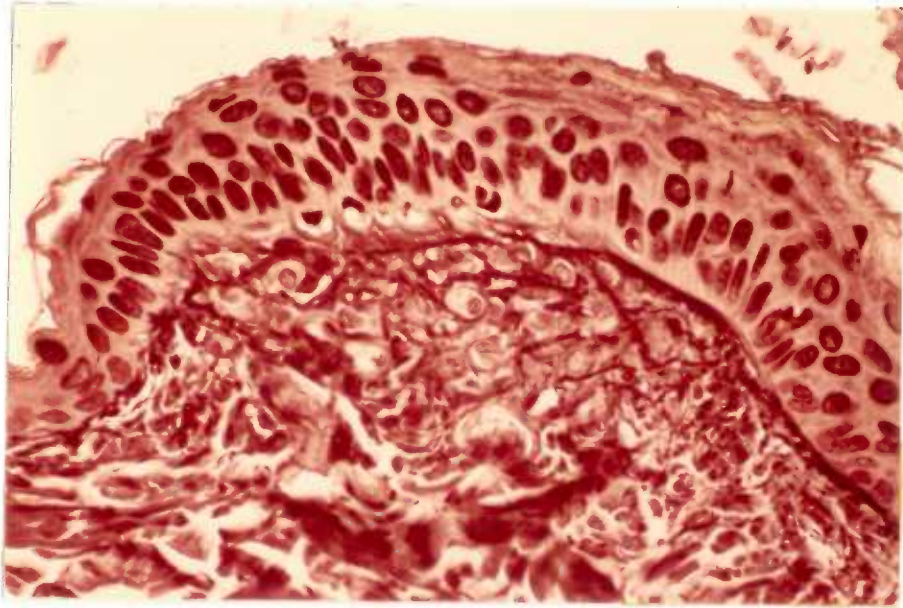
figure XIII
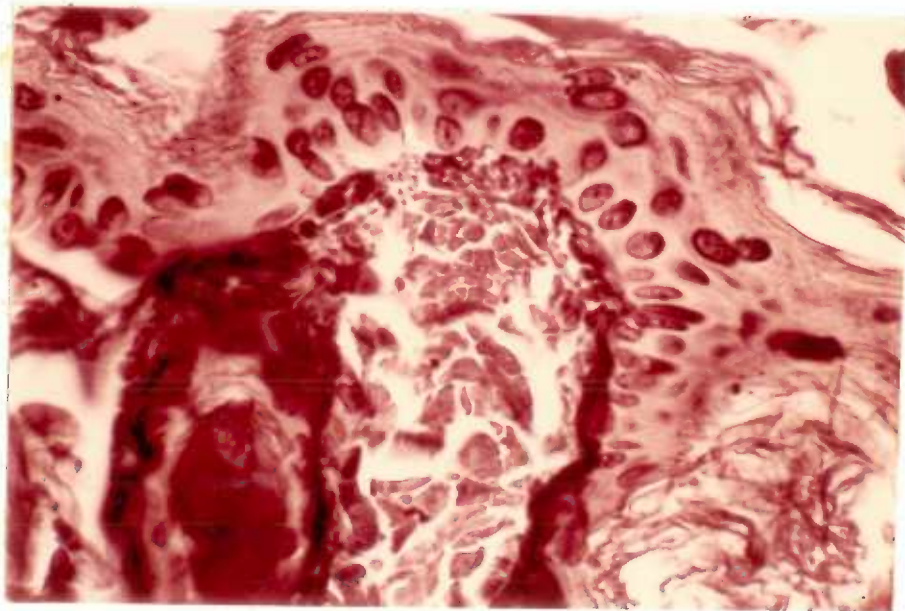
figure XIV

Figure XV: Locus of dermal change in rat skin treated with anthramine

for fourteen weeks. The area of MCI positivity is associated with

epithelial acanthosis and hydropic degeneration of epithelial germinal

cells. There appears to be increased cellularity of the dermis here.

MCI-PAS x 400.

Figure XVI: Same locus as in Figure XV turned 180°. Area of LFB

negativity corresponding to MCI positive area. LFB-PAS x 400.

Figure XVII: Same locus as in Figures XV and XVI. Increased

orceinophilia and thickened and disrupted basement membrane are

seen associated with epidermal thickening. SOAB x 400.
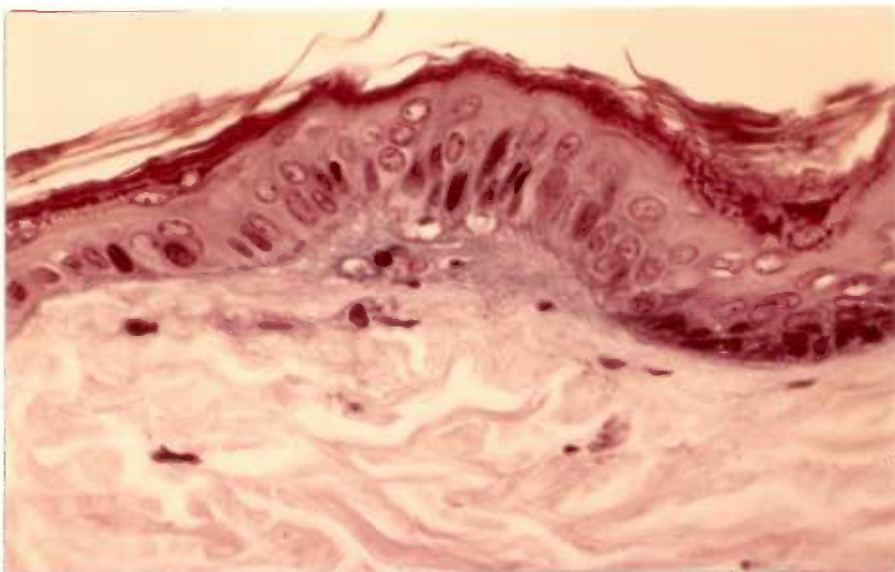
figure
XV
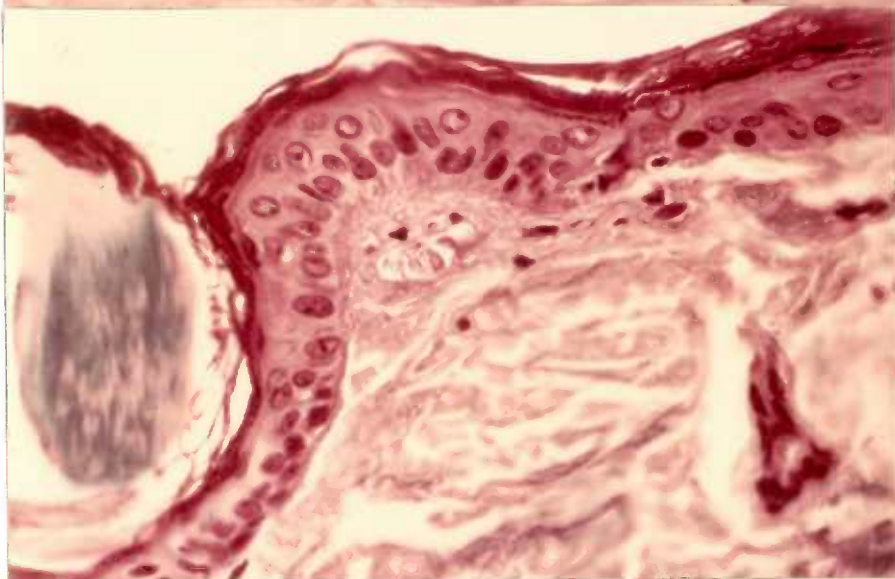
figure
XVI
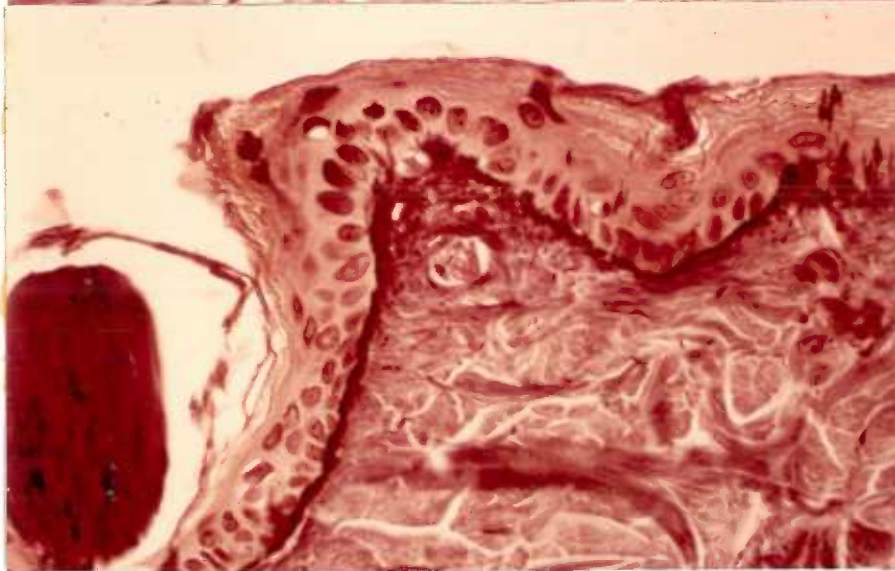
figure
XVII

FIGURES XVIII, XIX, XX, XXI: Same locus is seen in all four
pictures. Rat skin painted for fourteen weeks with 2-anthramine.
As seen previously, all changes may be found in the same specific
area. In order of appearance, they are; rarefaction and fraying of
superficial dermal collagen (Figure XVIII), MCI positivity (Figure
XIX), increased orceinophilia (Figure XX) and LFB negativity
(Figure XXI). The epidermis is thickened with a thick and frag-
mented basement membrane. It shows cells undergoing hydropic
degeneration. The cellularity in the dermis is increased in this
area. These changes are qualitatively and quantitatively equivalent
to those seen in Figures XV-XVII. H&E, MCI, SOAB and LFB x 400.
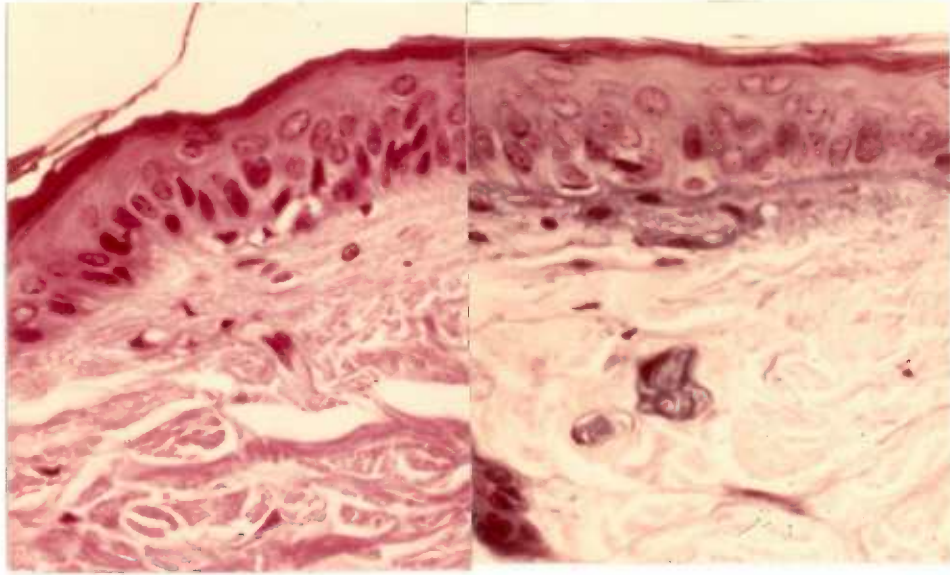
figure XVIII   figure XIX
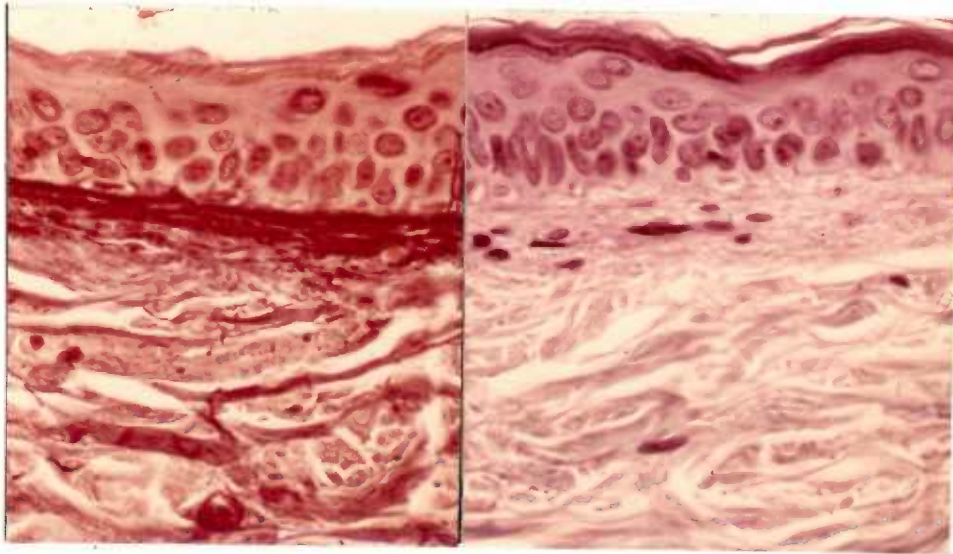
figure XX   figure XXI

B.   Collagen Metabolism in Precancerous Rabbit Skin

1.   Introduction

After the topical application of carcinogenic hydrocarbons, fibers of dermal collagen appear histologically to be fragmented, clumped, frayed, and disoriented, suggesting a defect in either the construction or destruction of this protein.   These fibers also demonstrate a loss of their normal elective affinity for Luxol fast blue stain which is thought to stain mature collagen. (56).

Fibroblasts normally synthesize collagen precursors on polyribosomes attached to the membranes of their rough endoplasmic reticulum.   Collagen precursors exceeding a molecular weight of 10,000 then pass through the Golgi zone, and, in the presence of ferrous iron, alpha-keto-glutarate, ascorbate, atmospheric oxygen and the enzyme(s) protocollagen hydroxylase, the proline and lysine are hydroxylated.   Once hydroxylated, the single collagen polypeptide chain, called an α chain, (which has a left-handed helical configuration), unites with two other α chains to produce the three stranded collagen molecule which is then secreted via vesicles into the extracellular space.   In the absence of oxygen or in the presence of an iron chelator, the protocollagen accumulates within the cell (78).   Replacement of iron and oxygen allows rapid hydroxylation and extrusion of protocollagen into the extracellular matrix, suggesting that the hydroxylations are

essential for passage of the molecules out of the cells producing them. Neither hydroxyproline nor hydroxylysine can be incorporated directly into the molecule. While the high concentration of hydroxyproline (14%) and of proline (15%) give the polypeptide chain a left-handed twist, the three-stranded molecule has a gradual right-handed twist. Initially, it is held together by hydrogen bonds. This triple helix can be considered the basic unit in collagen fiber formation, for it is by a gradual clumping of these molecules with an approximate one quarter overlay that the fiber is produced. Since the collagen molecule is about 2900 Å long, this overlapping of one molecule with its neighbor produces in the fiber a 640-700 Å repeating period which can be visualized with the electron microscope. After formation of the triple helix, the collagen begins a process of further hydrogen bonding and eventual intra- and inter-molecular cross-linking which adds stability to the molecule; as a result, the molecule has a very long half-life in vivo. The cross-linking is effected by the oxidative deamination of the ϵ-amino group of a projecting lysine residue to form α-amino adipic semialdehyde. A connective tissue enzyme, amine oxidase, catalyses this oxidation. The resulting aldehyde then condenses in a typical aldol condensation with another aldehyde on an adjacent chain in the same or another molecule. This covalent linkage results in a β-component with a molecular weight twice that of the α-component. In young animals the $\alpha:\beta$ ratios are high, indicating that little cross-

linking has taken place. In adults, however, the $\alpha{:}\beta$ ratio is low,
indicating a high degree of cross-linking and a grater stability of the
collagen. This "aging" is an important consideration when extraction
procedures are used to isolate collagen in various states. Cross-
linked collagen is insoluble. However, prior to cross-linking a com-
plete spectrum of aggregates are produced with an increasing size and
increasing degree of hydrogen-bonding occurring as a function of aging.
Neutral salts, by the establishment of a Donnan Equilibrium between
the inside and outside of a collagen aggregate, are able to draw water
into that aggregate. Influx of water creates a swelling pressure
which, if it exceeds the cohesive force of the hydrogen bonds, will
break those bonds and solubilize the aggregate. This is called the
"salting in" effect. Jackson and Bentley (75) showed that in young
collagen, where the hydrogen bonding is poorly developed, low con-
centrations of salt were sufficient to solubilize the protein, However,
as the collagen aged and the degree of hydrogen bonding approached
its maximum, ever stronger concentrations of salt were needed to
exceed the cohesive forces. Eventually, the aggregates reached an
age at which the bonds could be broken only with acids. Collagen
older than this (i.e., cross-linked collagen) was insoluble and
could be broken down only by gelatinization. One can think of col-
lagen fiber formation like the growth of a tree. An initial estab-

lishment of a "nucleus" of hydrogen-bonded molecules could, by the adsorption of free molecules or small aggregates of molecules, gradually increase in size until it became a macroscopically visible fiber, the outer shell of which was new, weakly-bonded collagen and the inner core of which was old, firmly cross-linked collagen. Between these two layers would be a spectrum of molecules of varying age, bonding and extractability. Since hydroxyproline always constitutes 14% of the mammalian collagen molecule, but is essentially absent from all other proteins, assay of this imino acid can be directly related to collagen concentration.

The present study represents an effort to elucidate the alteration in the morphology and tinctorial affinities observed in dermal collagen of skin undergoing carcinogenic treatment. Since it is felt that these specific changes could derive from either an alteration of the normal neosynthesis or aggregation of collagen or from an abnormal rate of breakdown of this molecule, (which normally has a very low turnover rate), these problems were specifically studied.

To study the influence of carcinogen painting on the synthesis of collagen, the animals should be subjected to the carcinogenic treatment and their ability to make collagen in the carcinogen-painted tissue then studied by measuring the uptake and incorporation of an acutely-administered, labeled collagen precursor. The ability of

the carcinogen-treated skin to aggregate new collagen into old fibers could be studied by fractionating this collagen into young, middle, and old-aged groups and comparing, in painted and unpainted animals, the rate of transfer of the labeled precursor from young to old collagen. These two facets of collagen production will be called collectively, ANABOLISM.

To study the influence of carcinogenic treatment on collagen destruction or CATABOLISM, the labeled precursor should be incorporated into the collagen before the onset of carcinogen painting. The amount of label remaining in that collagen after the carcinogenic treatment should be compared with that remaining in a similarly-injected, unpainted animal. Furthermore, the question of the route by which collagen is degraded (i.e., direct degradation or disaggregation) could be answered by noting the presence or absence of "leached" label in the extractable fractions of collagen. Furthermore, the inflammatory effects of the carcinogen should be separated from its carcinogenic effects by studying collagen metabolism in a parallel series of animals treated with a known irritant.

Finally, this type of study requires two controls, unpainted skin from the painted animal as well as the conventional unpainted animals. The reason for including the former is twofold. First, due to normal individual variation, this should provide a more mean-

ingful and readily comparable control than a separate unpainted animal. Second, comparison of the findings in the unpainted site (which never gets tumors) with those in the control animals would tell whether the effects on the painted site are peculiar to that site or merely the result of a general systemic reaction to the carcinogen unrelated to the carcinogenic process.

2.   Materials and Methods

Twenty-four New Zealand white rabbits weighing an average of 2.33 kg. were divided into two equal groups, the first to study breakdown, or CATABOLISM, and the second to study synthesis and aggregation, or ANABOLISM, of collagen during carcinogenesis. Each group was additionally subdivided into three subgroups, a carcinogen-treated group, a group treated with an irritant (or promoting compound) and a control group which was unpainted. As can be seen from Figure XXII, to study catabolism a single $300\mu c$ injection of ^{3}H-proline was administered intraperitoneally three days before beginning carcinogenic treatment. The carcinogenic treatments consisted of painting twice weekly, for three weeks, an 8 x 8 cm. area on the interscapular region of the back with a 1% solution of 9,10-dimethyl-1,2-benzanthracene (K & K Laboratories, Inc., Hollywood, California) in acetone. The irritant, dodecylbenzene ("Alkane 56"-Standard Oil Co. of California) an active fraction of

croton oil, was similarly applied to the second group of animals.

The third group of animals served as unpainted controls. After three

weeks, ten animals were killed and the painted area, and a similar-

sized area from the lumbar region were shaved, depilated with Surgex

(a chemical depilatory) and removed. Four-millimeter punch biopsy

specimens were taken for histologic examination from the middle of

each piece. The specimens were fixed in Helly's formaldehyde (9:1)

and stained with H&E, Giemsa, Luxol Fast Blue, Mowry's Colloidal

Iron and Silver Orcein-Aniline Blue, as described in Experimental

Section A. The remainder of the tissue was used to study collagen

metabolism. The collagen was first extracted from the tissues on

a shaker with 0.14M and 1.0M neutral salt solutions. Details of this

procedure are presented at the end of this section. The collagen

remaining after these extractions was arbitrarily referred to as

insoluble collagen, but included collagen soluble in stronger salt

solutions and weak acids and buffers as well as the truly insoluble

fractions.

The technique of Prockop, Udenfried and Lindstedt (132a) was

used for the isolation, quantification and radioassay of collagen

hydroxyproline in each fraction. This involves the oxidation with

chloramine T of all the amino and imino acids of a collagen hydroly-

sate. The amino acids are converted to α-hydroxy acids. These

and the oxidation products of the interfering radioactive proline are

extracted with toluene, the last extraction being kept as a blank for liquid scintillation counting. The water-soluble intermediate oxidation products of hydroxyproline remain in the aqueous phase which is then heated briefly to convert these products to pyrrole, which is soluble in and extracted with toluene. This reaction proceeds as follows:

HO—⟨ ⟩—COOH $\xrightarrow{(O)}$ HO—⟨ ⟩=COOH $\xrightarrow{}$ ⟨ ⟩—COOH $\xrightarrow{\Delta}$ ⟨ ⟩

HYDROXYPROLINE   PYRROLINE-4-HYDROXY-2-CARBOXYLIC ACID   PYRROLE-2-CARBOXYLIC ACID   PYRROLE
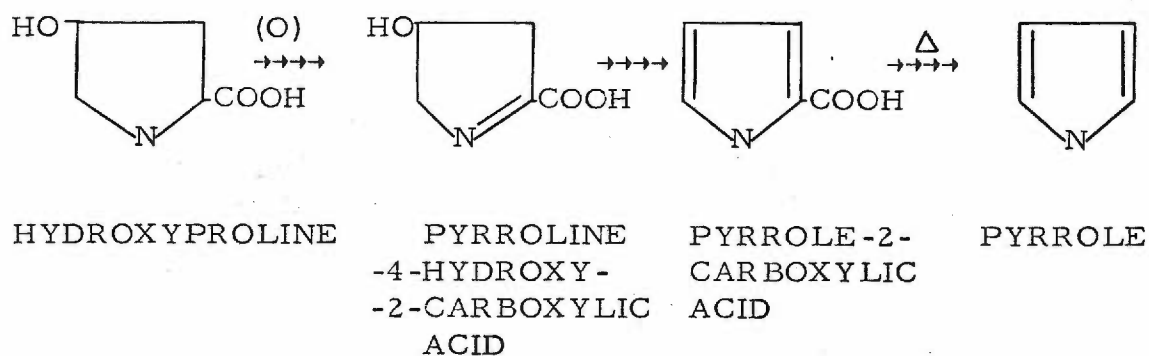
When extracted into toluene, the pyrrole (which is equivalent to hydroxyproline mole per mole) is assayed for quantity and radioactivity. Quantification was done by standard colorimetry, the pyrrole forming a specific chromophore with pDAB (Ehrlich's reagent). Colorimetry was performed on a Bausch & Lomb Spectronic 20 spectrophotometer and absorbancy read at 560mμ. Radioassay was carried out on a 720 Nuclear Chicago Liquid Scintillation Spectrometer. The counting was carried out at ambient temperatures, correcting for efficiency by the channels ratio method. The counting procedure is presented in greater detail in Appendix III.

