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## Alteration of Proline Hydroxylase Activity by Corticosteroids

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ALTERATION OF PROLINE HYDROXYLASE ACTIVITY  
BY CORTICOSTEROIDS

BY

KENNETH ROBERT CUTRONEO

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE  
REQUIREMENTS FOR THE DEGREE OF  
DOCTOR OF PHILOSOPHY  
IN  