Materials: The following solutions were used in the analyses:
0.2M Chloramine T (Eastman Organic Chemicals) in distilled water,
prepared fresh daily; 0.2M sodium Pyrophosphate, pH 8.0 in distilled
water; 1.0M sodium thiosulfate in distilled water; toluene (AR-Mallinckrodt); 1.0M Tris buffer, pH 8.0 in distilled water.

p-Dimethylaminobenzaldehyde, or Ehrlich's reagent, (AG-Matheson, Coleman and Bell) was prepared in the following manner:
27.4 ml concentrated sulfuric acid was added slowly to 200 ml absolute
ethanol in a beaker and the mixture cooled; in another beaker, 120 gm
of p-DAB were added to 200 ml of absolute ethanol; the acid-ethanol
mixture was then slowly stirred into the other mixture. This solution
can be stored in the refrigerator for several weeks. The crystals
which form when the solution is cooled can be readily dissolved by
warming under tap water.

The phosphor solution used here contained 0.1 gm of 1,4-bis
2-(5-phenyloxazolyl) benzene (Scintillation Grade, Packard Instrument Co., Inc.) and 4.0 gm of 2,5-diphenyloxazole (Scintillation
Grade, Packard Instrument Co., Inc.) in 1.0 liter of toluene. Protected from light, this solution is stable for long periods.

The tritiated proline (L-proline-3,4-^{3}H) was provided by New
England Nuclear Corporation. It had a specific activity of five
curies per millimole.

The sodium chloride solutions used in the collagen extractions were adjusted to a pH of 7.7 with 0.2M phosphate buffer.

ISOLATION OF COLLAGEN

Sacrifice (5cc Na-Nembutal)
↓
Shave
↓
Depilate with Surgex
↓
Remove 8 x 8 cm painted and unpainted pieces of skin

- -

↓   ↓

4 mm punch biopsy specimen   Remainder
↓   ↓
Histology   Freeze, liquid N

↓
Crush in mortar and pestle, weigh
↓

- -Centrifuge ←←← Extract, 40 ml 0.14M NaCl (pH 7.7) x 24 hr 4°C
supernate   "   ↓
← ← ← Extract - same 24 hr
    "   ↓
← ← ← Extract - same 24 hr

Pool (.14M salt soluble collagen

Dialyze against 0.1M acetic acid until salt free   Extract, 40 ml 1.0M NaCl (pH 7.7) x 24 hr 4°C

↓   ←←   supernate - -Centrifuge ←←←

Clarify dialysate by centrifugation   "   ↓
← ← ← Extract - same 24 hr
    "   ↓
← ← ← Extract - same 24 hr

Pool (1.0M salt soluble collagen)   Insoluble residue

75

Evaporate under
N$_2$ on water bath

Transfer to hydrolysis
tubes with 10 ml of 6N
HCl and hydrolyse at
140°C x 3 hr

Wash (shake quickly in
distilled water, centri-
fuge, discard supernate)

Add 10 ml dist. water,
cap with tinfoil, auto-
clave at 124°C x 3 hr

Clarify by centrifugation
(20,000G)

To an aliquot of gelatin,
add an equal quantity of
of 12N HCl and hydrolyse
at 140°C x 3 hr

collagen hydrolysate

ISOLATION, QUANTIFICATION AND RADIO ASSAY
OF HYDROXYPROLINE AS PYRROLE

Hydrolysate
↓
Transfer to evap. dish with D.W.
↓
Evap. on water bath
↓
Transfer to a 200 x 25 mm screw cap culture tube with 4-6 ml D.W.
↓
Adjust pH to 8 ± 0.5 with NaOH (phenolphthalein) and make up to 10 ml with D.W.
↓
Add 2 ml pyrophosphate buffer (pH 8.0)
↓
Oxidize: add 2 ml Chloramine T (0.2M), let stand at room temp. 20 min.
↓
Stop oxidation with 20 ml 1M sodium thiosulfate (oxidation products of hypro water-soluble at this stage)
↓
Readjust pH to 8.0
↓
Add 6 ml tris buffer, 10 ml $CHCl_3$ and shake vigorously about 100 x
↓
Centrifuge briefly at low speed to separate phases
↓
Aspirate $CHCl_3$ from bottom of tube (remove completely--it quenches)
↓
Saturate with excess NaCl
↓
Extract aqueous phase with 10-15 ml toluene 4 more times with shaking and centrifugation as before
⋮
↓

Aqueous phase

↓

Boil for 25 minutes in water
bath (converts intermediate
oxidation products to pyrrole)

↓

Cool under tap

↓

Add 11.5 ml toluene and shake

↓

Transfer 10 ml of clear toluene phase
into counting vial

+

1 ml phosphor

↓

Count in liquid scintillation
counter

Toluene from final
extraction

↓

10 ml into counting   Remainder
vial (=blank for   discard
scintillation
counting

0.5 ml of toluene phase
(free of any water)

+

4 ml fresh toluene

+

2 ml pDAB

↓

Develop color for 15 minutes

↓

Read OD at 560 mμ and
relate to standard curve
made with redistilled
pyrrole

3.   Results

   Painting with dodecylbenzene produced a severe inflammatory

reaction characterized by extreme hyperkeratosis, scabbing, slough-

ing, and induration. A study of collagen metabolism in this skin was

therefore not attempted.

   Painting with dimethylbenzanthracene, however, did not pro-

duce such drastic irritation and studies on collagen metabolism were

performed in these specimens. Collagen concentrations in the 0.14M

neutral salt-soluble (Figure XXIII), 1.0M neutral salt-soluble (Figure

XXIV) and insoluble (Figure XXV) fractions were all seen to be less in

the painted than in the unpainted sites when determined on a weight

per weight basis. In each fraction, this decrease in collagen was

statistically significant. On a weight per surface area basis, however,

there were no differences between painted and unpainted sites in the

0.14M salt-soluble (Figure XXVI), 1.0M soluble (Figure XXVII) or

insoluble (Figure XXVIII) fractions.

   The average weight per unit surface area of the skin from the

painted sites was seen to be nearly twice that of unpainted skin

(Figure XXXI). Grossly, the painted skin appeared edematous, some-

what indurated and occasionally erythematous and hyperkeratotic.

   A comparison of the specific activities (as disintegrations per

minute per microgram of hydroxyproline) in painted versus unpainted

skin showed no consistent differences in the catabolism of dermal collagen. However, changes in anabolism occurred as shown in Figures XXIX and XXX. When the unpainted values were set at 100% the specific activities of both the extractable and non-extractable fractions from the painted site (Figure XXIX) were increased in all animals. Radioactivity per unit area of skin, also expressed as percent of unpainted (Figure XXX), was increased in both extractable and non-extractable collagen from painted skin. Finally, the absolute radioactivity data as DPM/cm^2 are presented in Figure XXX. Here it is seen that there is a great difference between different animals receiving the same treatment in the amount of label incorporated.

4.   Discussion

The increase in the weight of the skin per unit surface area in the painted site (Figure XXXI) can be accounted for by mere edema or swelling (i.e., thickening). The equal amounts of collagen per unit surface area of skin in painted and unpainted sites (Figures XXVI-XXVIII) can also be explained by mere swelling of the skin (which would not change the total amount of collagen present per unit surface area). Finally, the lesser amount of collagen present in a gram of painted than in a gram of unpainted skin (Figures XXIII-XXV) would again merely indicate that a unit wet-weight skin in the painted site contains more of diluent and proportionately less collagen than a

corresponding unit wet-weight skin in the unpainted site. The decrease in the collagen content of the painted site on a weight per weight basis and the increase in the weight per area of painted skin offset each other (ca. 46% decrease in the former and ca. 54% increase in the latter). Thus, it appears that the collagen was diluted by an increased quantity of another dermal component. This dilution could derive from either an increase of interstitial fluid or an increase in some other non-collagenous component or both.

Although there are indications that increased water of hydration represents only a small share of the diluent, the biochemical data of Dobson (29a), Prodi and Laschi (133b), Kirchheiner (84a), Adams (2), and Sasaki (148) all indicate a significant increase in the amount and synthetic rate of MPS in precancerous and cancerous tissues. Since it is a known property and proposed function of MPS to hold water in tissues, one would suspect that both MPS and tissue water would be involved in this dilution.

The collagen in the painted site was seen to have a greater specific activity than that in the unpainted site after the administration of an acute label (ANABOLISM - Figure XXIX). Specific activity, however, is influenced by the amount of "cold" (unlabeled) collagen present prior to administration of the label since this unlabeled collagen will dilute the new labeled collagen. Since the amount of

collagen which appears to differ in painted and unpainted sites on a weight basis (due to an increase in another dermal component), is actually equal on an area basis, the specific activities were related to the amount of collagen present on an area basis as total DPM/cm^2 (Figure XXX). As seen, although the numbers of animals are insufficient for statistical verification, there is an increase in the activity incorporated per area of skin. Since the amount of collagen per surface area is equal, we must propose that either (a) in the painted site cells produce more collagen than those in unpainted skin, or (b) there is an increased number of collagen forming cells in the painted site.

We know that irritating substances which cause inflammation, such as croton oil (73), cristobalite (SiO_2), quartz and carbon (21) and a carageenan (34) stimulate collagen formation. Thus, it is a distinct possibility that DMBA, by virtue of its irritancy, could stimulate collagen synthesis by the fibroblast.

The histological studies, although not designed to be quantitative, have clearly demonstrated an increased dermal cellularity, including increased numbers of fibroblasts. However, the collagen-producing ability of these cells is uncertain. Any attempt to correlate the increased incorporation of radioactive precursor with dermal DNA content would be difficult to interpret due to the concom-

itant increase in dermal mast cells. Thus, at present, although (a) is a distinct possibility, it is impossible to exclude (b) in an explanation of these radioactivity data.

A final point from the ANABOLISM data is that concerning extracellular aggregation. It would appear that there is a greater rate of transfer of collagen from the extractable pool to the non-extractable pool (i.e., aggregation) in painted than in unpainted sites, since, as seen in Figure XXIX, there is a greater percentage elevation of the specific activity of the non-extractable collagen than of the extractable collagen in the painted site. However, when one looks at the total activity per unit surface area, (Figure XXX), which, as mentioned, is a more realistic figure than specific activity, it appears that the transfer of activity in the painted site in animals #2 and #3, is depressed by comparison with the unpainted site. However, the number of animals in which such a comparison can be made obviously does not permit any conclusions regarding aggregation.

In the CATABOLISM portion of the experiment, unpainted controls showed an absence of activity in the soluble pool three weeks after injection of the label. This would be expected since it has been shown that a labeled proline precursor incorporated into newly synthesized collagen is aggregated into the insoluble pool within a few days (75). The experimental results, however, cannot be interpreted due to the loss of several fractions.

While this experiment suggested that some alterations in collagen metabolism were associated with carcinogenesis, several circumstances precluded a conclusive interpretation. Although the rabbit-DMBA model has the advantage of a short induction period and provides large quantities of skin for analysis, the rabbit is a delicate laboratory animal. From the onset of this experiment most of the animals were unhealthy. Most had mucoid enteritis at some time during the experiment and were variously affected. A study of protein metabolism is of dubious significance in an animal thst is not gaining weight constantly, since Gross (53) and Wirtschafter and Bentley (176) have shown that the synthesis of collagen is directly dependent on growth. Additionally, since substances causing inflammation stimulate collagen formation, it is difficult to attribute the alterations in collagen synthesis observed in painted skin to either the irritancy or to the carcinogenicity of the DMBA. This study was also deficient since the small number of surviving animals often prevented statistical analysis of the data.

Although the study provided no definitive answers, it served to point out a more productive approach to this problem. A system of carcinogenesis has to be established in which the carcinogenic effects of the compound used are clearly independent of (and, therefore, free of) any inflammatory effects. This system must employ animals in good health during the entire precancerous period so that differ-

ences in growth rate will not mask any effects of the carcinogen on the metabolism of collagen. The design of such an experiment will be discussed in the following section.

5. Summary

The metabolism of dermal collagen was studied during cutaneous carcinogenesis in the rabbit. The carcinogen, dimethylbenzanthracene, and the promoter, dodecylbenzene, were applied topically for three weeks and their effects noted by (1) an assay of total hydroxyproline in soluble and insoluble collagen of painted and control sites, and (2) the incorporation of an acute label and the loss of a chronic label of tritiated proline in painted and control sites. While the dodecylbenzene proved too irritating for a meaningful study of protein metabolism, the DMBA was less so. An apparent decreased concentration of collagen in all of its fractions in the DMBA-painted sites on a weight/weight basis was found to be due to a dilution effect since the weight/area ratio in painted skin was greater than in unpainted skin, while the collagen/area ratio in painted and unpainted sites was equal. An increase in the incorporation of acutely-administered ^{3}H-proline in painted sites was observed. It was concluded that this effect on collagen synthesis could be due to the irritating rather than the carcinogenic effects of DMBA.

FIGURE XXII: This shows the experimental design. To study the effect of painting on collagen catabolism, the labeled proline was injected three days prior to the start of DMBA painting, whereas, to study its effect on collagen anabolism, an acute proline label was administered three days prior to sacrifice at the end of the painting period. The collagen was processed as shown on the right. The details of these procedures are presented at the end of the materials and methods section. The locations of the eight cm^2 painted and unpainted sites are shown in the diagram.
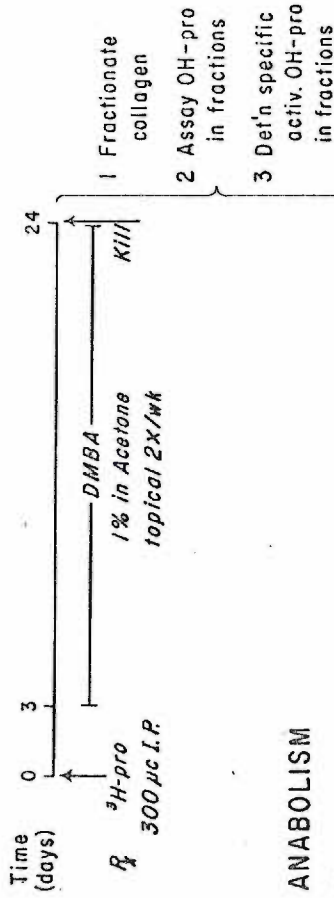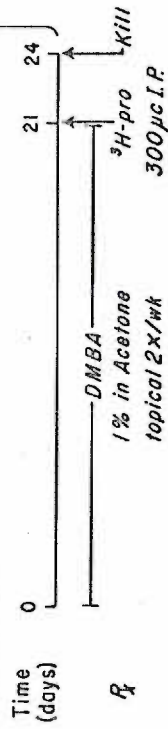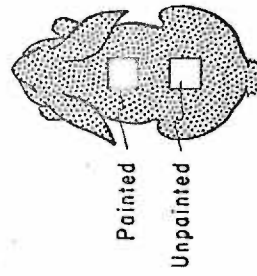
figure XXII

FIGURE XXIII: This shows the concentration of collagen in the 0.14M

neutral salt soluble pool of the DMBA-painted and adjacent unpainted

sites on a weight basis. The unpainted values are set at 100% and

the painted values presented as a percentage thereof in order to

eliminate the confusion of individual variation. The absolute values

as μg hydroxyproline/mg. (wet weight) skin x 100 are set atop each

bar. The difference between painted and unpainted values is statis-

tically significant.

DMBA: COLLAGEN CONCENTRATION — 0.14 M EXTRACTS

An#	1	2	3	4	5	6
D =	2.00	2.50	1.65	2.05	4.62	2.19

Painted □   Unpainted ▨

[OH-proline] — γ / mg skin × 100

Significant decrease in painted area · A = < .01

[OH-proline] — % of unpainted
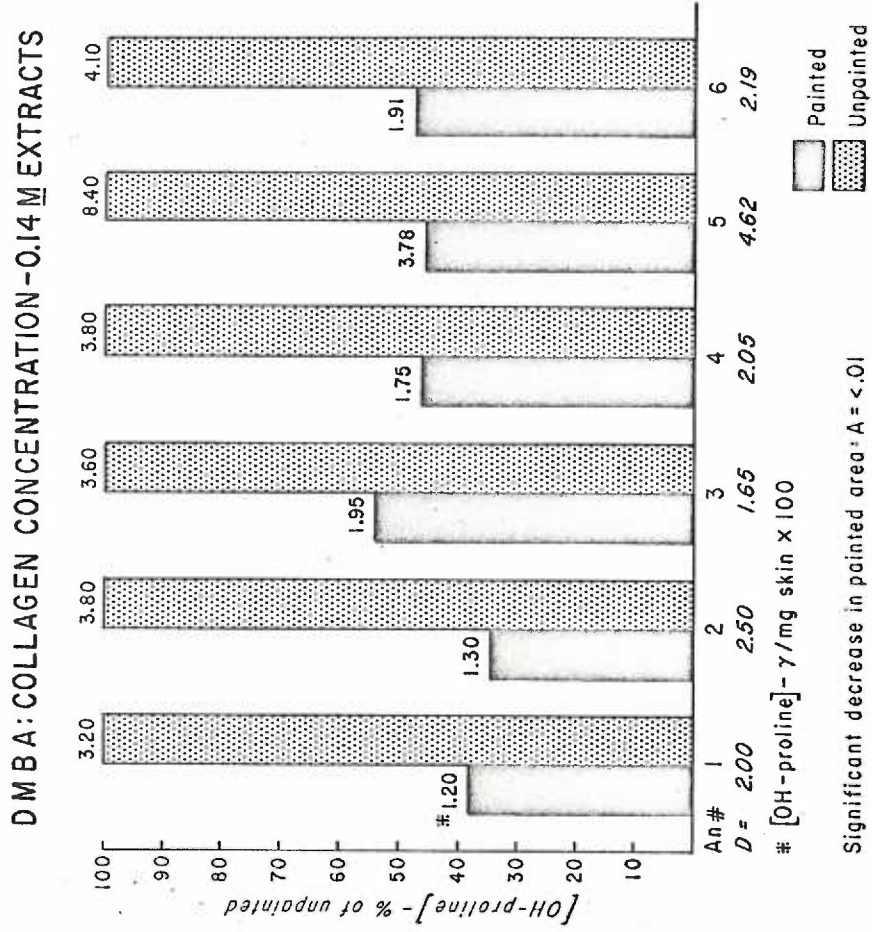
figure XXIII

FIGURE XXIV: This shows the concentration of collagen in the 1.0M neutral salt soluble pool in DMBA-painted and adjacent unpainted sites on a weight basis. Again, the difference between P and U is significant.

90

figure XXIV

FIGURE XXV: This shows the concentration of collagen in the insoluble pool in DMBA- painted and adjacent unpainted sites on a weight basis. Again there is significantly less collagen in the painted site.

figure XXV

FIGURE XXVI: This shows the concentration of collagen in the 0.14M neutral salt soluble pool in DMBA-painted and adjacent unpaited sites on an area basis. There is no significant difference between P and U.

DMBA : COLLAGEN CONCENTRATION - 0.14M EXTRACTS

figure XXVI

painted
unpainted

ℓ OH-p/cm² skin surface

animal no.

94
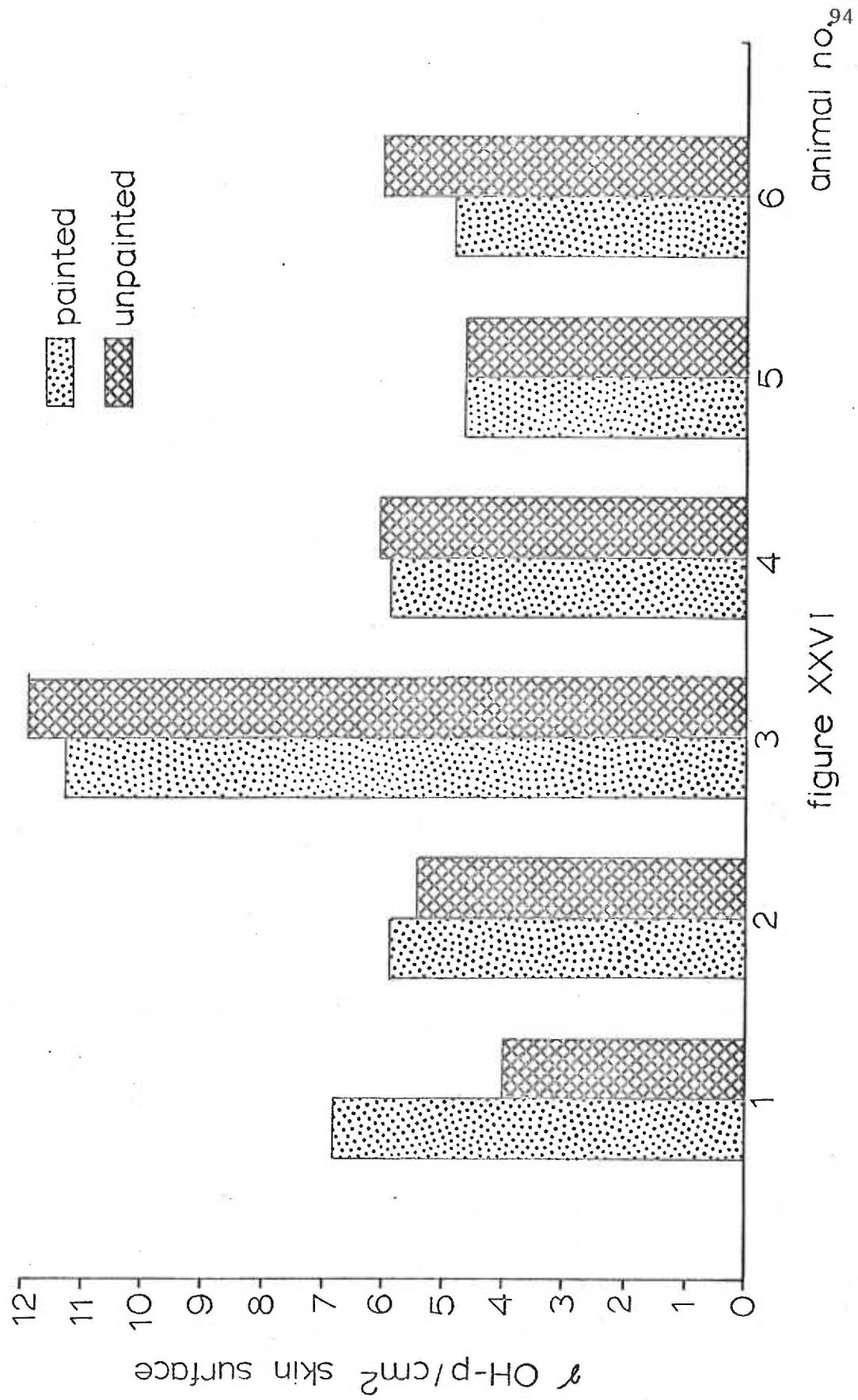
FIGURE XXVII: This shows the concentration of collagen in the 1.0M neutral salt soluble pool in DMBA-painted and adjacent unpainted sites. Again, there is no difference between P and U.

DMBA : COLLAGEN CONCENTRATION-1.0M EXTRACTS
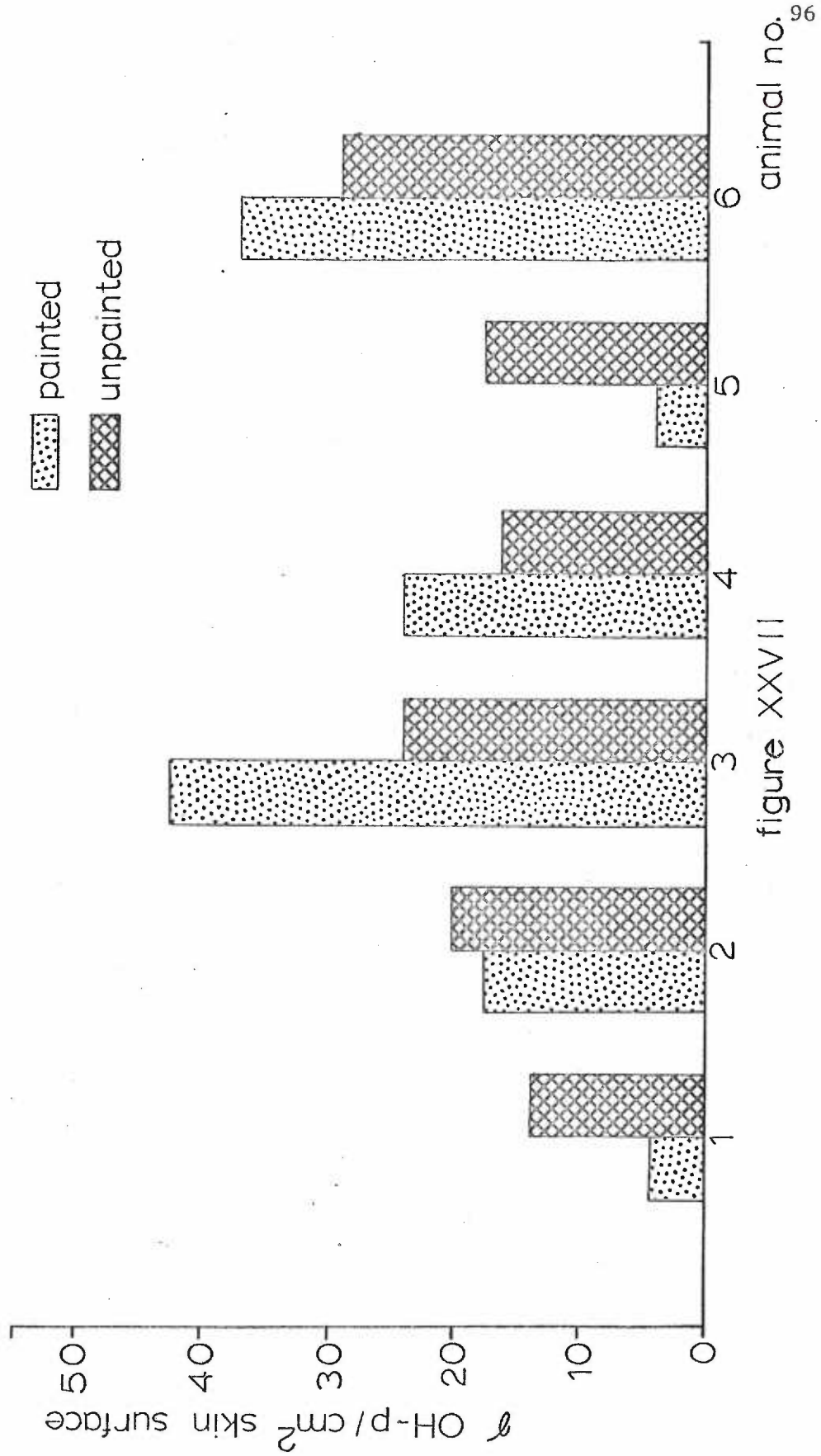
figure XXVII

FIGURE XXVIII: This shows the collagen concentration in the

insoluble pool in DMBA-painted and adjacent unpainted sites.

Again, there is statistically no difference between P and U.

DMBA : COLLAGEN CONCENTRATION – INSOLUBLE RESIDUES

figure XXVIII
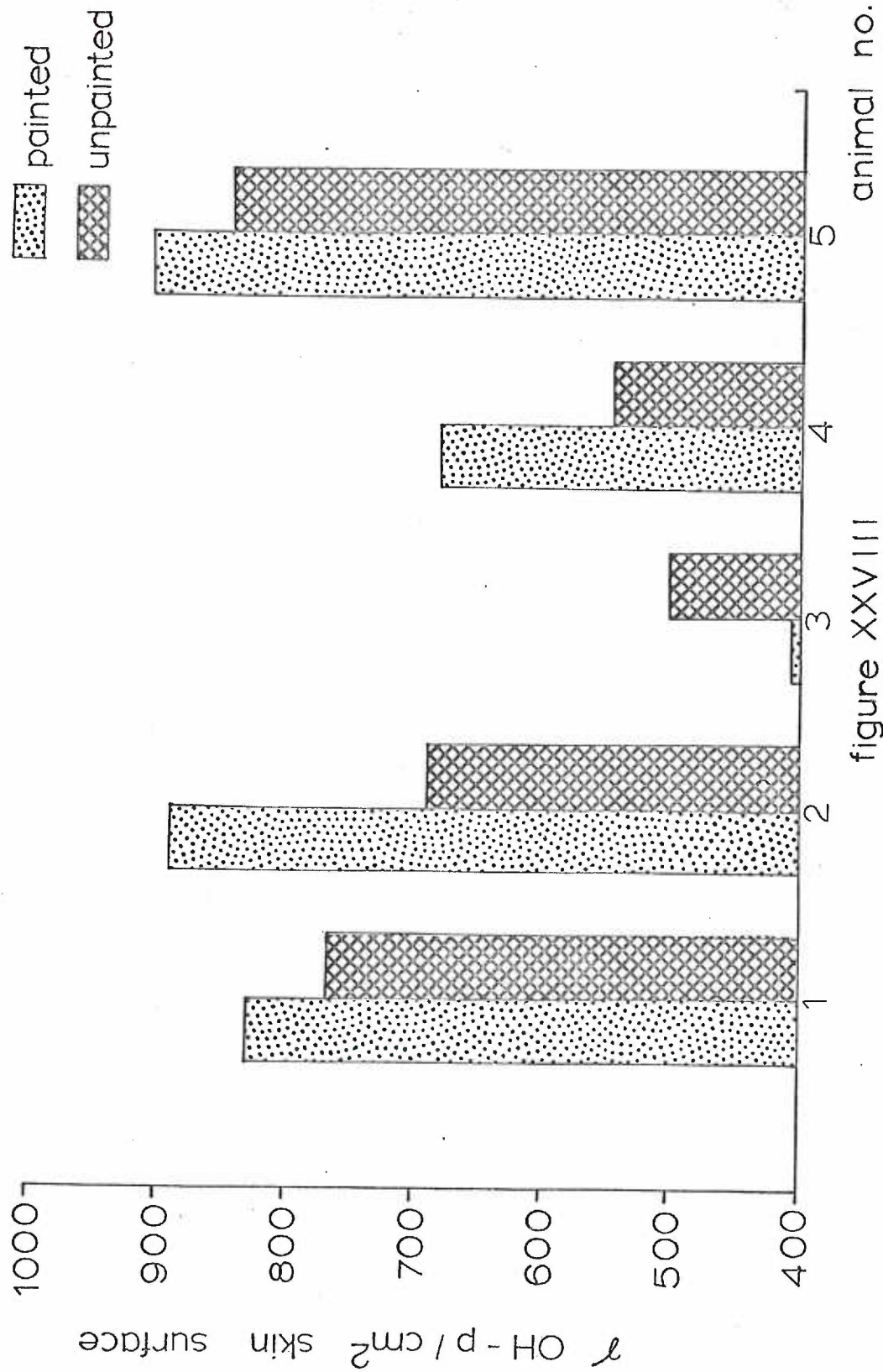
FIGURE XXIX: This shows the ratio of specific activities of collagen hydroxyproline in DMBA-painted and adjacent unpainted sites. The unpainted values have again been set at 100% and the painted values of extractable (dotted = 0.14M + 1.0M NSS) and non-extractable (cross-hatched = insoluble) collagens shown as percentage deviations therefrom. In all cases there is an increase in specific activities of the painted site. The relatively greater increase of activity in the non-extractable pool would indicate a greater rate of aggregation from extractable to non-extractable collagen in the painted site than in the unpainted site.

ANABOLISM: SPECIFIC ACTIVITY RATIOS

A. Increased synth.(E↑)

B. Increased aggr.
(N↑>E↑)

extractable collagen(E)
non-extractable collagen(N)

U = 100%

animal No.

figure XXIX

% of unpainted

100
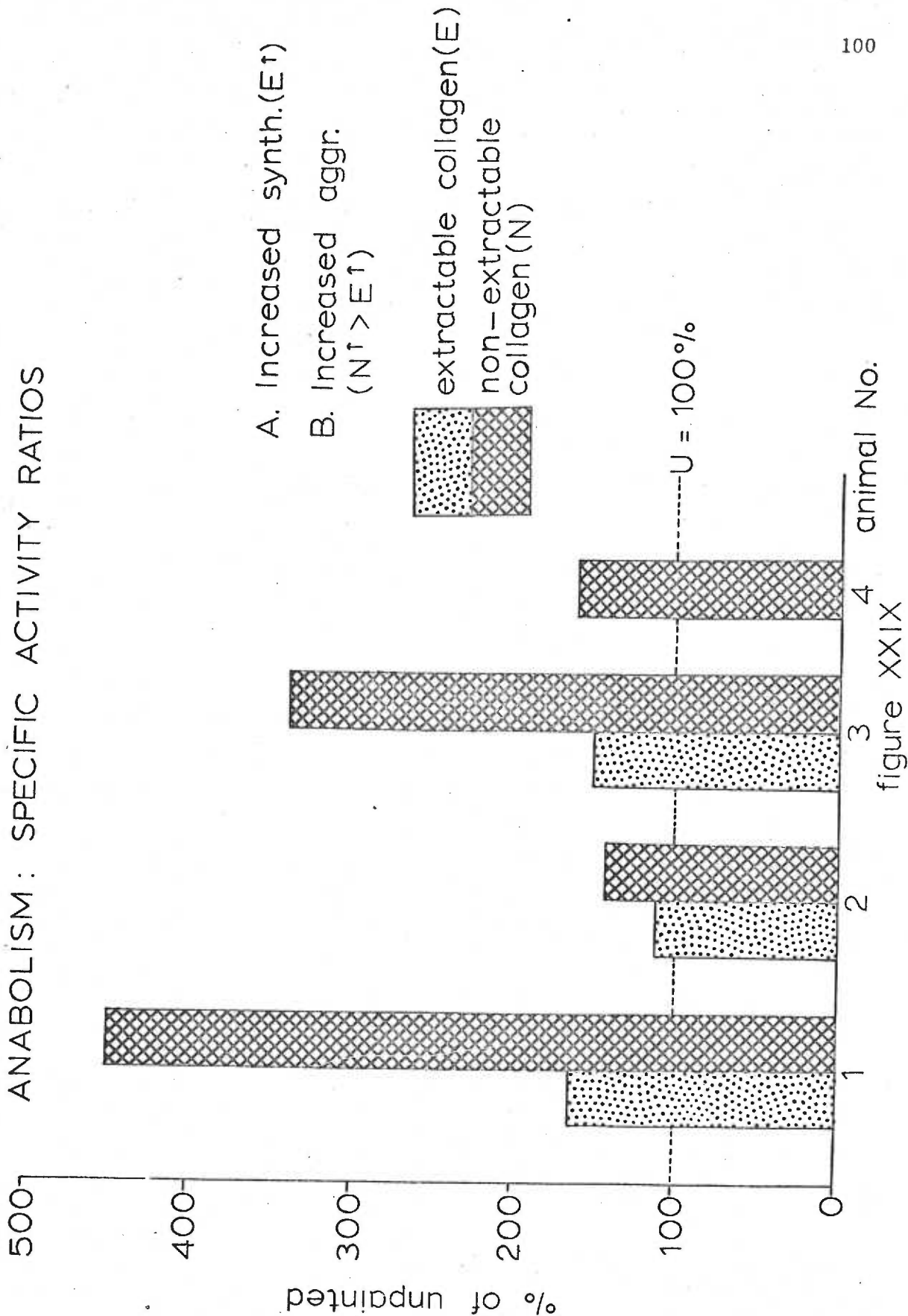
FIGURE XXX: This shows the ratios of total radioactivity per unit

area incorporated into DMBA-painted and unpainted sites on the

same animal after administration of an acute ^{3}H-proline label. The

unpainted values are set at 100% and the painted values expressed

as percent of unpainted. The absolute values are shown in the table.

Extractable (0.14M + 1.0M-soluble) collagen is represented by

dotted bars and non-extractable (insoluble) by cross-hatched bars.

There appears to be a greater incorporation of label into the collagen

in the painted site (i.e., all painted values exceed unpainted values).

However, in animals #2 and #3, where a comparison is possible, it

appears that the rate of aggregation (transfer of label from soluble

to insoluble) is depressed in the painted site.

102
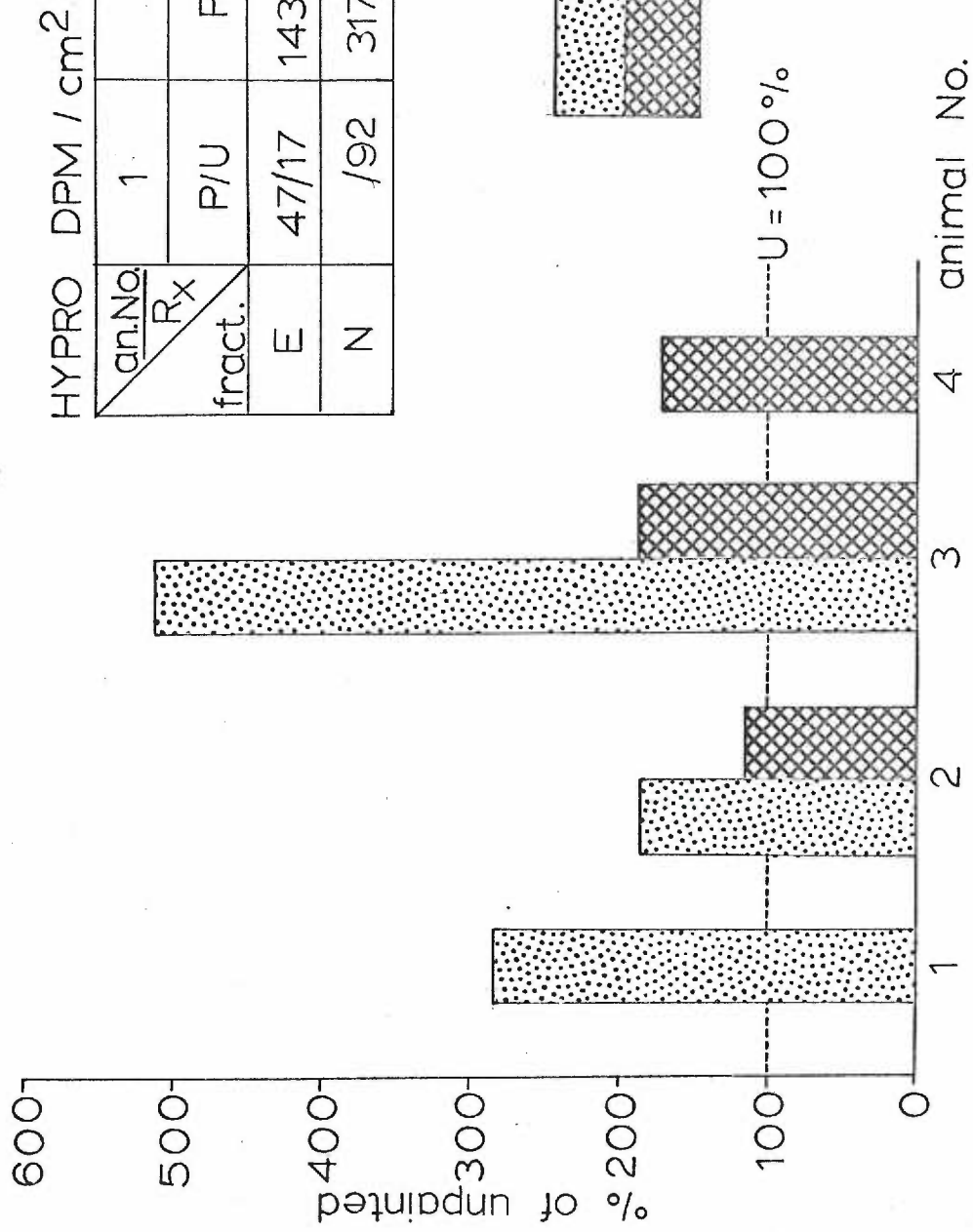
HYPRO   DPM / cm²

HYPRO an.No. Rx fract.	1		2		3		4	
	P/U		P/U		P/U		P/U	
E	47/17		143/77		16/3		/95	
N	/92		317/274		30/16		472/273	

extractable collagen (E)
non-extractable collagen (N)

HYPRO   DPM / cm²

% of unpainted

U=100%

figure XXX

animal No.
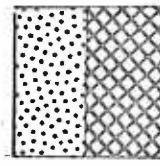
FIGURE XXXI: This shows that the weight per surface area of
painted skin is greater than that of unpainted skin. This most
likely represents an inflammatory reaction resulting from the
irritant qualities of DMBA.

BIOPSIES   weight / area : RATIOS of U/P

figure XXXI

painted   unpainted

animal No.

weight/area   % of painted   P = 100
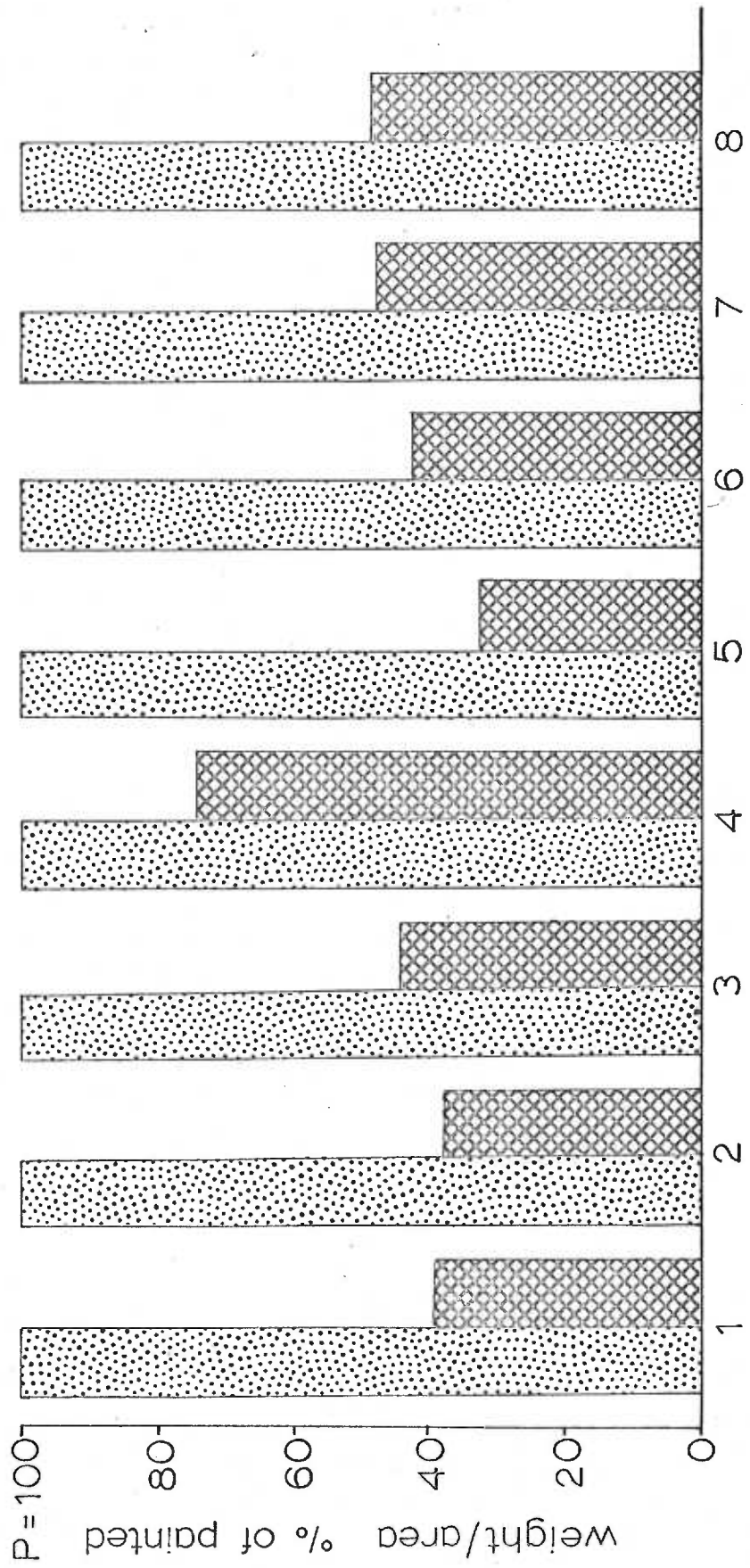
C.   Collagen Metabolism in Precancerous Rat Skin

1.   Introduction

The histology and histochemistry of the dermal response to carcinogens has been described in Experimental Section A. The collagen alteration is particularly marked consisting of fraying, fragmentation, clumping, rarefaction and a loss of normal affinity for certain dyes. These alterations occur consistently in every animal-carcinogen system studied and precede epidermal neoplasia. To determine the biochemical reasons for these changes, a study of possible alterations in the metabolism of collagen in painted skin was needed.

Initial efforts to define these alterations conclusively, failed (Experimental Section B). Although the experiment suggested that alterations in collagen synthesis did exist, it was impossible to determine whether these alterations were due to the carcinogenic initiating qualities or to the non-carcinogenic inflammatory-promoting qualities of the carcinogen (DMBA). The reasons for these problems were inherent in the design of the experiment and were discussed, (Discussion-Experimental Section B).

To recapitulate briefly, they included primarily, in addition to the above mentioned use of a carcinogen with irritating qualities, the use of an insufficient number of animals for meaningful statis-

tical analysis, and the choice of an animal which, due to the instability of its growth characteristics, is particularly unsuited to studies of protein metabolism.

To circumvent these problems, several alterations in the experimental design had to be made. The experiment should employ large numbers of metabolically-stable (i.e., healthy) young, growing animals and a non-inflammatory carcinogen. Frequent samplings of the collagen should be made to establish metabolic trends so that findings would not be "taken out of context" as in the rabbit experiment. Again, two controls should be used, a contralateral unpainted site and a separate unpainted animal, for the reasons discussed in the rabbit experiment. The experiment should monitor growth rate (by body weight gain) in painted and control animals because of the close relationship between growth rate and collagen synthesis. The inflammatory effects of the carcinogen should also be monitored since it has been shown that inflammation can result in collagen dilution.

To study the influence of carcinogen painting on collagen breakdown (or Catabolism) the collagen should first be uniformly labeled with radioactive proline over a period of several days and the painting then begun after sufficient time had elapsed for the incorporation of that label into the insoluble collagen pool. The amount of label remaining in the insoluble pool could then be measured at intervals

throughout the course of the carcinogenic treatment and compared with the amount of label remaining in the insoluble collagen of unpainted animals. The localization of any observed alteration of collagen breakdown could be determined by comparison of the results obtained in the painted site with those in a contralateral unpainted site and the skin of a separate, unpainted animal. This should show whether the effects of the carcinogen on collagen catabolism are limited to the painted, precancerous site or are merely a part of a generalized systemic reaction. The specific route of collagen breakdown, i.e., back through the extractable pools (disaggregation), or through direct degradation, can be determined by observing whether or not any label lost from the insoluble pool during treatment shows up in the extractable pools. Here also, results in the painted site must be compared with both contralateral unpainted and control tissues.

Collagen buildup, anabolism, or synthesis and aggregation can be studied by the administration of an acute label to painted animals at periodic intervals throughout the painting period. Again, a sufficient time for incorporation of label into the insoluble pool should be allowed to elapse between injection and sacrifice. Comparison of the total amount of label incorporated into painted, contralateral unpainted and control skin will provide an indication of the relative

rates of collagen synthesis in these tissues. Fractionation of the collagen into salt soluble and insoluble fractions and assay of the amount of label in each will give an idea of the rate of transfer of label from soluble to insoluble (aggregation) during the incorporation period. This rate of aggregation can then be compared in painted, unpainted and control sites.

The present study represents an attempt to determine in pre-cancerous skin any differences in collagen synthesis and aggregation and/or breakdown, either directly or by disaggregation. The study incorporates the above improvements in experimental design, and employs the non-inflammatory carcinogen, 2-amino anthracene in metabolically-stable, young, growing rats.

2.   Materials and Methods

Experimental Procedures: The design of this study is shown in Figure XXXII. To study collagen breakdown or CATABOLISM, a uniform label of $60\mu c$ tritiated proline was injected intraperitoneally daily for five days to 75 male Sprague-Dawley rats about ninety days of age. After three days, which was sufficient to aggregate 90% of the label into the insoluble collagen pool, twice-weekly painting of the entire left dorsum with 1% solution of the carcinogen, 2-amino-anthracene in acetone, was begun. Five of the painted animals were

killed biweekly and three unpainted controls were killed every four weeks. Painted, contralateral unpainted and control tissues were shaved, depilated, removed, freed of subcutaneous fat and muscle, frozen in liquid nitrogen, pulverized in a steel mortar, and the collagen extracted at 4°C with 0.14 and 1.0M neutral salt solutions leaving an insoluble residue fraction. The hydroxyproline in each fraction was assayed and its specific activity was then related to a unit weight of tissue. The objective was to determine whether or not the rate of collagen breakdown was altered by the carcinogenic treatments and to determine whether this breakdown occurred via the extractable pool.

The second portion of the experiment was designed to study collagen synthesis and aggregation. Seventy-five Sprague-Dawley rats about ninety days of age were painted twice weekly for fourteen weeks. Painted animals were killed biweekly and unpainted controls every four weeks. Labeled proline was administered three days before death, the time necessary for incorporation of the label into all fractions of the collagen. The tissues from painted, unpainted and control sites were processed as before, and the total activity in each fraction related to unit weight. This portion of the experiment was designed to uncover any differences in the rate of synthesis or defects in the normal pathway of aggregation.

The details of the separation, quantification and radioassay of the collagen hydroxyproline are presented at the end of this section. The isolation of collagen was as in the rabbit experiment, with the exception that a small amount of the crushed tissue was used for measuring hydration by wet weight-dry weight ratios. The separation and isolation of the hydroxyproline from the collagen, however, employed a technique different from and better than that previously used. This latter technique which we used to isolate hydroxyproline as pyrrole, (as described in Experimental Section B), was abandoned because of its length, cost, and questionable reproducibility. Thus, a method originally proposed by Moore and Stein (40B), was modified so that hydroxyproline could be rapidly isolated from proline in a large number of whole hydrolysates by chromatography on Dowex cation exchange resins. This technique, which is presented in detail in Appendix I, gave rapid and reproducibly high yields of pure hydroxyproline from whole hydrolysates. The technique involves an initial nitrous acid treatment of whole hydrolysates to convert the amino acids to hydroxy acids. The imino acids are either unchanged or converted to their N-nitroso derivatives by this process. The hydroxy acids are extracted with ether and discarded, leaving the water soluble imino acids and their N-nitroso derivatives. These are solubilized, put on Dowex cation exchange columns and eluted under

a pressure of about 250 mms of mercury with a total of 120 ml of 1.5N HCl. The separation of proline from hydroxyproline with both purified compounds and nitrous acid-treated whole hydrolysates gave good resolution (a peak separation of at least 10 ml) as shown in the appendix.

The radioactivity of the samples was counted on a Packard Tri Carb liquid scintillation spectrometer, model 3310, for intervals which varied inversely with the activity of the sample as discussed in Appendix III.

The colorimetric assay of the hydroxyproline was carried out in triplicate on a Technicon Autoanalyzer as described by Grant (49). The assay involves an oxidation of the hydroxyproline with Chloramine T. The excess Chloramine is destroyed with perchloric acid. The hydroxyproline chromogen is then reacted with p-dimethylamino-benzaldehyde. Absorption is measured at 562mμ. All samples were measured in triplicate.

Data were subjected to paired and unpaired t-tests for significance and analyzed for standard deviation and standard error of the mean. 95% was chosen as the limit of confidence. Statistical analyses were carried out on an Olivetti Underwood Programma 101 table computer with programs developed by the manufacturer.

Materials:

Hydroxyproline Assay: p-dimethylaminobenzaldehyde (AG Matheson Coleman and Bell) 5% in n-propanol (solution should be clear and very pale yellow--store in refrigerator); perchloric acid-27 ml of 70% AR perchloric acid diluted to 100 ml with water; Chloramine T (1.41 gm) dissolved in 80 ml water and diluted with 120 ml methyl cellosolve (ethylene glycol monomethyl ether) and 200 ml buffer-made fresh daily; buffer--50 gm citric acid monohydrate, 12 ml glacial acetic acid, 120 gm sodium acetate (3.H_2O) and 34 gm sodium hydroxide made up to 1 liter in distilled water, pH adjusted to 6.0, stored under toluene at $4^\circ C$; samples diluted to a final concentration of less than $10\mu g/ml$ (since measurements above this concentration become increasingly less accurate).

Radioassay: Phosphor solution used contained 0.1gm of 1,4 bis 2-(5 phenyloxazolyl) benzene (scintillation grade, Packard Instrument Co., Inc.) and 4.0 gm of 2,5 diphenyloxazole (scintillation grade, Packard Instrument Co., Inc.) made up to 1 liter in toluene, protected from light.

Tritiated proline (L-proline (UL)-^{3}H, SA = 750mc/mM) was provided by the Nuclear Chicago Corp. Before the addition of 14 ml of scintillation fluor to each counting vial, the samples were solubilized with 1 ml of a 1M solution of Hydroxide of Hyamine 10-x,

p-(diisobutyl-cresoxyethoxyethyl) dimethylbenzylammonium hydroxide , in methanol, (Packard Instrument Co., Inc.).

Chromatography: Resin used was Dowex AG 50 W-X4 200-400 mesh H+ analytical grade cation exchange resin (BioRad Laboratories); the Chromatographic columns were 50 cm long x 1 cm inside diameter with a 50 ml reservoir at the top and a plastic stopcock, (Glassbowing Dept., Research Instrument Service, University of Oregon Medical School, Portland, Oregon). Preparation of the columns and regeneration of the resin will be discussed with the technique in Appendix I.

Carcinogen: 2-aminoanthracene ("2-anthramine"- Aldrich Chemical Co., Inc.).

Collagen Extraction: The collagen extractants used were as in Experimental Section B for rabbit skin collagen.

ISOLATION OF COLLAGEN

Sacrifice (5cc Nembutal)
↓
Shave
↓
Depilate with Surgex
↓
Remove painted and unpainted
pieces of skin
↓
- -

↓	↓
4mm punch biopsy specimen	Remainder
↓	↓
Histology	Freeze, liquid N
	↓
	Crush, mortar and pestle
	↓
	Divide
	↓
	→→Weigh→Dry→Weigh
	↓
	Weigh
	↓

Dialyze against ←←←←←←← Pool .14M salt ←←← $\begin{smallmatrix}\text{Supernate}\\\text{Centrifuge}\end{smallmatrix}$ ←←Extract, 40 ml 0.14M
0.1M acetic acid   soluble collagen   NaCl (pH 7.7) x 24 hr,
until salt free   4° C
↓   ↓
Clarify dialysate   ←←Extract - same 24 hr
by centrifugation
↓   ←←Extract - same 24 hr
Evaporate under
N₂ on water bath   ←←Pool 1.0M salt ←←← $\begin{smallmatrix}\text{Supernate}\\\text{Centrifuge}\end{smallmatrix}$ ←←Extract, 40 ml 1.0M

Dialyze against ←←←←←←←Pool .14M salt ←←← Supernate/Centrifuge ←←Extract, 40 ml 0.14M
0.1M acetic acid soluble collagen NaCl (pH 7.7) x 24 hr,
until salt free 4° C
↓ ↓

Clarify dialysate ←←Extract - same 24 hr
by centrifugation ↓
↓ ←←Extract - same 24 hr
Evaporate under ↓
N₂ on water bath ←←Pool 1.0M salt ←←← Supernate/Centrifuge ←←Extract, 40 ml 1.0M
↓ soluble collagen NaCl (pH 7.7) x 24 hr
Transfer to hydrol- 4° C
ysis tubes with ↓
10 ml of 6N HCl and ←←Extract - same 24 hr
hydrolyse at 140° C ↓
x 3 hr ←←Extract - same 24 hr
↓ ↓
↓ Insoluble residue
↓

```
↓                                              ↓
↓                                              ↓
↓                                              ↓
↓                                              ↓
↓                                         Wash - shake quickly
↓                                         in dist. water,
↓                                         centrifuge,
↓                                         discard supernate
↓                                              ↓
↓                                         Add 10 ml dist. water,
↓                                         cap with tinfoil, auto-
↓                                         clave at 124°C x 3 hr
↓                                              ↓
↓                                         Clarify by centrifuga-
↓                                         tion (20,000 G)
↓                                              ↓
↓ →→→→→→→→→→→→→→→→→→→→ Collagen Hydrolysate ←← To an aliquot of gel-
                                         atin add an equal
                              ↓          quantity of 12N HCl
                              ↓          and hydrolyse at
                         Evaporate       140°C x 3 hr
                              ↓
                    Dilute to known volume
                    with D.W.
```

ISOLATION, QUANTIFICATION and RADIOASSAY

of HYDROXYPROLINE

HYDROLYSATE
↓
Transfer aliquot (in D.W.) to hydrolysis tube
↓
Dry under N_2 in boiling water bath
↓
Add excess HONO; let stand at room temperature for ten minutes,
heat at $140^\circ C$ until light yellow (don't cap tightly)
↓
Dry under N_2 in boiling water bath
↓
Dry under vacuum over P_2O_5 for two days
↓
Extract with 5 ml anhydrous ether and discard ether
↓
Dissolve sample in 0.5ml 70% EtOH to desalt
↓
Transfer, evaporate under N_2 and dissolve in 1 ml 0.1 N HCl
↓
Separate pro from hypro on Dowex cation exchange column
eluting with 1.5N HCl
↓
Pool hypro fractions in evaporating dish and dry on steam bath
↓
Dissolve in a known volume of distilled water

↓   ↓
↓   ↓
Aliquot for quantification   Aliquot for radioassay of hypro
of hypro   ↓
↓   Dry
Dilute   ↓
                                            Add scintillation fluor
↓   ↓
Assay hypro colorimetrically   Count activity in liquid scintillation
                                            spectrometer

3.   Results

Figure XXXIII shows the total radioactivity, as well as the distribution of activity, in a gram of skin, i.e., (DPM/μg OHp) x (μg OHp/gm dry wt.), in painted, contralateral unpainted and control sites of chronically labeled animals throughout the course of the experiment. P. and U bars each represent the average of five animals, whereas, C bars represent the average of three animals. The amount of insoluble collagen (which is far greater than the amount of soluble collagen) is represented as a tenth of its real value so that soluble collagen activity can be fit on the same graph. At time zero, approximately 90% of the label resides in the residue fraction. However, with time, nearly all of the label (ca. 98.8% at twelve weeks) is transferred to the residue (insoluble) pool. After six weeks of painting, the activity present in painted (P), contralateral unpainted (U) and control sites on separate unpainted animals (C) begins to differ. An obvious stairstep discrepancy appears between P, U and C where the total activity in P is always greater than that in U, which, in turn, is greater than that in C. This could be due to P having a lower turnover rate which would imply a lower rate of catabolism of collagen in the painted site. For example, if the collagen turnover (i.e., synthesis and degradation) rate in the painted site was depressed in comparison to that in the unpainted site, one could expect

that radioactivity incorporated into the collagen in the painted site at an earlier date would be lost at a slower rate than in the unpainted site; if this were the case, more radioactivity would remain in the collagen with the lower turnover rate and one would, therefore, expect a higher specific activity of that collagen. If this interpretation is correct, it would appear that collagen does not break down via the extractable pool, since the soluble collagen of unpainted and control skins, which would have greater turnover rates, does not contain more activity than the soluble collagen of painted skin. However, interpreting these data as representing altered rates of collagen breakdown is most likely incorrect. If the activity in the control from the beginning of the experiment to the end is considered, there is an apparent and considerable loss of activity from the control skin collagen. Since collagen does not turn over appreciably in fourteen weeks, a neosynthesis of "cold" collagen diluting the "hot" pool must be postulated to explain this diminution of activity per gram of skin. Thus, the greater amount of activity observed in the painted site collagen would indicate a reduced rate of collagen synthesis in this site (and a reduced dilution of the activity present in a gram of painted skin). That this is indeed the case can be verified by studying the ANABOLISM portion of the experiment. Figure XXXIV shows the total radioactivity and the distribution of activity after an acute label

in painted, contralateral unpainted and control sites throughout the course of painting. Again the amount of insoluble collagen activity has been represented by a tenth of its real value so that changes in soluble collagen activity can be shown on the same graph. As can readily be seen, from the fourth week on, there is a precipitous decline in the incorporation of radioactivity in all three sites. Total radioisotope incorporation into the painted site consistently appears to be less than incorporation into the unpainted site which, in turn, is consistently less than incorporation into the control site on an unpainted animal. It appears that from the fourth week on, the extractable activity in P is less than that in U. From the second week on, it appears that the non-extractable activity in P is less than in U.

While extractable activity does not appear to consistently differ between U and C, their non-extractable activities differ at eight and twelve weeks, with less activity being incorporated into U than into C. This could indicate a systemic effect of the carcinogen, since the unpainted site always has less activity than the control skin. However, differences between U and C in the rates of synthesis could also be explained by the observed differences in growth rate between painted and unpainted animals, since growth profoundly influences collagen synthesis (23, 75). Thus, collagen CATABOLISM (Figure XXXV) and collagen ANABOLISM (Figure XXXVI) were corrected for growth by means of a growth factor

derived from their relative weights at each time point. This correction factor is justifiable since the weight of the skin represents a constant proportion of the total body weight to at least 56 weeks of age in the rat (10), and the body weight at this age is a function of protein synthesis. The growth correction was applied as follows:

Eight Weeks:

Average body weight: Painted animal = (P and U sites)= 380 gms
Unpainted animal = (C sites)= 442 gms

Growth factor $= \dfrac{380}{442} = 0.845$

Total activity C (Catabolism) $= 1.92 \times 10^5$ DPM/gm dry wt. skin

Total activity C (Catabolism) corrected for growth $= (1.92 \times 10^5) \times (0.845) = 1.79 \times 10^5$ DPM/gm dry wt. skin

Figure XXXV shows the CATABOLISM figures corrected for growth. As can be seen from the growth curve in the upper right-hand corner, after the fourth week the growth of painted and unpainted animals becomes increasingly discrepant. At ten weeks of painting, the painted animals ceased growing. Although the experiment was concluded at fourteen weeks, most of the animals had by then received lethal doses of carcinogen and during the next two months died of renal failure. Due to the dependence of collagen metabolism on growth, the period of significance in this study is from 0-10 weeks

of painting, while the animals are still growing. When the activity

per weight of skin is corrected for growth, there is statistically no

difference between U and C at any point. The difference between P

and U persists, and can best be explained as a depression of collagen

synthesis in the painted site.

By reference to figure XXXVI showing collagen ANABOLISM

corrected for growth, it is seen that here also correcting for depres-

sed growth of the painted animals equates U and C. However, this

figure demonstrates a significant depression of collagen synthesis

localized to the painted site at two, six and eight weeks. The signifi-

cant difference between U and C at twelve weeks reflects the influence

of growth on collagen synthesis, since collagen synthesis in the painted

animals at this point has virtually ceased.

Figures XXXVII-XXXIX show the total concentration of collagen

in the 0.14 and 1.0M salt-soluble and insoluble fractions in P, U and

C sites during the course of the experiment. The concentration of

0.14M salt-soluble collagen reflects the depressed synthesis in the

painted site. The significant difference between C and U at twelve

weeks is, as seen before, merely a manifestation of discrepant growth

rates in painted and unpainted animals. The apparent decreased con-

centration of 1.0M soluble collagen in the painted site is statistically

insignificant. Any effect of the depressed synthesis on collagen

concentration in the insoluble pool is unapparent, most likely due to the large size of this pool compared with the soluble pools.

Finally, Figure XXXX shows the wet weight-dry weight ratios in painted and unpainted sites during the course of painting. Each bar represents the mean of ten animals and, as can be seen from the standard errors of the mean, there is no difference between painted and unpainted sites in the degree of hydration. Increased hydration of tissues is generally regarded as a hallmark of inflammatory edema and is thus an indication of the degree of inflammation in a tissue (57). At the fourteen week point, as can be seen, there suddenly develops a great variability of hydration from one animal to the next. Most likely this has nothing to do with inflammation but is due to the severe dehydration preceding death. This dehydration was visible grossly and probably represented a manifestation of the renal failure.

4.   Discussion

In contrast to rabbit-DMBA carcinogenesis where an obvious inflammatory reaction occurs, presentation of the collagen concentration and radioactivity on an area basis here was considered unnecessary since it was seen that anthramine produced no inflammation as determined by equal percentage hydration in painted and unpainted skin and

by the absence of collagen dilution (i.e., total collagen per gm dry weight was equal in painted and unpainted sites).

While the data were, of necessity, discussed in the results section, certain points are worthy of repetition. First, it was seen that an apparent decrease of normal collagen breakdown resulting from the carcinogenic treatment, in reality, merely reflected a depressed neosynthesis of collagen. Collagen newly synthesized throughout the course of the Catabolism study is unlabeled. Thus, the greater the collagen synthetic rate of the skin, the smaller will be the specific activity of its collagen as a result of dilution of the pre-existing "hot" collagen by new "cold" collagen. This interpretation was verified by the Anabolism study which showed a uniform depression of collagen synthesis in the painted site.

In the Anabolism study, it appeared that the carcinogen-produced depression of collagen synthesis was not limited to the painted site, but was found in the contralateral unpainted site as well, since radioactive incorporation in this site was uniformly lower than that in the control. If this were so, it would indicate a systemic influence of the carcinogen on collagen metabolism. Since the contralateral unpainted site never gets tumors, it would indicate that the influence of the carcinogen on collagen metabolism had nothing to do with its carcinogenic properties. However, since it is known that the growth

rate has a great influence on collagen synthesis, it was probable that the different rates of incorporation of labeled collagen precursor into contralateral unpainted and into control skins resulted merely from different growth rates of the animals. Correcting for the differences in growth rate equated incorporation in unpainted and control sites demonstrating a localization of the effect of the carcinogen on collagen metabolism to the painted site.

Several important points were established via the collagen fractionation. First, depressed collagen synthesis was reflected in the lowered concentration of 0.14M neutral salt soluble collagen in the painted site. This fraction represents the most recently synthesized collagen and is the smallest fraction; thus it is the most sensitive indicator of collagen synthesis. Collagen concentrations in the 1.0 and insoluble pools which are relatively much larger were uninfluenced by a depressed synthesis of this magnitude.

In the Anabolism study it was seen that the incorporation of radioactivity was equally depressed in all fractions of the painted site demonstrating that while synthesis was depressed, extracellular aggregation was normal.

The utility of the rat-anthramine system for the study of the biology of cutaneous carcinogenesis is promising. It is hoped that an optimal concentration/skin area relationship can be established for the carcinogen that will not interfere with its potency,

but will circumvent its toxicity while still providing ample tissue for analytical purposes.

5. Summary

The metabolism of dermal collagen was studied during the initial stages of cutaneous carcinogenesis induced with topically-applied 2-anthramine in young Sprague-Dawley rats. Collagen catabolism was determined by measuring the loss of chronic label of tritiated proline throughout the course of carcinogenic treatment. Collagen anabolism was studied by measuring the incorporation of acutely administered tritiated proline at biweekly intervals during the treatment. Collagen aggregation and disaggregation were studied by fractionation into salt-soluble and insoluble fractions and measurement of the transfer of label from one fraction to another. Total collagen concentration in each fraction was monitored through-out the course of treatment, as was the percentage hydration. Painted sites were compared with contralateral unpainted sites and with control sites on untreated animals. It was found that while collagen catabolism and aggregation were unaffected, the carcinogen induces both a depression of dermal collagen synthesis localized to the area of painting and a systemic toxic reaction. The depression of collagen synthesis coincides with the onset of the histopathological changes in

the dermis (i.e., at about six weeks of painting). The changes in

collagen synthesis were reflected by the amount of collagen extract-

able with 0.14M neutral buffered salt solutions (i.e., the "new"

collagen) which, from the sixth week on, was decreased in the painted

site. The changes were shown not to result from an inflammatory

reaction.

FIGURE XXXII: This shows the experimental design. As can be seen from the diagram, each painted animal was painted on one side only, the contralateral site serving as a control. The experiment was divided into two portions: The CATABOLISM portion of the experiment to study both the rate and route of collagen degradation employed prelabeling the collagen over a period of five days; an incorporation period of three days was then followed by twice weekly anthramine painting and biweekly sacrifice. The collagen was then processed as shown on the right, the details of which are presented at the end of the materials and methods section. The ANABOLISM portion of the experiment, designed to study the relative rates of synthesis and aggregation of collagen in painted versus contralateral unpainted and control skin, employed an acute labeling of the collagen three days prior to sacrifice at biweekly intervals throughout the period of painting. The collagen was processed as in the catabolism portion of the experiment. At four week intervals in both experiments, labeled but unpainted control animals were killed.
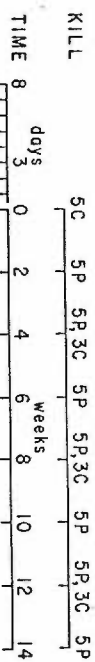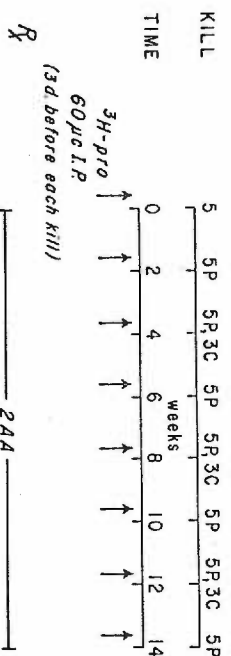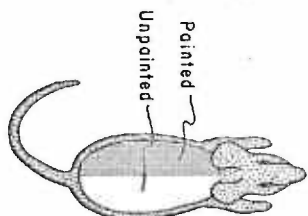
figure XXXII

FIGURE XXXIII: This shows the distribution of label in a gram dry weight of skin from pain ted (P), contralateral unpainted (U) and control (C) skin in the CATABOLISM experiment. The insoluble collagen has been reduced to one tenth its actual value in order to be represented on the same graph with the soluble collagens. While there is no consistent difference between the various treatments in the amount of collagen present in the extractable pools, there does appear to be less insoluble collagen in C than in U, and less in U than in P from the sixth week on.

COLLAGEN CATABOLISM

= .14 M salt soluble collagen
= 1.0 M " "
= insoluble collagen/10
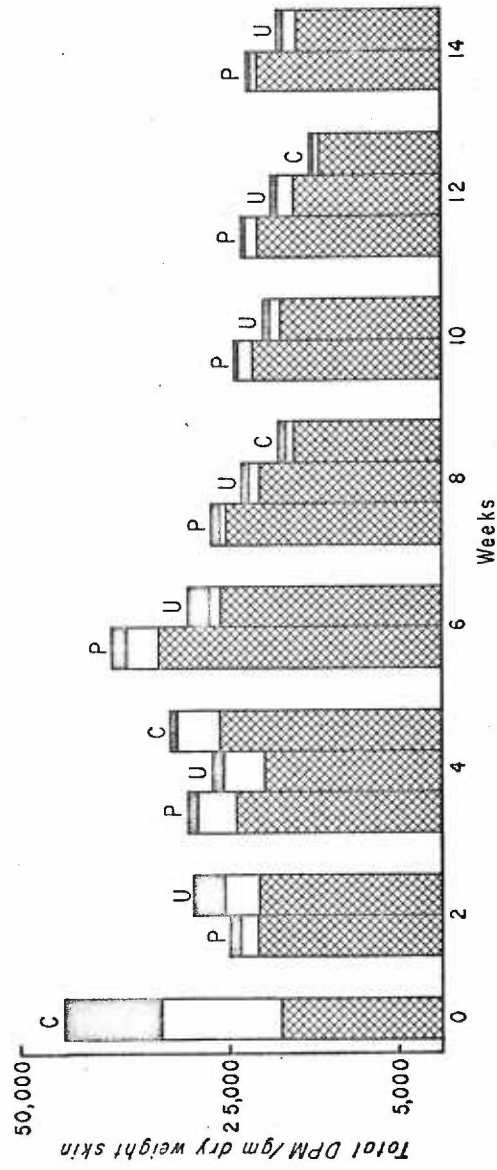
figure XXXIII

FIGURE XXXIV: This shows the distribution of label in a gram dry weight of skin from painted (P), contralateral unpainted (U) and control (C) skin. Here from the sixth week on there appears to be less soluble collagen in painted than in contralateral unpainted skin. There is also less incorporation of label into the insoluble pool of painted than unpainted collagen and less into painted than into control.

COLLAGEN ANABOLISM

= .14 M salt soluble collagen
= 1.0 M " "
= insoluble collagen /10

75,000

50,000

25,000

5,000

Total DPM/gm dry weight skin

C

P U

C

U P

U P

C U

U P

U P

C

P U

0   2   4   6   8   10   12   14

Weeks
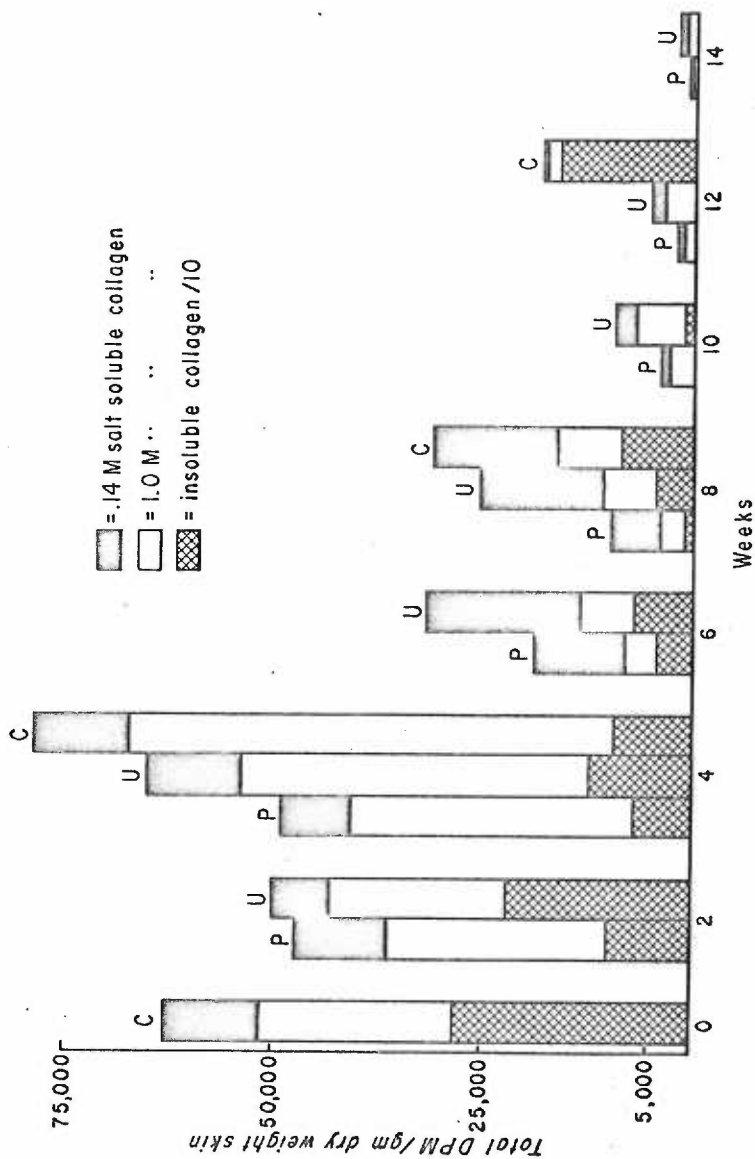
figure XXXIV

FIGURE XXXV: Since the growth rates of painted and control animals differed widely as shown by the graph at the upper right corner, and since growth is a direct reflection of collagen and other protein synthesis, the total radioactivity/gm skin was corrected by a growth factor derived from the relative weights of painted and unpainted animals at each time point. As seen, this procedure approximates U and C showing that the apparent systemic effect of the carcinogen (seen in Figure XXXIII) is merely the result of different rates of growth and thus different degrees of dilution of the pre-existent "hot" collagen pool by "cold" collagen. While, from this graph, one might expect a slower rate of turnover of collagen in P than in U, it is readily seen from Figure XXXVI that this discrepancy is merely the result of a greater collagen synthetic rate in U.

COLLAGEN CATABOLISM – CORRECTED FOR GROWTH

figure XXXV

134
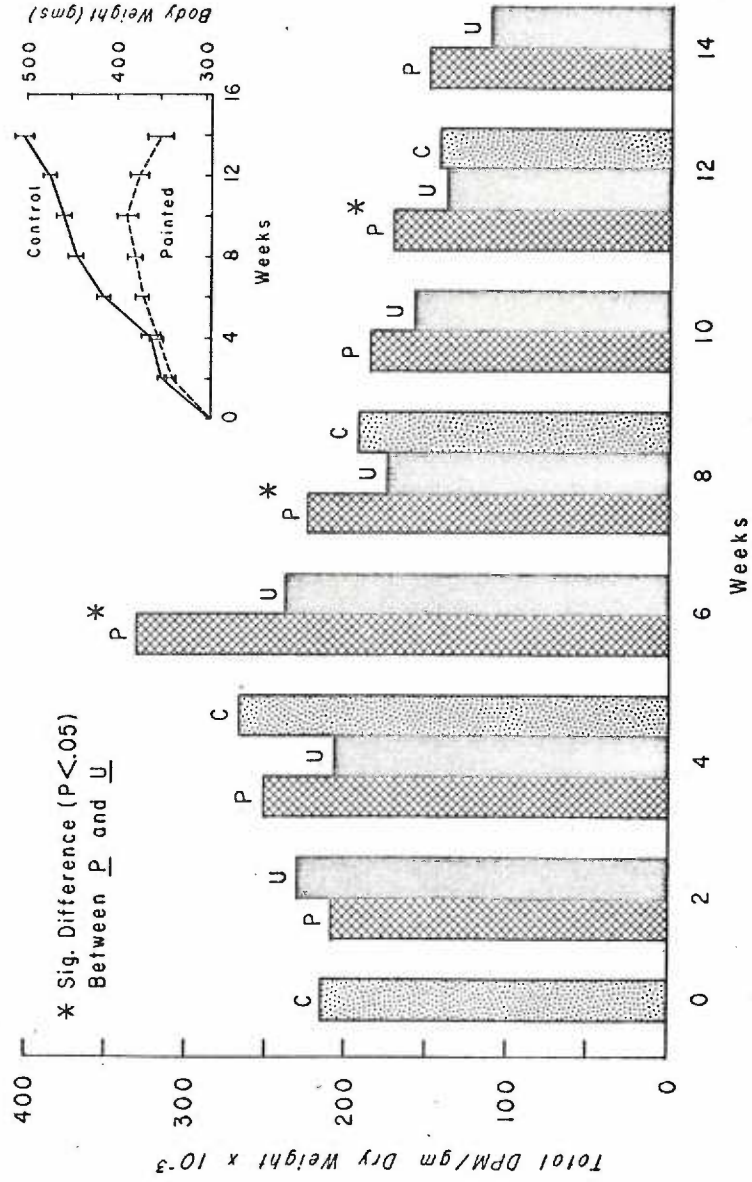
FIGURE XXXVI: This figure shows the total activity per gram of skin corrected for growth in the Anabolism experiment. Again, correcting for growth approximates U and C until twelve weeks at which point the painted animals are losing weight rapidly and collagen synthesis has come to a virtual standstill. Throughout the significant portion of this experiment where the painted animals are gaining weight (i.e., up to ten weeks of painting) there is depressed incorporation of label into the collagen of the painted side.

COLLAGEN ANABOLISM – CORRECTED FOR GROWTH
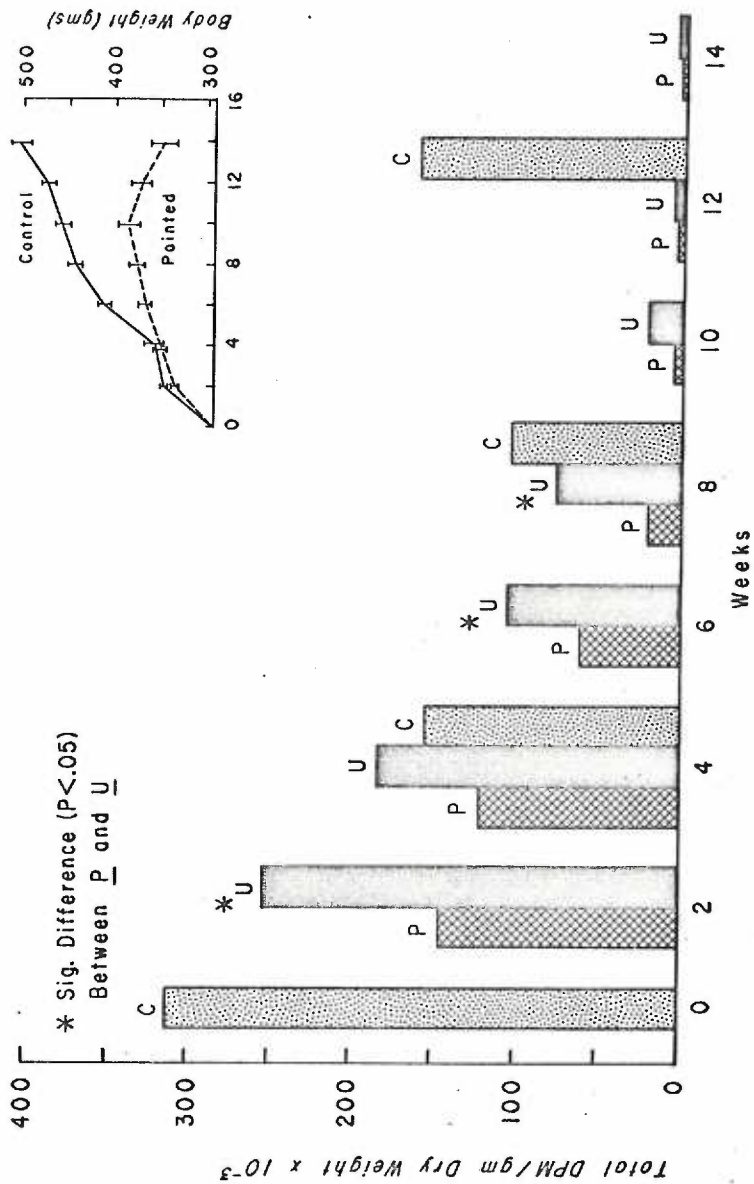
figure XXXVI

FIGURE XXXVII: This shows the concentration of collagen in the 0.14M neutral salt soluble pool in painted versus contralateral unpainted sites. Each painted and each unpainted point represents the mean of ten samples, whereas, each control point represents the mean of six samples. Collagen concentration is expressed as μg hydroxyproline per unit dry weight. The significantly lower concentration of collagen in the painted site from four weeks on is consistent with the observed depression of synthesis or tritiated proline incorporation in this site.

O.14 M NEUTRAL SALT EXTRACTABLE COLLAGEN
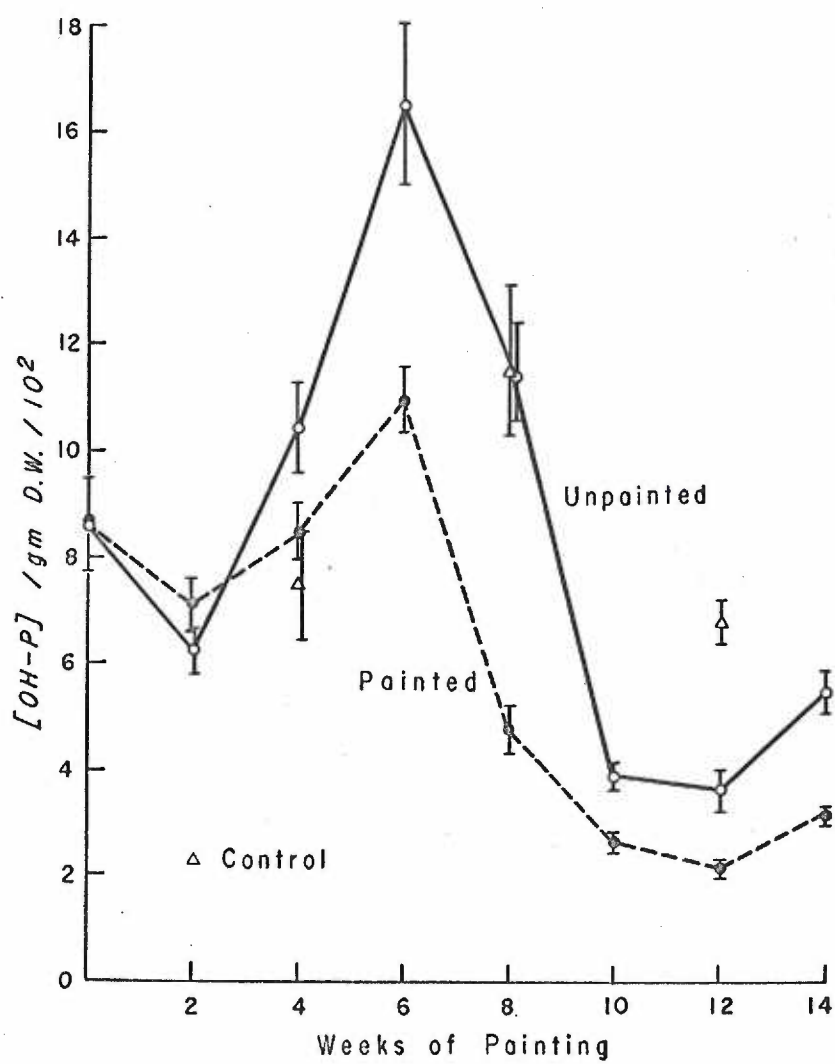
figure XXXVII

FIGURE XXXVIII: This figure shows the collagen concentration as μg hydroxyproline per unit dry weight skin in the 1.0M neutral salt soluble pool. At no time is there a significant difference between P and U.
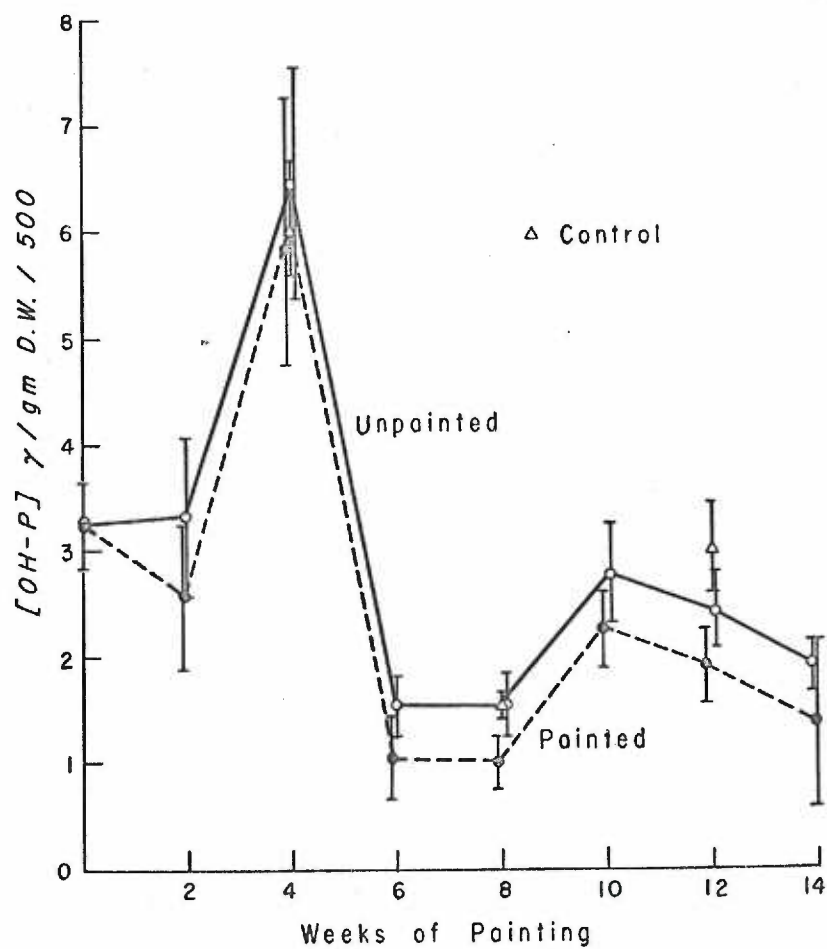
figure XXXVIII

FIGURE XXXIX: This shows the collagen concentration as μg

hydroxyproline per unit dry weight skin in the insoluble pool. As

can be seen, there is no significant difference between P and U

throughout the painting period.

INSOLUBLE COLLAGEN
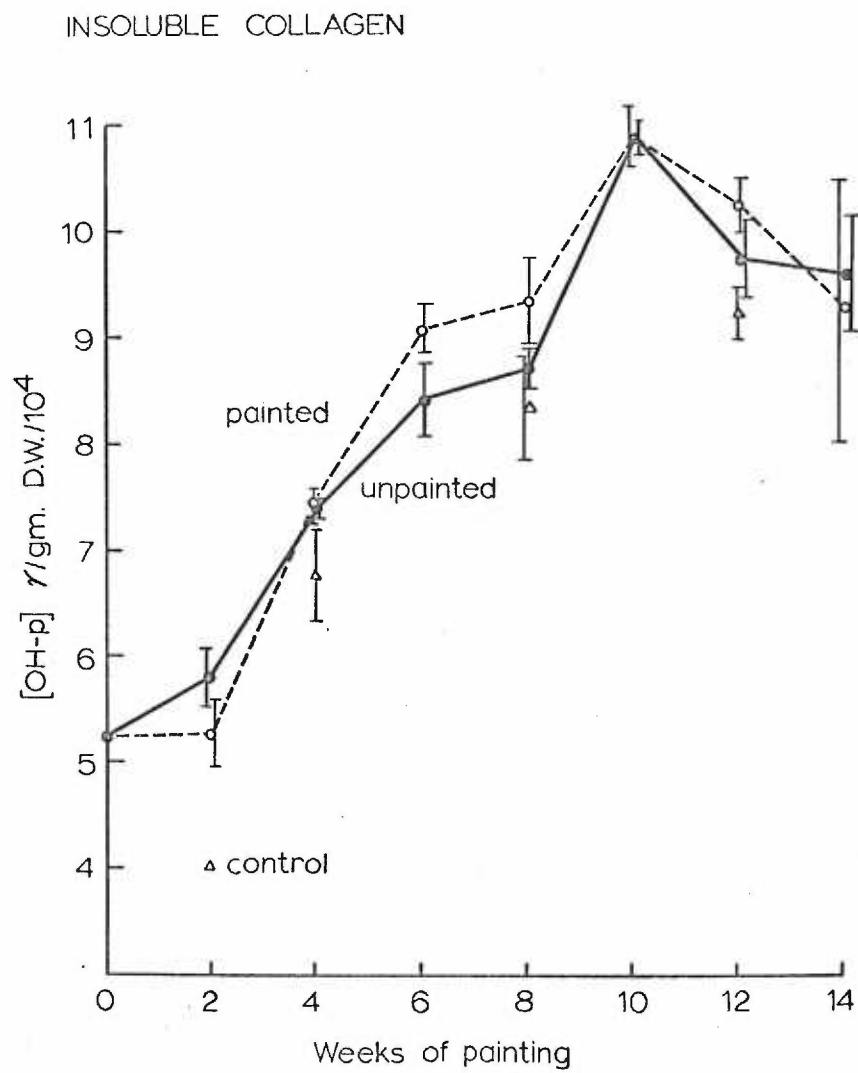
figure XXXIX

FIGURE XXXX: This figure shows the inflammatory reaction of painted versus unpainted skin throughout the course of the experiment. As can be seen there is no difference between the two sites at any point. Each bar represents ten animals. The scatter seen suddenly at fourteen weeks is most likely due to the beginning kidney involvement which varied considerably from one animal to the next at this point. Percentage hydration (i.e., $\frac{\text{Wet weight-dry weight}}{\text{Wet weight}} \times 100$) was used as a measure of the inflammatory response here.
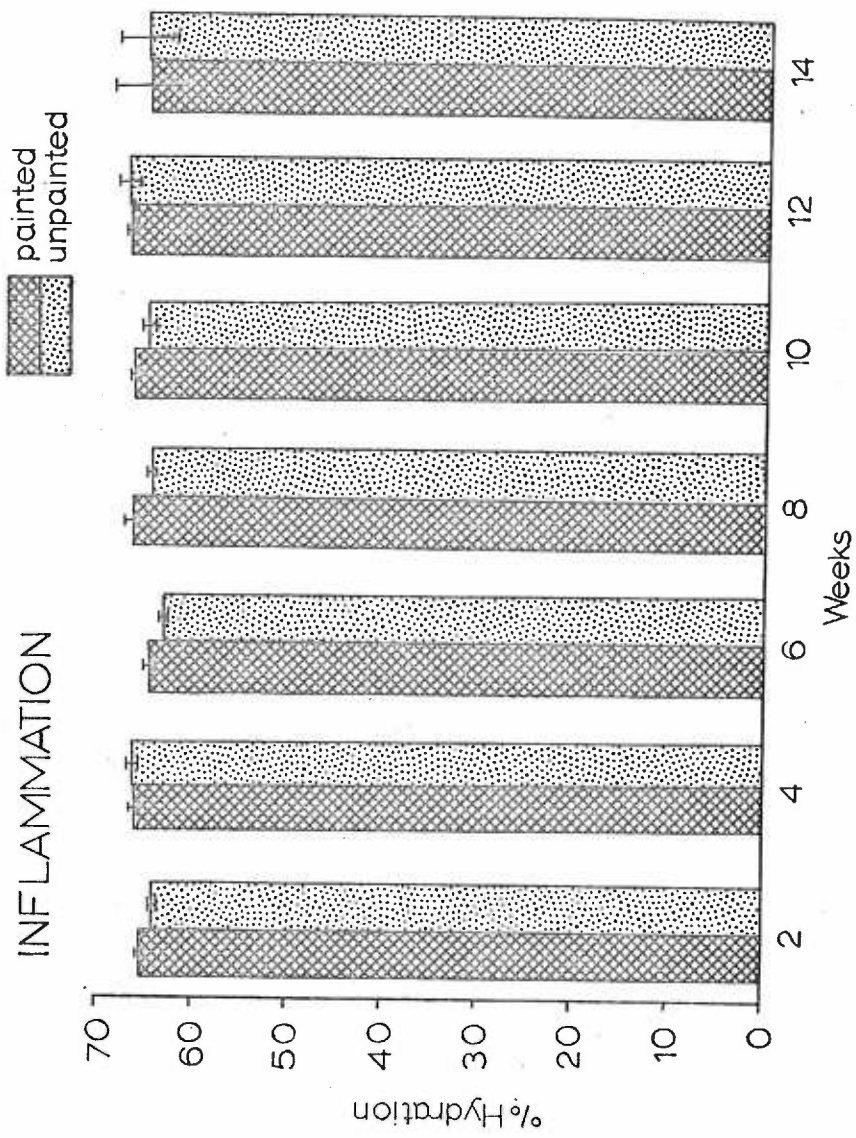
figure XXXX

III. GENERAL DISCUSSION

If stromal changes are related to epidermal carcinogenesis,
their effects must be mediated through an alteration in epithelio-
mesenchymal interaction. The influence of mesenchyme on its
associated epithelium and vice versa have been extensively studied
and several important principles have been proposed. In order to
appreciate these principles and to apply them to the pathogenesis of
cancer, the work in this field will be reviewed. Experiments indicat-
ing a functional dependence between epithelium and mesenchyme will
be discussed first, followed by a consideration of the specificity
involved in epitheliomesenchymal interaction. Specific physical and
diffusable mesenchymal factors influencing epithelial morphogenesis
and their proposed mode of operation will then be reviewed. Next,
an attempt will be made to apply the results of these studies both to
an understanding of those stromal alterations which were observed
during chemical carcinogenesis and to a theory of the manner in
which they might influence or relate to this process. Finally, brief
consideration will be given to possible avenues of future research
which may be pertinent to the resolution of such hypotheses.

A functional dependence exists between epithelium and mesenchyme
in skin as well as all other tissues combining these two elements. For
example, Moscona showed that chick epidermis separated from its

connective tissue by tryptic digestion and cultured alone in vitro

does not undergo mitosis and keratinizes progressively and dies

within a few days (112). Szabo (160) obtained similar results with

embryonic and adult mouse skin. Wessels has demonstrated that

in tissue culture chick epidermis depends on the close proximity of

dermis for normal differentiation and even survival. Other isolated

epithelia show similar dependence on contact with connective tissue.

Grobstein (52b) working with kidney and saliva gland, Auerbach with

thymus (4), Saunders (149b) and Zwilling (178a-c) with limb bud,

Koch with tooth germ (85) and Muthukkaruppan with lens (115) have

established, by various in vitro techniques, the absolute dependence

of epithelium on mesenchyme for growth, orientation, motility and

differentiation.

Although the requirement of an epithelium for mesenchyme is

invariable, the specificity of this requirement varies considerably.

For example, in tissue recombination studies Grobstein (52a) has

observed that mouse salivary gland epithelium combined with any

mesenchyme other than salivary does not undergo normal morpho-

genesis. However, despite this organ specificity, the salivary

mesenchyme can be heterotypic with regard to the species of deriva-

tion. Mouse pancreatic epithelium, on the other hand, behaves

oppositely since in vitro induction of its growth and differentiation is

promoted by all embryonic mesenchyme (47). Most other tissues, including skin, appear to be intermediate in their specificity requirements. McLoughlin (102 a, c) combined trypsin-separated chick epidermis with various mesenchymes and found that all evoked epidermal growth and differentiation. However, epidermis grown on proventriculus initially fails to keratinize and secretes mucus while on gizzard mesenchyme the epidermis secretes mucus or becomes ciliated. On heart myoblasts the epidermis spreads out rapidly to an "extremely attenuated single layer of squamous cells" that do not keratinize. On limb mesenchyme, the epidermis keratinizes normally. Sengel demonstrated the applicability of this phenomenon to normal epidermal development. The results of combining dorsal dermis and tarso-metatarsal epidermis and the converse suggested that the epidermis develops anatomically according to the nature of the dermis, but that its histological differentiation conforms to its origin. Sengel (153) considers the development of the feather germ the result of numerous reciprocal inductions during which the dermis and epidermis alternate in the role of inductive and reactive tissues. This concept has been confirmed by Croisille and LeDouarin (24) who showed that hepatic mesenchyme behaves differently in the presence or absence of epithelium. The essential role of ectoderm in the induction of limb outgrowth has been demonstrated by Saunders (149a).

In the chick limb bud, materials which form the parts of the future limb are laid down in proximo-distal sequence apparently through induction by the apical ectodermal ridge (AER), a ridge of thickened ectoderm capping the apices of limb primordia. If the AER is excised as the bud is elongating, terminal limb defects result. The defects are restricted to successively more distal levels the older the embryo at the time of the operation. Zwilling (178a) has shown that grafting an additional AER to the appendage bud results in the outgrowth of a double limb.

The specific mesenchymal factors which have been shown to influence epithelial morphogenesis are of two types, physical and diffusable. Physical factors will be considered first. The mechanisms proposed for their interaction with epithelia will be reviewed, followed by examples of their influence on epithelial morphogenesis. In the light of these studies, the possible role of the dermal collagen alterations on epidermal carcinogenesis will be analyzed. Physical components of the dermis can effect epidermal morphogenesis through three well-recognized mechanisms. Contact guidance, originally described by Harrison in 1914 (60), remains perhaps the most impressive evidence that the substratum plays an important role in the movements of cells. If cells are grown on glass or plastic fibers or on glass which has been scored with fine parallel grooves, individ-

ual cells assume elongate spindle shapes which conform precisely to the longitudinal axis of the fibers or the grooves (173a). Although various explanations for this phenomenon have been proposed, none has received wide acceptance. Therefore, it seems simplest to say that the cell merely seeks the greatest possible contact between its "ruffled membrane" (leading edge) and the substratum. Migration of pigment cells along blood vessels, movement of nerve axons along blood vessels and the movement of neural crest cells ventrally along the neural tube perhaps represent in vivo examples of this phenomenon.

Another aspect of cellular responsiveness to physical factors is termed contact inhibition, (1a). When a moving fibroblast encounters another fibroblast, movement in that direction stops. However, at a free surface a new ruffled membrane will form and the cell will move in that direction. An exception to this phenomenon is the behavior of the sarcoma cell. Abercrombie and Ambrose (1b) have shown that when a population of sarcoma cells and a population of normal fibroblasts collide in culture, they infiltrate each other. Two populations of fibroblasts on the other hand, exhibit normal contact inhibition with no infiltration. Santesson (147) in a study of benign and malignant epithelial tumors found that when made to collide in vitro, the benign tumors, unlike the malignant ones, did not infiltrate normal fibroblasts .

Cells will not move unless adherent to a substratum. The adhesiveness of the cell surface and the nature of the substratum are both involved. Based on studies in which a mixture of amphibian cells in culture sorted out according to type, Holtfreter (71) proposed the term, selective intercellular adhesion, to describe the sticking-together and later release of cells. The timing of this adhesion would contribute to the cellular pattern of a tissue. Although the mechanism of this adhesion is unknown, electron microscopy has shown an extra-cellular gel at the point of adhesion. This selective adhesion is considered necessary for the maintenance of an integrated epithelial sheet and can account for the stopping of a migrating cell at its proper destination.

There are numerous examples of the influence of interaction with physical substrata on the migration, orientation, mitosis and differentiation of epithelia. For example, Wessels (174b) spread epidermis on a Millipore filter with holes 0.15 mm in diameter. Despite the uniform presence of nutritional factors, the basal layer was oriented and incorporated tritiated thymidine only where it was in contact with the filter but did not incorporate thymidine over the holes. Van Scott (167) maintains that the orientation, differentiation and proliferation of adult skin requires only a suitable attachment surface. Epithelial outgrowths from adult skin explants on a glass or plastic surface

maintained proliferation-maturation gradients after the original explant (containing dermis) was removed. Normal growth, organization and keratinization of versene-separated chick epidermis was shown by Dodson (30b) to be maintained on boiled dermis or even on collagen gel. Further indications of the importance of physical factors in epitheliomesenchymal interaction and of the possible influence which collagen can exert on epithelial morphogenesis have been provided by Kallman and Grobstein (80a). These authors cultured epithelium from mouse salivary rudiments separated from salivary mesenchyme by a filter. An increased organization of epithelial cell ribosomes soon developed which coincided with the appearance of collagen fibrils in association with the basal lamina. When the salivary mesenchyme was labeled with radioactive proline or glycine before culturing, the label accumulated specifically at the basal surface of the epithelium. This localization did not occur if the epithelium was labeled with the same compounds. Collagenase treatment of the epithelium removed the labeled products and destroyed morphogenesis while growth was unaffected. Hyaluronidase had no effect. When cultured with salivary mesenchyme, salivary epithelium undergoes dichotomous branching to form secretory acini and ducts. Collagen fibrils appear along the ducts and in clefts between acini, but morphogenetically active areas are free of fibrils. In this con-

nection, the dermis in old burn scars, in chronically sun-damaged skin or during wound healing, all of which are predisposed to epidermal invasion, possesses degenerated collagen (45). Vernoni (169) has shown epithelial cancer is more easily induced in wounded than in normal skin. The confinement of basal cell epithelioma by a collagen capsule, the geometric surface patterns imposed by collagen in invertebrates (170a), scale patterns imposed by collagen in fishes (173b), and the encapsulation, septation and ensheathment of normal tissues attest to the role of collagen in tissue morphogenesis.

How then could collagen influence the carcinogenic process? Due to its stability, the contribution of the collagen molecule is most likely physical. Collagen alterations during carcinogenesis in skin (93, 58, 26c, 48, 33, 166) and other organs (116, 130, 14) and the findings of collagen alterations in tumors (3, 44, 55, 116, 48) have been reviewed in the GENERAL INTRODUCTION. The present studies have indicated that application of a carcinogen to the skin depresses collagen synthesis, while collagen aggregation and breakdown are unaffected. Histologically, the change in dermal collagen preceding epidermal neoplasia consists of fragmentation and rarefaction with occasional fraying of the fibers. Since epidermal cells are inherently motile as shown in wound healing studies, this motility is most likely restrained under normal circumstance by physical

factors. If a defect develops in the substrate, as, for example, from a depression of new collagen synthesis, the epidermis could initiate a downward migration through the gaps so produced. This migration would be followed by an increased rate of mitosis of adjacent remaining surface epidermal cells, as has been shown in wound healing studies (175). These studies have demonstrated that whatever stimulates epidermis into activity does so by causing cells to move, not primarily by causing them to divide. It may be that the papillary layer controls the epidermis by inhibiting the migratory activity of cells. By analogy, in carcinogen-treated dermis, the gaps developing in the papillary layer could lead to increased mitosis by stimulating migratory activity of epidermis. In the normal epidermis, migration away from the dermal source of nutrition leads to maturation and cell death. Thus, by reverse analogy, a cell infiltrating the dermis might be expected to remain immature (hence motile) and have increased longevity.

Probably the most convincing evidence that diffusable substances are important in tissue development is provided by the numerous in vitro experiments in which an epithelium on one side of a cell-free Millipore filter is induced to undergo morphogenesis when an appropriate mesenchyme is placed on the opposite side. Chemotaxis, the attraction or repulsion of living cells to or from a

chemical stimulus, could be operative in the normal behavior of epidermal cells. Under these circumstances, the direction of cellular movement could be influenced by a concentration gradient of the chemotactic substance. Several clearly established examples of chemotaxis exist in plants, i.e., the attraction of bracken sperm to the archegonium, and the attraction of myxamoebae of the cellular slime molds to an aggregation center. However, many suspected examples of chemotaxis in animal cells are based largely on the end result (i.e., an accumulation of cells at the destination). These examples have not withstood critical analysis, with two exceptions. Twitty and Niu (164) confined several Triturus pigment cells in a capillary tube and found that they moved apart, whereas a single cell remained stationary. By culturing cells under a cover slip in a viscous medium, Harris (59) showed that individual leukocytes and monocytes were attracted to a wide variety of micro-organisms, but lymphocytes were not. If morphogenesis is visualized in terms of chemotaxis, a ready explanation for cellular segregation can be achieved by postulating the production of a chemotactic agent by a cluster of cells and subsequent migration of like cells along the concentration gradient thus produced. Nevertheless, although chemotaxis provides an attractive explanation of tissue interaction in morphogenesis, the evidence remains unconvincing. In animals, a

variety of endogenous compounds have been shown to exert specific

growth-promoting influences on various embryonic and adult target

tissues. Levi, Montalcini and Booker (89c) isolated a "nerve growth

factor" (NGF) from mouse salivary gland. They proposed that NGF

must function during normal development since anti-NGF antibodies

presented to chick embryos in ovo selectively abolished sympathetic

ganglion formation and nearly completely inhibited the development

of the sympathetic nervous system. This protein has also been

isolated from some mouse sarcomas (20d, 89b) and snake venom

(20a). NGF is a normal constitutent of the sympathetic cells and the

blood and body fluids of a variety of vertebrates, including man (89a).

Another protein, Embryonic Growth Factor (EGF), isolated

and characterized by Cohen (20a) causes a precocious opening of

eyelids and eruption of teeth in the mouse or rat fetus, but unlike

NGF, there is no evidence that EGF plays a role in normal ecto-

dermal development.

Rutter, Wessels and Grobstein (144b) have demonstrated a

cell-free material which duplicates the activity of mesenchyme in

stimulating the growth and differentiation of pancreatic epithelium.

Wessels (174b) has shown that this compound furthers columnar

orientation and proliferation of epidermal basal cells but stresses

that an essential prerequisite to this interaction is contact of these

cells with a suitable substratum.

A final growth factor, embryo extract obtained by homogenization of whole chicken embryos, stimulates mitoses in various cell types and may contain some growth hormones.

The influence of acid mucopolysaccharides or acidic glyco-saminoglycans (GAG's) on morphogenetic processes has been extensively investigated, but reports of their effects on normal and neoplastic epithelium have been equivocal. Takeuchi (161a) and Morrison et al (111) found these substances and extracts containing them to be stimulatory to the growth of sarcoma and HeLa cells respectively, while other have provided substantial evidence of their inhibitory effects on various tumor and normal cell types (113, 5a, 60). Perhaps the most extensive studies on the influence of GAG's on cell division have been performed by Lippman, who observed mouse L-cells and Ehrlich Ascites carcinoma cells, both in vitro and in vivo, (90b). Sulfated GAG's were found to inhibit mouse L-cell division and Ascites growth. Protein polysaccharides from bovine nasal cartilage and nucleus pulposus had a stimulatory effect. Polysaccharide-treated L-cells produced large malignant tumors when innoculated into mice, whereas untreated L-cells produced no tumors. Histochemically, these treated cells showed the polymers adsorbed to the cell surfaces. Chemical analysis of wash solutions after incubation with test sub-

stances also indicated that the cells had adsorbed polysaccharides. These data suggest that glycosaminoglycans exert an influence on epithelial cell division. Since cell differentiation occurs only after cell division, the potential effect of acidic GAG's on differentiation is readily appreciated. If the polysaccharide adsorbs to the plasma membrane, the motility of the cell could be influenced as well.

Finally, Hauschka and Konigsberg indicated the indispensible role of collagen in multiplication and differentiation of myogenic cells (61).

Various non-specific diffusable compounds have been demonstrated to exert a critical and specific influence on epithelial behavior. Moscona (112b), for example, demonstrated that variation of CO_2 concentration from the physiologic level of 5% sensitizes chorion to otherwise ineffective stimuli. Murray and Benitez have shown that deuterium oxide can mimic the effects of NGF on nerve out-growth (114), and Barth has shown the varied differentiation of presumptive epidermis in response to various electrolytes (7).

Several factors or conditions are necessary to permit these diffusable substances to exert their influence. First, adequate substrate is essential for a respondent cell to demonstrate its competence (174b). In the absence of proper substrate, regardless of stimulus, the cell will die and at no time will it be capable of

responding. Secondly, for a diffusable substance to exert a directive influence, it must either share an extreme specificity with its target tissue or else it must have restricted mobility. Imagine the problems that would arise in the minute but extremely heterogenous embryo if this were not the case! The problem of mobility has been studied by Lash (88) in the spinal cord-metanephrogenic mesenchyme induction system and by Wessels (174b) in the dermo-epidermal system. The effectiveness of diffusable inducers was limited by diffusion distance (i.e., filter thickness) and by pore size. In this regard, in the studies reported here, an initial locus of cancer in the epidermis has been observed and the area in which the stromal changes are noted is generally well circumscribed and small, (Figures I-XXI).

A third prerequisite for inductive expression of diffusable substances is the matter of timing. Normally, the inducer must be present for some hours before the repsonding component is sufficiently established on its new course to become independent of the inducing component. Beyond this step the response appears to be gradually stabilized (52d). Generally, there is a lag period between the combination of interactive tissues and their response to each other which Grobstein attributes to production, transit or response time. In this regard it is interesting to note that the observed

depression of collagen synthesis was a transitory rather than constant finding.

Operationally, it is difficult to separate hormones, inducers and growth substances. The cell is probably transformed at an early stage from non-differentiated to covertly differentiated and what is commonly regarded as induction is likely a non-specific "unmasking" resulting in overt expression of covert differentiation. Support for this idea is offered by the non-specificity allowed the evocator of a differentiated response as seen, for example, in the similarity of effect of D_2O to NGF (114) in nerve multiplication and differentiation or the differentiation of presumptive epidermis into nerve, pigment or ciliated cells in the presence of $Na+$, $K+$, $Mg++$ or HCO_3^- (7), or the multiplication and differentiation of myo-genic cells into myotubes in the presence of collagen but not in its absence (61). The information content of these substances is dubious. They would rather appear to release or to cue preprogrammed integrated sets of synthetic processes permitting the cells to express potentials determined by previous events (72). From here, chains of inducers might exist whose activities are serially connected; thus, subsequent inductions depend on precedent inductions within a given system. For induction to occur, there must be both inductor tissues and "competent" or respondent tissues. Inductive interaction

appears to be as necessary for the maintenance of tissue equilibrium as it is for its initial establishment.

The question should be asked, what diffusable dermal components change in carcinogen-treated skin and how might they influence epidermal carcinogenesis? While none of the dermal components undergoing change could be considered diffusable in the strict sense of the word, those under consideration here are nevertheless semi-labile, and either produce or are capable of influencing the mobility of diffusable substances. Thus, stromal changes involving the acid mucopolysaccharides, the basement membrane, the dermal vasculature and the cells of the dermis shall be discussed here.

The acid mucopolysaccharide (AMP) alterations appeared first as an increase in Mowry's colloidal iron positivity. Alcian blue positivity of the AMP in the dermis and the connective tissue surrounding epidermal tumors was partially sensitive to hyaluronidase digestion and was fully removed by magnesium chloride concentrations in excess of 0.8M. These findings indicate an increase in dermatan sulfate and chondroitin-4-sulfate and/or chondroitin-6-sulfate and hyaluronic acid. The altered Mowry positivity could also indicate an altered solubility or precipitation of the AMP which in normal skin is washed out by the histochemical procedure. This altered solubility could possibly be due to protein-binding by the AMP, and

if so, would have relevance to the demonstration by Lippman that, by themselves, AMP inhibit epithelial growth while mucoprotein is stimulatory. Furthermore, any changes in dermal AMP also relate to the collagen changes histologically and perhaps also those seen chemically, in that Keech (81) has shown that chondroitin-4-sulfate, chondroitin-6-sulfate and keratosulfate accelerate the formation of fine regular fibrils, while heparin retards collagen deposition and produces thick, irregular fibrils. Dermatan sulfate and hyaluronate had no effect. These polysaccharides are thought to influence nucleation in a collagen solution by virtue of their shape and charge. In this way the AMP could exert its effect indirectly through the collagen (substrate) alteration. The increased amount of chondroitin sulfate in the carcinogen-treated dermis might be responsible for the altered morphology observed with light microscopy. Another possible effect of altered dermal AMP is a disruption of the normal diffusion gradients existing between blood vessels and epidermis. A hampered diffusibility of nutrient could predispose to "epidermal groping" (i.e., chemotaxis), for nutrient as manifested by its down-growth and infiltration, or alternatively a more favorable diffusion rate produced by the AMP alteration could result in an enhanced response to serum growth factors (89a). It is also possible that by-products of AMP degradation merely serve to evoke a covertly dif-

ferentiated potential in the epidermal cell. That such a growth potential exists normally in the epidermis has been shown by Gillman (45), who described epidermal growth in wounded skin resembling cancer; the obvious difference here is control. Finally, the importance of AMP in epidermal development has been stressed by Sengel (153), who has shown that during feather formation, AMP becomes concentrated in the mesenchymal condensations (which evoke feather formation) and finally localizes in the base of the dermal papilla; the latter structure has maximal inducing and mitogenic activity. Thus, altered stromal AMP could lead to aberrant epidermal behavior in various ways.

The basement membrane, while neither diffusable nor stromal, is of particular significance in a discussion of diffusable substances in epitheliomesenchymal interaction because of its interface position. The observed basement membrane alterations consisted of clumping, thickening and fragmentation, localized to the area of maximal stromal change. Since basement membrane generally appears wherever epithelium and mesenchyme come into contact, changes in this structure could result in or from an altered epitheliomesenchymal interaction.

If one considers that the basement membrane is produced by the epithelium (125) or by a conjoint effort of epithelium and mesen-

chyme (104), it is easy to regard alterations in this structure as resulting from defective epithelio-mesenchymal interation. Two studies indicate that the basement membrane is indeed derived from the epithelium. In proline tracer radioautography studies, Hay (63) demonstrated a proteinaceous component of the basement membrane excreted by epithelial cells. Kallman and Grobstein (80b) have shown that the epithelium contributes a polysaccharide component to this structure.

If one considers the basement membrane as a "gateway" for the passage of dermal inducers, then it is logical to imagine that alterations in that structure (i.e., thickening, fragmentation) would result in defective epitheliomesenchymal interaction. Several findings support this idea. During embryogenesis, induction appears to require a thin basement membrane, as in the first sites of epitheliomesenchymal interaction in chick embryos (83) or sites of limb induction in amphibian larvae (6, 82). At metamorphosis, in the latter, the larval epidermis changes to the glandular adult type only when the normally thick basement membrane has been attenuated with the consequent accumulation of dermal fibroblasts which are then able to establish a closer relation with the epidermis (82). Thus, the first thickening of the basement membrane may signal the end of an epitheliomesenchymal interaction. If the basement membrane is

considered to be semi-permeable, then gaps in the basement membrane could allow passage of either abnormal products of dermis or normal trophic substances too large to filter through the intact basement membrane. It could also allow direct contact with carcinogen-transformed fibroblasts.

Dissolution of the basement membrane has been reported by Balinsky (6) to lead to epidermal-dermal interactions essential to formation of a supernumerary limb in amphibian embryos. Thus, it is likely that disruption of the basement membrane allows enhanced contact of epidermis with its abnormal dermis. However, neoplasia resulting from breaks in the basement membrane is unlikely, since small breaks are commonly observed in benign conditions such as psoriasis (126).

Finally, direct action of carcinogen on the basement membrane could lead to alteration of the epithelial cell surface which is in direct contact with intercellular cement substance (i.e., sialomuco-proteins) confluent with epithelial basement membrane. It is possible that the loss of contact inhibition in malignant tumor cells which are often associated with a disrupted basement membrane (162, 29b) derives from this source. In this regard, Dodson (30c) showed that chick epidermal orientation and differentiation on collagen occurred only after establishment of a PAS-positive basal lamina.

Although, as mentioned, the basement membrane is in a strategic location to control permeability and hence other activities of epithelial cells, it poses no barrier to cell movement, since, as shown by French, Florey and Morris (39), entire cells may traverse it.

The vascular changes seen in this study during carcinogenesis consist of an increased tortuosity and mild engorgement of dermal capillaries. The possible epidermal influence exerted by an alteration of the dermal vasculature has been suggested by Ryan (145). He envisioned a constant downward growth of epithelium which seeks out its source of nutrition, the dermal capillary, and orients itself accordingly. Several findings indicate that an altered blood supply might influence carcinogenesis. Goldberg (46) studying the effects of carcinogenic coal tars, noted that the initial applications produced a sharply circumscribed dilatation of the dermal capillaries and venules. Continued painting led to continued dilatation, until it finally became irreversible. Eventually, permanent dilatation led to the development of many angiectases. Carcinomata arose at the sites of maximum concentration of ectasias. Non-carcinogenic irritants produced no ectasias. Orr (119b), studying phenol red vital staining during chemical carcinogenesis, suggested that the development of functional ischemia preceded tumor formation. On

the basis of these and other studies, Warburg, Posener and Negelein (171) and Urbach (165a) regard the cancer cell as a select strain, differing from normal cells in its ability to carry out anaerobic glycolysis. Urbach further considers the carcinogen to impart to normal cells this altered metabolic capability via a genetic alteration, and this alteration gives those cells a biological advantage in an environment favoring anaerobic rather than aerobic metabolism. However, if this is a heritable alteration, it is difficult to imagine the selective growth advantage that it could offer a cancer cell in a distant but normal metastatic site.

Moscona (112b) has shown that deviation of the CO_2 concentration of ambient air from the physiological level of 5% sensitizes chorion to otherwise ineffective stimuli. For example, more than 5% permits it to secrete mucus in response to excess Vitamin A; less than 5% allows it to keratinize in response to high O_2 levels; but as long as 5% CO_2 is present, chorionic epithelium is unaffected by these stimuli. Thus, alteration of normal dermal vasculature could produce, merely by fluctuations in CO_2 levels, significant epidermal modulation, a consequence of which could be hyperplasia.

An altered dermal circulation might also affect the perivascular fibroblasts which, by virtue of inadequate nutrition, might alter their collagen or AMP synthesis and thereby stimulate epidermal

infiltration or growth.

Finally, the observed increase in dermal cellularity could influence carcinogenesis by means of diffusable substances. The cellular response consisted of a mild mastocytosis and fibrocytosis. This increased cellularity could arise as a consequence of an inflammatory response to the carcinogen. However, two factors argue against this: 1) The hypercellularity occurred with 2-amino anthracene, a non-irritating carcinogen and, 2) The hypercellularity was confined to localized areas. The mastocytosis could, by increased heparin production, elicit collagen changes (81). This could secondarily lead to an epidermal response via the substrate effect already discussed. Increased fibroblastosis could represent an inductive interaction between stroma and the localized area of pre-neoplastic epidermis, or it could represent the formation of an altered clone of fibroblasts which could, in turn, evoke an alteration in epidermal behavior. The possible inductive or evocative function of fibroblasts in epidermal hyperplasia or neoplasis has a precedent in amphibian limb morphogenesis (6), (82), and chick feather germ formation (83), where, as mentioned, morphogenesis is immediately preceded by basement membrane attenuation and the local accumulation of dermal fibroblasts.

The possibility that an altered clone of fibroblasts predisposes to epidermal neoplasia is also possible since transfilter induction

of hyperplasia in normal epithelium by cancer cells has been demonstrated. A recent report of epithelial carcinogenesis induced by the subepithelial implantation of embryonal cells would add further credence to this possibility (11). McLoughlin, with reference to her epithelio-mesenchymal-recombination studies concludes that "it is probable that fibroblasts are responsible for the characteristic effects of each type of mesenchyme on the epidermis, since in most combinations the fibroblasts were specifically attracted by the epidermis which usually becomes completely surrounded by them."

A critical analysis of the contribution of the altered stromal components to the neoplastic process must address itself to the question of whether or not these stromal alterations are actually related to carcinogenesis, directly or indirectly. Although all stromal alterations could be expected to influence epidermal behavior, none has been shown to impart a permanent and heritable neoplastic quality to normal epithelium. Epithelial cells have a covert potentiality for invasive growth which, as Gillman has shown, becomes overt during wound healing. Thus, simple modulation can produce a phenotypic "cancer cell." Since modulation can be triggered by a number of non-specific stimuli, it is quite possible that abnormal products of carcinogen-altered fibroblasts could provide such a non-specific stimulus. Neoplastic alteration of the epithelium,

however, is not a simple phenotypic modulation but rather a permutation in genotype. This permutation necessarily involves a heritable alteration, as the cancer cell is known to metastasize, survive, and retain its neoplastic qualities in the absence of the initiating stimulus. The connective tissue alteration could participate in this neoplastic permutation by "predisposing" the epithelial cell to a malignant somatic mutation. This seems unlikely due to the multiplicity of neoplastic foci in the epidermis, all of which behave in a very uniform manner. The other alternative is that proposed by Pitot and Heidelberger (129) which essentially involves a permanent metabolic alteration without somatic mutation. This theory suggests how a perpetuated change could result from a transient interaction of a carcinogen, (which in this case could be an altered connective tissue factor), and a cytoplasmic protein. It is proposed that the carcinogen combines with and thereby inactivates the repressor of an enzyme-producing operon allowing production of the enzyme. The enzyme then catalyzes a reaction, the end products of which activate a second repressor regulating production of the original repressor. This results in complete derepression of enzyme production. The alteration is heritable in that daughter cells will contain the end product for this derepression. The process is reversible by removal or inactivation of such an end product. The model can explain how

cancer cells can revert to normal provided that no secondary genetic change has taken place. It is consistent with the demonstration (62) that hydrocarbon-induced tumors have a normal chromosomal karyotype. Several investigators using different chemical classes of carcinogens in different organs in different species have demonstrated that (1) in vivo carcinogens are covalently bound to soluble proteins of the target tissue in a fashion quantitatively related to their carcinogenic activities; (2) the proteins to which the carcinogens are bound exhibit similar electrophoretic behavior, and (3) these proteins are absent from the tumors that are induced. That this protein most likely does not derive from the dermis, however, has been shown by Heidelberger (64) who, using tritiated carcinogenic hydrocarbons in autoradiography studies in mouse skin, showed that almost all protein to which hydrocarbons are specifically bound is located in epithelial cells. This would argue more for a direct effect of carcinogen on the epithelial cell, rather than an indirect influence via an altered dermal constituent.

What then, is the role, if any, of the dermal changes in the carcinogenic process? It is difficult to postulate a direct effect of the stromal change on neoplasia, although an indirect effect is possible. The stromal changes, in a manner similar to, but independent of, the epidermal changes, could be a manifestation of an influence

of the carcinogen on the fibroblast. In the Pitot-Heidelberger model system, the carcinogen then could be regarded as inactivating the normal repressor for production of an enzyme used in the production of hyaluronate or chondroitin sulfate, or could serve to activate the repressor produced by the collagen regulator gene. This would fit with the reduction of collagen synthesis seen in carcinogen-treated skin. Another possibility, and perhaps one more consistent with the carcinogen binding by epidermal rather than dermal protein, is that the carcinogen inhibits the production of previously-repressed epidermal substances which in turn produce directly or indirectly the various observed stromal alterations. In this connection, the faulty production of basement membrane by epidermal cells would be indicative of a defect in the normal epidermal cell machinery. The observed connective tissue lysis by malignant epithelium (162) would add additional support to such an idea.

Although this is, of course, speculation, it does pose questions which are amenable to experimental investigation. For example, the influence of carcinogen-exposed fibroblasts on unexposed epithelium or conversely, the influence of carcinogen-exposed epithelial cells on unexposed mesenchyme could be studied to determine (1) whether the epithelial and stromal alterations are related or unrelated, and (2) whether pre-cancerous epithelium can induce the connective

tissue changes, or (3) whether the carcinogen-altered fibroblast induces the epithelial carcinogenic response. Epitheliomesenchymal interaction in young versus old animals must also be investigated since it has recently been shown that connective tissues different in AMP ratios and in aggregative states of collagen yield different types of tumors in response to the same carcinogen. This finding alone precludes total exclusion of the stroma as an active participant in epithelial carcinogenesis. Whether these clear-cut stromal differences arise independently or are caused by the aging epidermis must be studied as well.

IV.   SUMMARY

Dermal changes during cutaneous carcinogenesis were studied

histologically and histochemically in rats using the carcinogen, 2-

anthramine (2AA), in rabbits using 9,10-dimethylbenzanthracene

(DMBA) and in rhesus monkeys using 2-AA, DMBA and methyl-

cholanthrene (MC), alone and in combination with a promoter, dode-

cylbenzene. Regardless of the carcinogen or animal species the

dermal response was predictably consistent. It involved alterations

in all dermal components. The collagen alteration included a rare-

faction, fragmentation and fraying of the fibers limited to numerous

loci of change within the superficial dermis. This gradually spread

to involve the entire dermis. Normal Luxol fast blue affinity was

lost in the areas undergoing morphological change. The mucopoly-

saccharide alteration accompanied the collagen changes both in time

of onset and in location. The alteration consisted of an increased

Mowry's colloidal iron positivity which was initially diffuse, but soon

became concentrated in the areas of initial epidermal infiltration. This

was considered to be due to either an increased quantity or decreased

solubility of the dermal mucopolysaccharide. However, the possibility

that this substance represented an epithelial mucin was not ruled out,

as it was often seen concentrated in the intercellular cement substance

of the epidermis. Staining by Alcian blue in varying concentrations of

magnesium chloride showed abolition of staining in peritumoral

dermis between 0.4 and 0.8M $MgCl_2$. By comparison with model

tissues containing predominantly or exclusively single mucopoly-

saccharide species, this was interpreted as an indication of the pre-

sence of dermatan sulfate (CS-B), chondroitin-4-sulfate (CS-A),

chondroitin-6-sulfate (CS-C) and/or hyaluronic acid (HA). After

extraction with 0.4M $MgCl_2$, treatment of the sections with hyaluron-

idase to which CS-A, CS-C and HA are sensitive, but to which CS-B

is resistant, resulted in a loss of roughly 2/3 of the alcianophilia.

This indicates that while the polysaccharide present in the carcinogen-

treated dermis consists partially of dermatan sulfate, that chondroitin

sulfate or hyaluronate or both are also present. A change in dermal

elastin, as seen with silver orcein-aniline blue staining, consisted of

a markedly increased orceinophilia in the immediately sub-epithelial

layers of the dermis. This was regarded as an increase of elastin

in this area. The dermal vasculature became engorged with increased

tortuosity, a common feature. An increased dermal cellularity

consisted of a mild mastocytosis and fibrocytosis. Finally, the

basement membrane underwent early and marked change consisting

of thickening and fragmentation. Two points are noteworthy: (1)

These changes were always confined to the exact same locus, and

(2) though these changes were always associated with neoplastic infil-

tration by the epidermis, their initial appearance preceded any evident epidermal change.

Since the collagen alteration appeared to be possibly a defect in the normal metabolism of this protein, a biochemical study of its synthesis, extracellular aggregation and breakdown was undertaken. Using radioactive proline as a tracer in the rabbit-DMBA system, an apparent decreased concentration of all fractions of collagen from the painted site was shown to be due most likely to a dermal inflammation. An increased incorporation of radioactivity by the collagen in the painted site was shown to arise most likely from either the irritancy of the carcinogen, since irritants are known to stimulate collagen synthesis, or from the increased number of fibroblasts present in carcinogen-treated skin. However, certain inherent weaknesses in the experiment itself precluded any definite conclusions regarding collagen metabolism. Thus, a similar study of collagen metabolism using large numbers of metabolically-stable rats was undertaken using the non-irritating carcinogen, 2-anthramine. The metabolism was studied at biweekly intervals throughout the first fourteen weeks of painting, which, in this model, is considered the induction period. At the end of this time the experiment was discontinued due to the toxicity of the carcinogen. This study, prior to the onset of the toxic response, showed a depression of collagen synthesis localized to the

painted area. Collagen aggregation and collagen breakdown appeared to be unaffected. No inflammatory reaction occurred in the painted site. The onset of the biochemical change coincided with the onset of the histological and histochemical changes (at about six weeks of painting).

The relationship of the dermal alterations to epidermal neoplasia was discussed from the standpoint of their etiological significance in this process. A unifying hypothesis was presented and discussed in the light of the observed stromal changes. Finally, possible future research in this area was outlined.

APPENDIX I. Separation of Proline and Hydroxyproline by Cation

Exchange Chromatography (110)

Preparation of Nitrous Acid:

Slowly pipette cold sodium nitrite solution into cold concentrated

hydrochloric acid, in an ice bath. Use 3 mls. of 40% sodium nitrite

for each 10 mls. of hydrochloric acid. Cover, cool and allow salt to

settle. Pipette off the center layer of the supernatant. Use this in

the nitrous acid reaction. Make fresh daily.

Nitrous Acid Treatment of Hydrolysates:

Hydrolyze the protein and evaporate to dryness with a stream of

nitrogen. Add excess nitrous acid reagent (1 ml./mg. protein) to

the hydrolysate residue and let stand at room temperature for 10

minutes; then heat at $140°$ C until the color is only faintly yellow--

DO NOT cap tightly. Evaporate off the hydrochloric acid-nitrous

acid mixture with a stream of nitrogen. By this treatment most of

the imino acids are unchanged (a little is nitroso-derivative) whereas

amino acids become α-hydroxy acids.

Preparation for Chromatography:

The residue from the nitrous acid treatment should be dried

under vacuum over P_2O_5 for a period of not less than two days. The

sample is then extracted with 5 ml. of anhydrous ether (containing

sodium chips to keep it dry) and the ether aspirated off and discarded;

the α-hydroxy acids being soluble in ether are removed in this way whereas the imino acids and their N-nitroso derivatives are left behind. After ether extraction, take up the residue in 0.5-1.0 ml. of 70% ETOH to desalt, evaporate and take up in 1 ml. of 0.1 N HCl.

Chromatography on Cation Exchange Columns:

Prepare a surry of Bio Rad AG 50W-X4 200-400 mesh H+ analytical grade cation exchange resin. This resin is reconstituted by washing in three changes of an equal volume of 6 N HCl, (being allowed to sit in the second change overnight), after which it is rinsed with distilled water to a neutral pH. The columns (50 cm. long x 1 cm. diameter with 50 ml. reservoir at the top) are plugged at the bottom with a lightly-packed 80 mg. piece of glass wool, and packed with 30 ml. of the slurry. When not in use, the resin should be kept under distilled water, and once reconstituted it should never be allowed to dry out.

The sample is applied with a dispo-pipette evenly over the surface of the resin which is kept flat with a Whatman #2 filter paper disc. When the sample has soaked into the resin (but before the surface of the resin can dry) add 2 ml. distilled water and follow this with a 30 ml. water wash. At the end of the water wash elute with 120 ml. of 1.5 N HCl. The fractions collected should be approximately as follows:

ML: 1-20-----bed volume of column--water
        20-50-----water wash
        50-70-----1.5 N HCl comes off--<u>no</u> imino acids
        70-100----all hypro
        100-110----<u>no</u> imino acids
        110-150----all proline

It is important that the surface of the resin be kept flat and that the columns be kept vertical.

The column is run approximately 45 minutes under an air pressure of 240 mm. Hg. The air pressure is applied to a series of columns by means of a distributor and is regulated through a manometer as shown in Figure XXXXI. The acid is evaporated from the fractions and the residue diluted to an appropriate volume for colorimetry. Proline and hydroxyproline concentrations are determined on an aliquot. The remainder of the fraction is dried and dissolved in hyamine hydroxide, scintillation fluor added and radioactivity determined by liquid scintillation spectroscopy. With regard to the colorimetry, determinations based on the nitroso derivatives give the same results as the imino acids themselves.

Typical separations are shown in Figures XXXXII through XXXXV. Regardless of whether the imino acids were purified compounds (Figure XXXXV) or obtained from hydrolysates of skin collagen (Figures XXXXII-XXXXIV), the technique behaved similarly. No attempt was made to obtain the hydroxyproline contained in the 10 ml.

fraction separating the two peaks, since quantitative recovery of hydroxyproline was unnecessary. When measured, recoveries of hydroxyproline were generally around 80%.

FIGURE XXXXI: This shows the apparatus used for the rapid elution

of Dowex cation exchange columns. The mercury differential in the

manometer (a.) was held constant at 240 mm. with line air regulated

by means of a screw valve (b.). This air pressure was distributed

to each of thirteen columns via a glass distributor (c.) as shown.

The reservoir (d.) on the column holds approximately 100 ml. of

eluent although generally not more than 60 ml. is applied at one

time. The interface between eluent and resin is kept flat with a

small circle of Watman No. 1 filter paper cut specifically for this

purpose. Elution time is approximately 45 minutes. The Dowex is

reconstituted overnight in 6N HCl.

c.

d.

e.

b.

air

a.
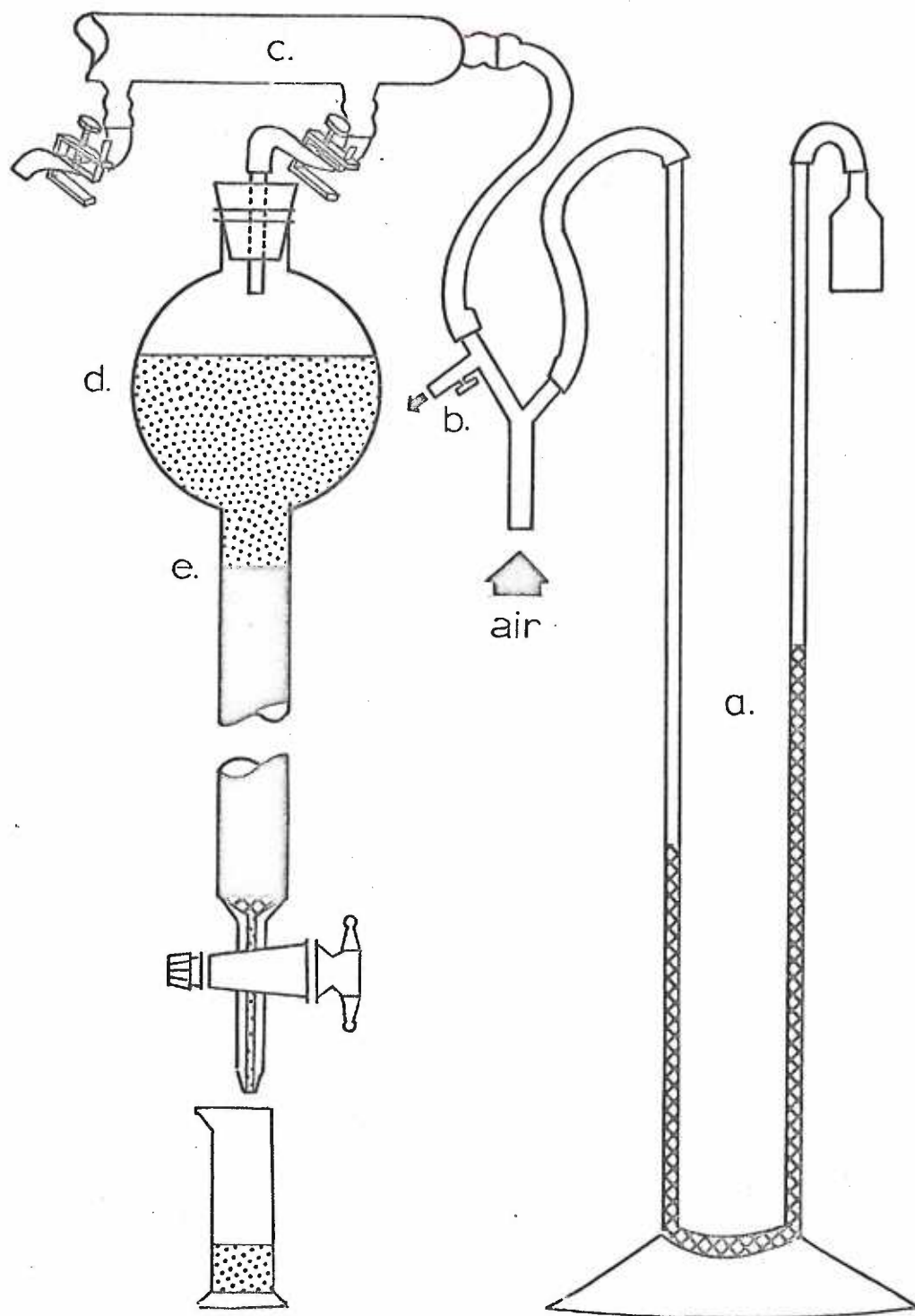
figure XXXXI

FIGURE XXXXII: This shows the separation of 600γ purified hydroxy-proline from 600γ purified proline on a Dowex cation exchange column eluted with 120 ml. 1.5 N HCl under an air pressure of 240 mm. of mercury as described in the text.

STANDARD 600 γ HYPRO & 600 γ PRO

figure XXXXII

184
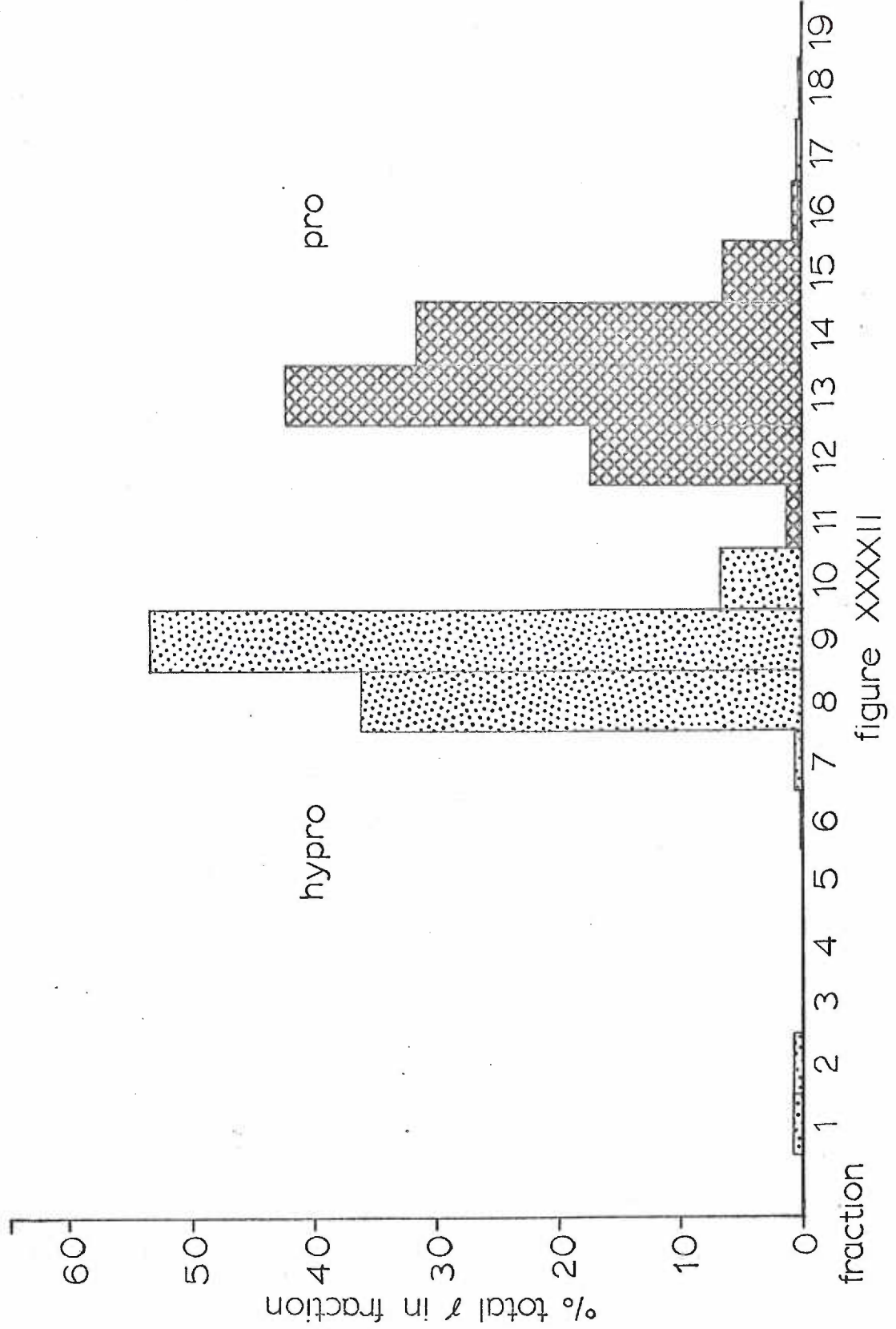
FIGURE XXXXIII: This shows the separation of proline and hydroxy-proline from rat skin collagen extracted with 0.14M buffered salt solution. Elution from Dowex columns was performed under pressure using 120 ml. of 1.5N HCl as described.

0.14M EXTRACT (648 ɣ) HYPRO
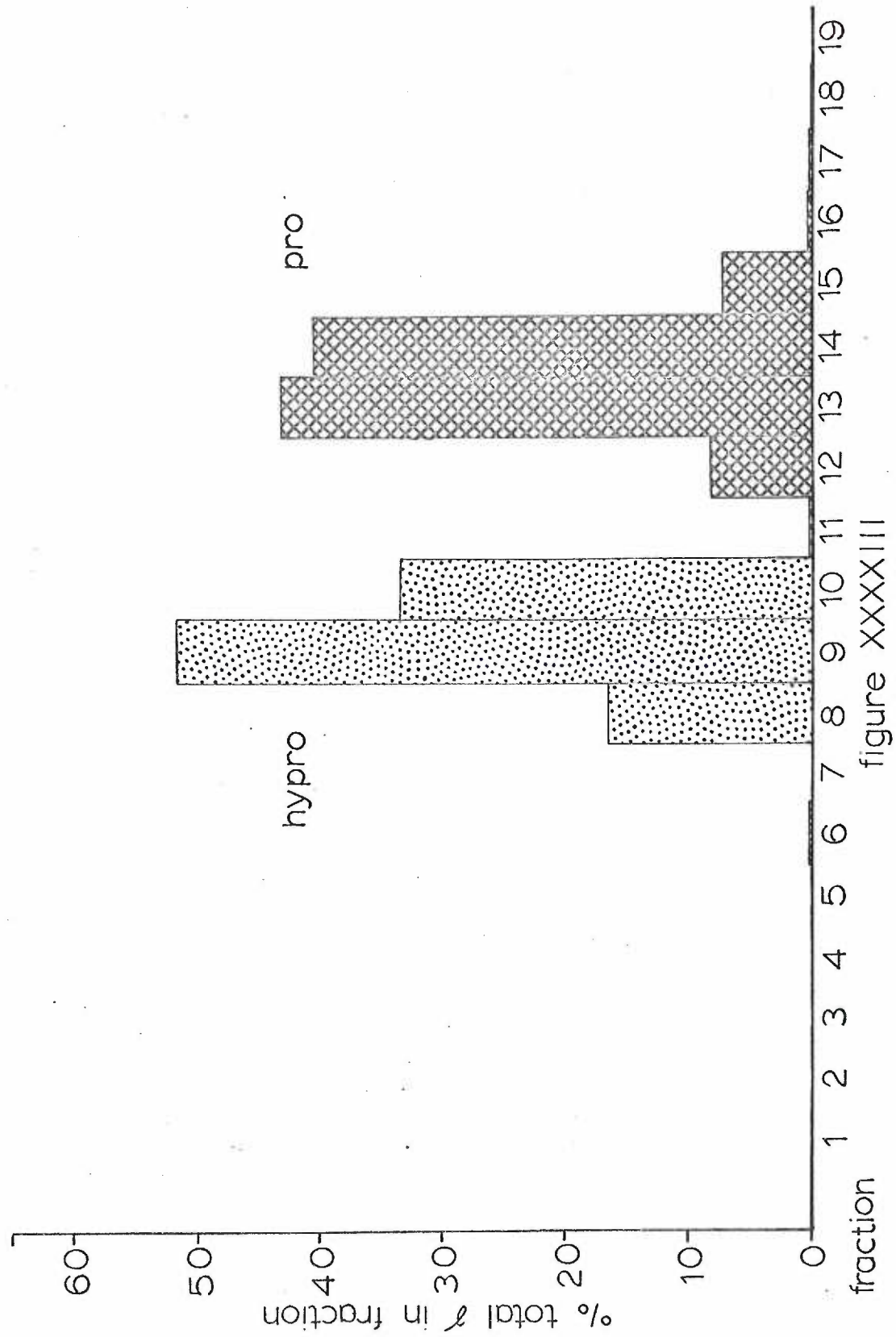
figure XXXXIII

FIGURE XXXXIV: This shows the separation of proline and hydroxy-proline from rat skin collagen extracted with 1.0M neutral salt solutions. The separation was performed as described in the text.

1.0M EXTRACT (531−570ℓ) HYPRO
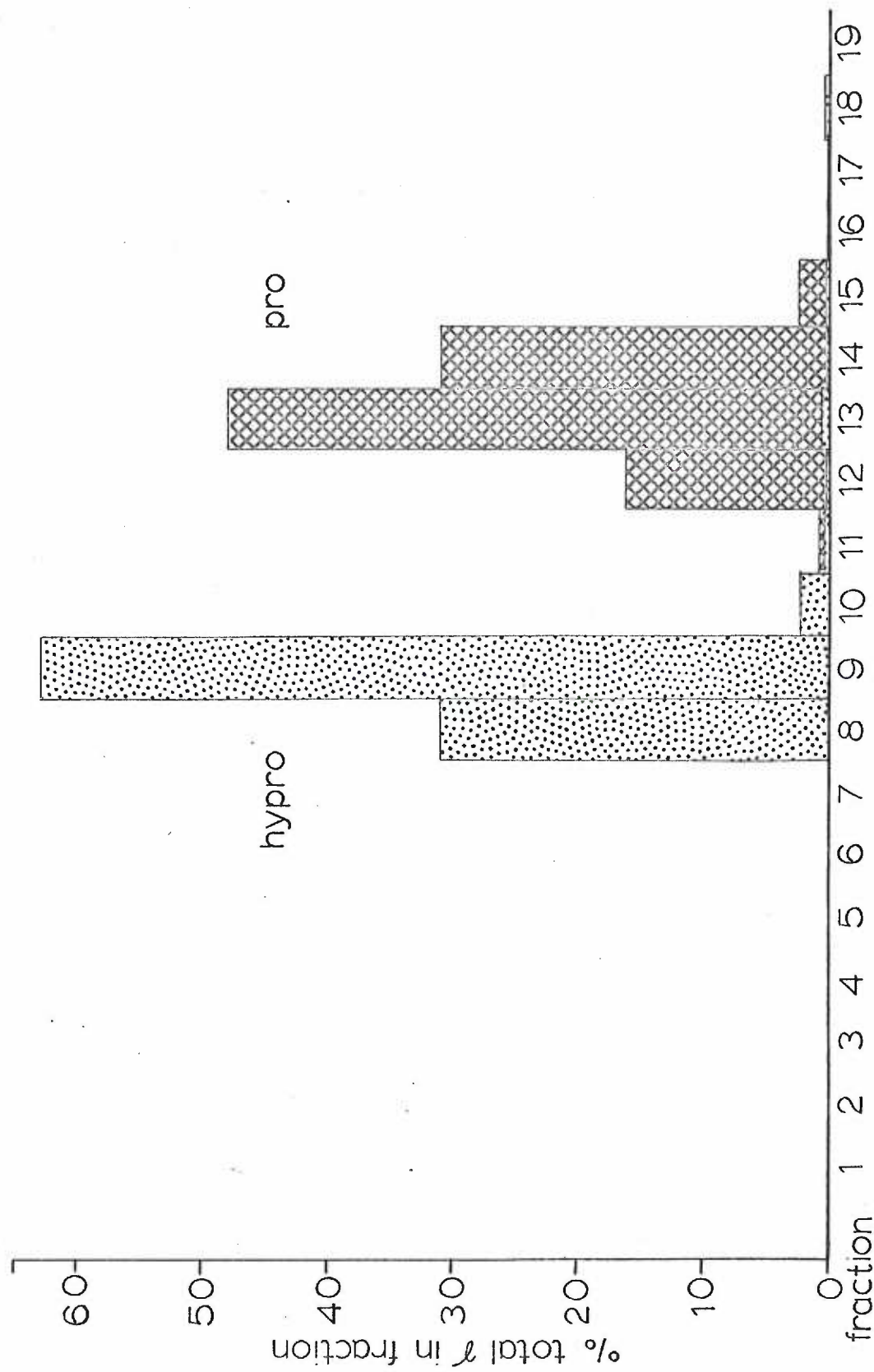
figure XXXXIV

FIGURE XXXXV: This shows the separation of proline and hydroxy-

proline from the insoluble fraction of rat skin collagen. The

separation was carried out as described in the text.

RESIDUE (530 - 600γ) HYPRO

figure XXXXV

190
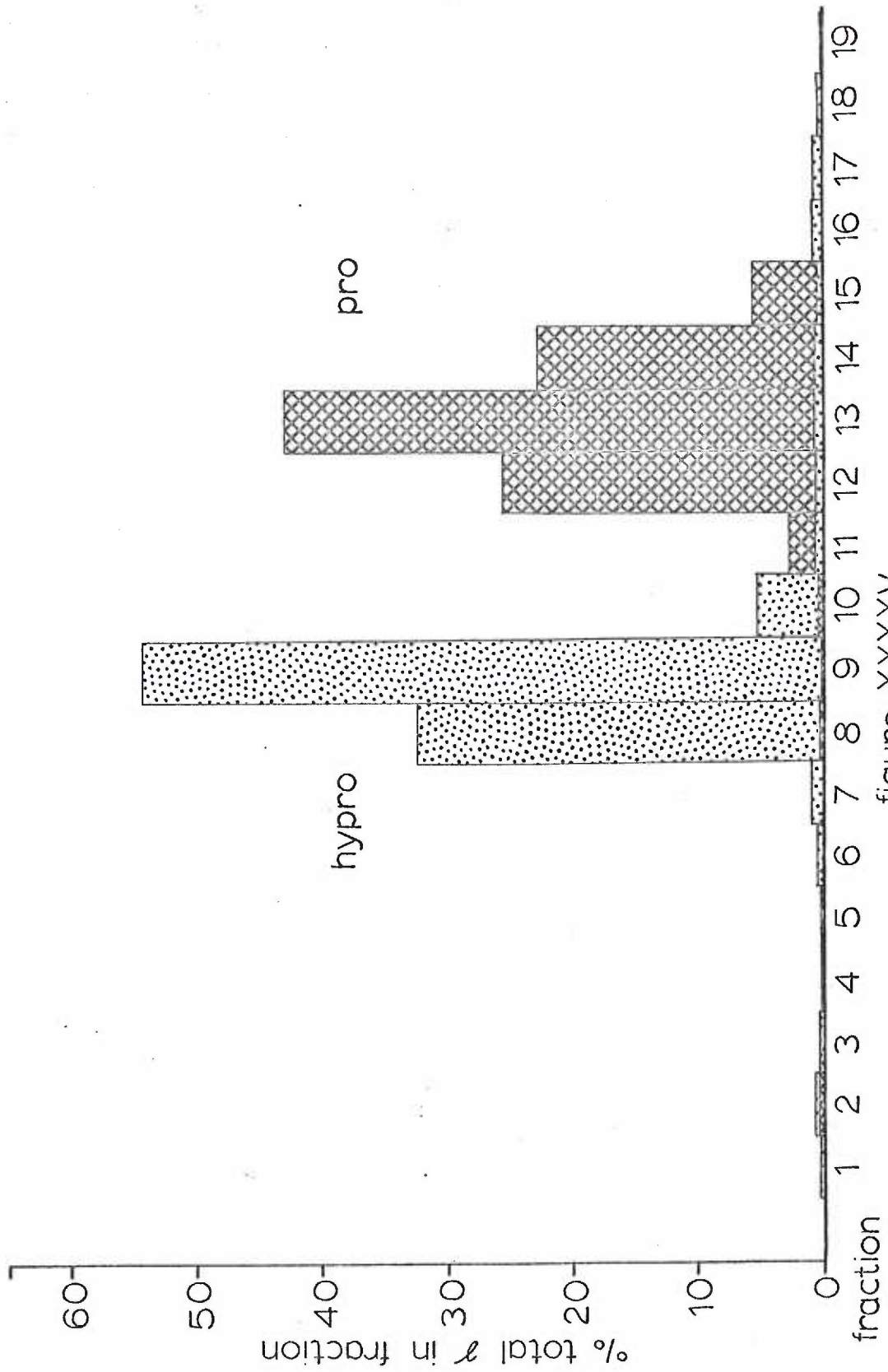
FIGURE XXXXVI: This shows the separation of 400γ hydroxyproline from 200γ of proline plus 3×10^6 DPM of tritiated proline (specific activity 5 c/mM) on a Dowex column eluted under pressure with 70 ml. of 1.5N HCl followed by 50 ml. of 4.0N HCl. Each fraction contains 10 ml. Fraction 10, which contains the majority of the hydroxyproline, contains 0.9% of the proline label while fraction 9 contains only 0.4% of the radioactivity. Thus, the hydroxyproline collected could be expected to be contaminated by 1.3% of the total proline. Fraction 11 which is not collected contains no measurable proline or hydroxyproline but 2.16% of the label.

STANDARD 400 γ HYPRO, 200 γ PRO, 3 X 10^6 DPM ^{3}HYPRO
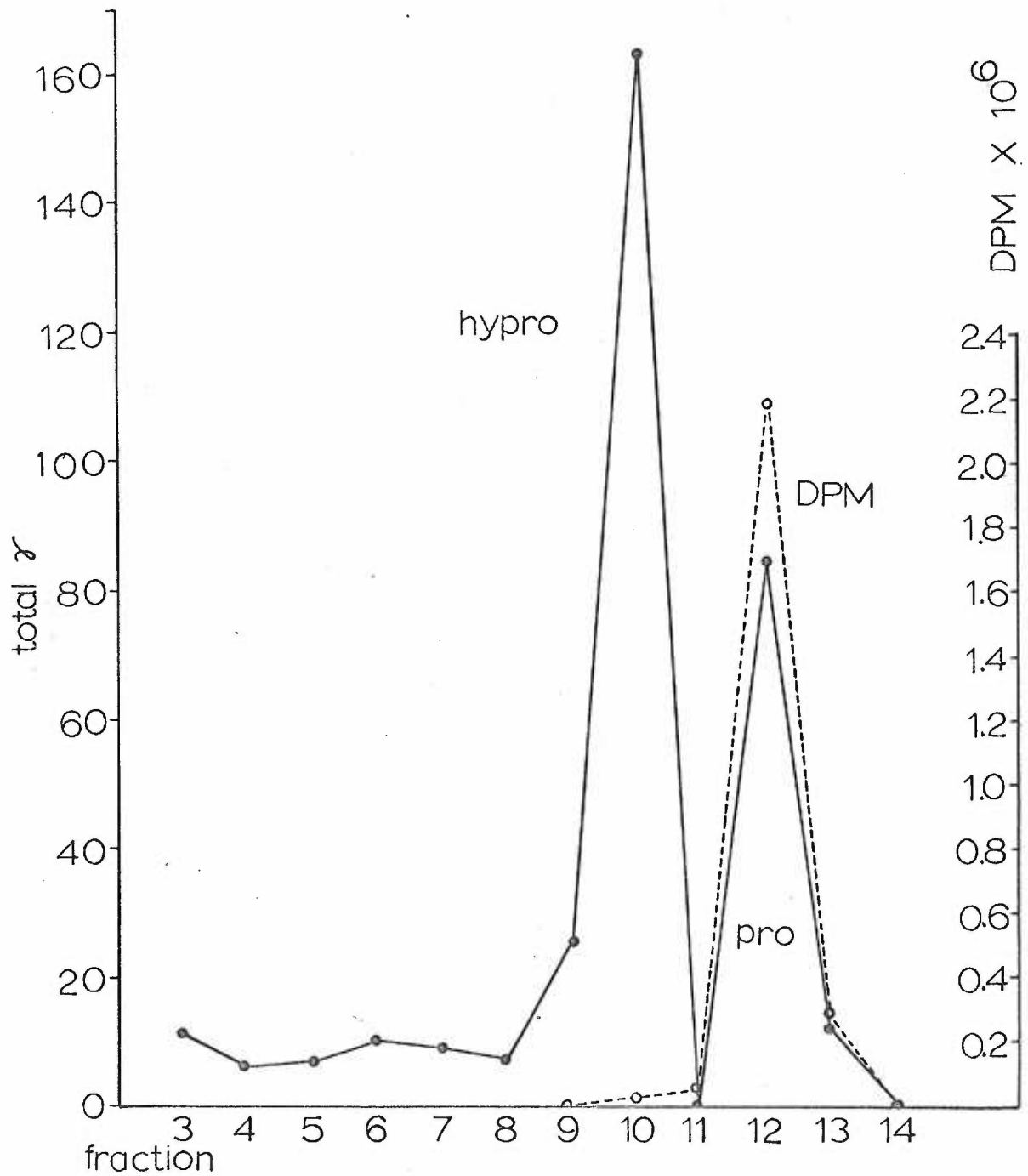
figure XXXXVI

APPENDIX II.   Technique for the <u>In Situ</u> Identification of Acid

Mucopolysaccharides.

Basing a histochemical technique on a well-known biochemical

technique, Scott et al (152b) have derived a simple method for the

in-tissue identification of specific acid mucopolysaccharides.   These

substances are polyanions with carboxyl and sulfate radicals pro-

viding the chief reactive sites.   In practice, the technique involves

an observation of the $MgCl_2$ concentration at which staining of AMP

by Alcian blue in a given tissue is prevented.   This salt concentration

will be determined by the specific AMP contained in that tissue

since the precipitability of the individual mucopolysaccharide species

(polyanions) with Alcian blue (organic cation) varies according to

their molecular configuration, charge density, etc.

The reaction between mucopolysaccharides and Alcian blue

conforms to mass action equilibria.   Scott et al (152b) have analyzed

the reaction in the following manner.   In abstract,

Given the reaction: $P^{Z-}ZM^+ + ZR^+ \rightleftharpoons P^{Z-}ZR^+ + ZM^+$

Where: P^{Z-} = Polyanion with Z neg. chgs.

R^+ = Precipitating cation (i.e., cetyl-pyridinium, Alcian blue)

M^+ = Counterion (i.e., a metal ion - Na, etc.)

According to the Law of Mass Action:

$$\frac{\left[P^{Z-}ZR^+\right]}{\left[P^{Z-}ZM^+\right]} \frac{\left[M^+\right]^Z}{\left[R^+\right]^Z} = K$$

$$\frac{\left[P^{Z-}ZR^+\right]}{\left[P^{Z-}ZM^+\right]} = K \left[\frac{R^+}{M^+}\right]^Z = E$$

E = the extent of pptn. or staining or the ratio of the insoluble to the soluble form (when R^+ replaces M^+ on a polyanion and R^+ is an organic cation, ppt'n ensues).

Plotting E vs. $\dfrac{\left[R^+\right]}{\left[M^+\right]}$ for different values of Z.
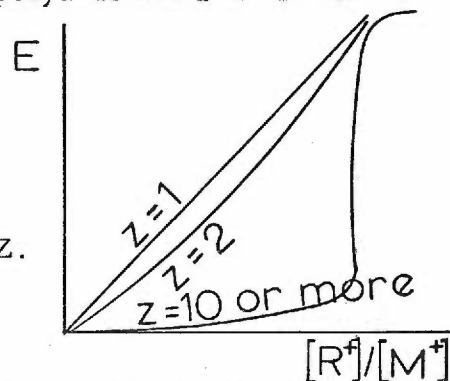
Thus, with ten or more negative changes, keeping the Alcian blue (or other organic cation = R^+) constant, and altering the counter cation (= salt, M^+) concentration, there is a critical electrolyte concentration, (CEC), at which the extent of ppt'n., E, suddenly changes.

In this case, E is a function solely of M^+ and the CEC is expressed in terms of M^+. Since with glycosaminoglycans, we are dealing with a Z value of ca. 100, such behavior could be expected.

Now, the position of the CEC on the X axis is dependent on the magnitude of K (equilibrium constant) which is in turn governed by the relative affinities of M^+ and R^+ for the ionic groups of the poly-anion. The ratio of the affinities is unlikely to be the same for all anions each of which should have a characteristic K (hence CEC) for a given pair of R^+ and M^+ or more specifically for a given M^+ (since as mentioned, R^+ is constant).

This interpretation is simplified and is subject to variations in charge density, interaction with neighboring bound ions and molecular weight.

As this treatment applies to the polyanionic mucopolysaccharides (P), the $-O-SO_3^-$, $-PO_4^-$, $-COO^-$ reactive groups provide the negative charge in the reaction, (Z^-), while the counterion (M^+), in this case is Mg^{++}. The precipitating cation (R^+) is Alcian blue, and the blue color or staining is due to a high value of E or high $\frac{R^+}{M^+}$ ratio. As M^+ is increased from a value of zero, the CEC's of the various mucopolysaccharides are successively reached and at those specific concentrations precipitation of the specific mucopolysaccharide by Alcian blue (i.e., staining) is prevented.

Due to the effects of other tissue constituents these substances do not behave as purified compounds <u>in situ</u>. Therefore, identification is in all cases tentative and must be based on similarity of staining in the tissue under study with model cells or tissues known to contain relatively pure single species of mucopolysaccharide (i.e., mast cells--heparin).

In practice, the technique is performed as shown here:

Materials:

a) Alcian blue -1% Alcianblau 8GS (Roboz Surgical Instr., Co., Washington, D.C.) in 3% acetic acid

b) Tetrahydrofuran - as supplied by Fischer Scientific Co.

c) Buffer - 0.1M sodium acetate buffer pH 5.6

d) Magnesium chloride - 5M stock

e) Staining solution - a series of coplin jars are set up to contain 2.5 ml of alcian blue solution plus 12.5 ml of buffer, to which is added sufficient $MgCl_2$ to attain the desired final concentration, and 35 ml of distilled water. The $MgCl_2$ concentrations used were OM, 0.2M, 0.4M, 0.8M and 1.2M.

f) Hyaluronidase - dissolve one vial Wyndase (hyaluronidase) 150 N.F. (TR) units in 1 cc distilled water

g) Formol alcohol - 90 ml. 80% ethanol plus 10 ml. formaldehyde

h) Kernechtrot solution (Nuclear Fast Red) 0.1% in 5% aluminum sulfate. Dissolve with heat, cool, filter and add crystals of thymol.

Method:

a) Tissue cut into pieces approximately 0.5 cm. on edge; fix in

formol alcohol for 12-24 hrs., dehydrate in tetrahydrofuran, clear, embed in paraffin and cut at 7μ in the usual manner.

b)   Deparaffinize, hydrate, stain for 2 hours in the above staining solution.

c)   Wash ten minutes in tap water and distilled water.

d)   Counterstain two minutes in Kernechtrot solution (0.1%).

e)   Wash briefly in distilled H_2O, dehydrate, clear and mount.

APPENDIX III. Radioactivity Determination by Liquid Scintillation

Spectrometry

Apparatus:

For the rabbit experiment (ESB), a Nuclear Chicago 720

series liquid scintillation spectrometer (Nuclear Chicago Corp., Des

Plaines, Illinois) was used and for the rat experiment (ESC) a Tri-

Carb liquid scintillation spectrometer model 3310 (Packard Instru-

ment Co., Downers Grove, Illinois) was used.

Twenty ml. low-potassium vials #6001009 with foil-lined,

toluene resistant caps (Packard Instrument Co.) were used throughout.

Materials:

The phosphor solution, as mentioned in the experimental section,

consisted of 0.1 gm. 1,4 bis $\left[2\text{-(5 phenyloxazolyl)}\right]$ benzene (Scintil-

lation grade - Packard Instrument Co.) + 4 gm. 2,5-diphenyloxazole

(Scintillation grade - Packard Instrument Co.) made up to one liter

in toluene and protected from light. 10 mL of this solution were used

with samples counted in the Nuclear Chicago instrument, whereas,

15 ml. were used with those counted in the Tri-Carb.

1 M Hydroxide of Hyamine 10-X in methanol was used as

supplied from Packard Instrument Co.

Techniques:

a)   Sample Preparation-Preparation of the samples differed in

ESB and ESC. In ESB (rabbit experiment) which employed the hydroxy-proline isolation procedure of Prockop et al (132a), the hydroxyproline, as discussed, was oxidized to pyrrole. Since pyrrole is directly sol-uble in toluene, fluor and sample were completely miscible. However, in ESC (rat experiment), the hydroxyproline isolated by chromatography was not soluble in toluene. It was, therefore, first dried in the count-ing vial under nitrogen and then dissolved by adding 1 ml. of hyamine hydroxide and heating at $60°C$ on a Blackstone ultrasonic generator for thirty minutes until the solution clarified. After the solution had cooled, the fluor was added and the samples dark-adapted in the spectrometer for a minimum of two hours and counted.

b) Counting procedure: The duration of the counting of each sample varied inversely with the radioactivity of that sample. This relation-ship was determined in each case from a curve[1] derived from the following equation:

$$t_s = \frac{K^2}{(\delta N_s - N_b)^2} (N_s + N_s X N_b)$$

where t_s = minimum time that a sample must be counted so that the standard error will be within $\pm \delta\%$ of the true count rate

δ = level of probability

N_s = total count rate

---

[1]This equation was originally developed by Dr. D.A. Rigas who has subsequently published a nomogram for radioactive counting statistics (139). The nomogram is flexible and easily read.

N_b = background count rate

K = standardized deviate or the normal curve function of the confidence level (i.e., 1.96 for 5% level of probability)

However, the curve derived from this equation is of somewhat limited value in that it does not allow one to use scintillation counters with varying background rates during the course of a single experiment and in that it assumes the same limits of confidence and tolerable fractional error in each use to which it is put. Specific activities were expressed as disintegrations per minute per microgram or micromole of hydroxyproline or pyrrole (which are interconvertible mole per mole). This figure was obtained as follows:

$$\text{DPM}/\mu g \text{ Hypro} = \frac{\text{Total CPM - background}}{\text{efficiency}} (=\text{DPM}) \times \frac{1}{\mu g \text{ or } \mu M \text{ Hypro counted}}$$

c) Efficiency Determination: Efficiency was determined in different ways in the two experiments. In ESB the efficiency was determined by the channels ratio method since the Nuclear Chicago liquid scintillation counter can be set to print out channels ratios. Beta particles emitted by a tritium atom have widely different energies (MEV). These are reflected in a spectrum of pulse heights registered at the scalers of a liquid scintillation counter during counting of a sample containing tritium. The channels ratio method of determining efficiency (17) depends on a downward shift of this pulse height spectrum as a

result of quenching according to the figure:
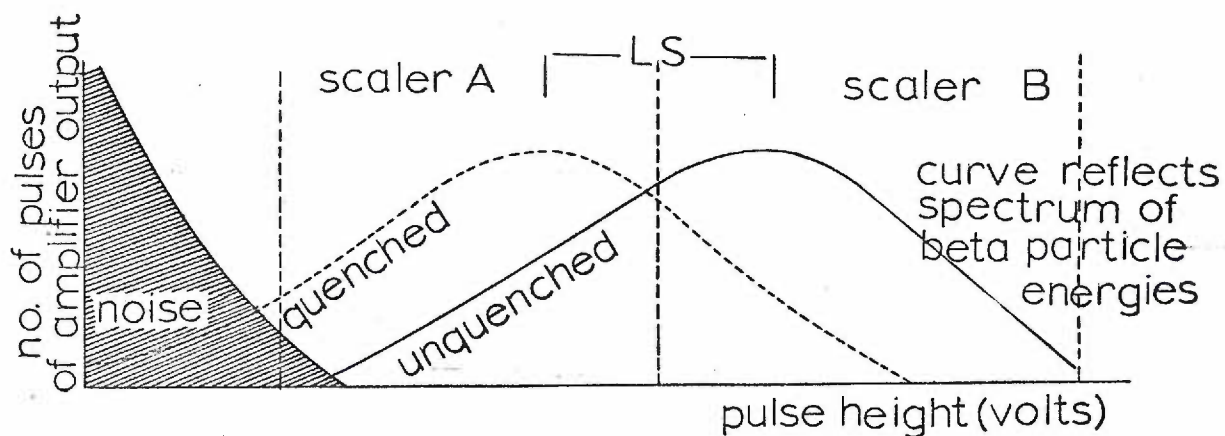
The greater the quenching, the further the energy spectrum will

show a left shift (LS) and the more the counts in the lower energy

channel will increase with respect to those in the higher energy

channel. The degree of this left shift will be reflected in the ratio of

counts observed in channel B to those in channel A (Channels Ratio

$R1 = B/A$). The larger this ratio, the less the quenching and the

greater the efficiency. The smaller the ratio, the greater the quench-

ing and the smaller the efficiency. By calculating the efficiency

(i.e., % efficiency = CPM/DPM x 100) of a series of increasingly

quenched standards, containing a known number of DPM, and noting

the channels ratios derived therefrom, one can draw a curve relating

channels ratio to efficiency. Thus, from the channels ratio of any

sample, one can, by reference to the curve, determine the efficiency

at which it was counted.

In ESC efficiency was determined by automatic External Stand-

ardization (67, 36). In this system, a radium probe is automatically placed next to each counting vial for one minute (at the end of the counting period) and the increase in the CPM over the normal rate registered. The radium probe produces this increase by the emission of γ rays which upon hitting any matter produce compton electrons which, within the counting vial, activate the phosphor in a manner similar to normal β particles. By relating this increase to a curve of efficiency V.S. increase prepared from a series of quenched standards, the counting efficiency can be determined.

Radioactivity was related to the amount of hydroxyproline or pyrrole in each case by taking a small aliquot of the sample for colorimetric assay.

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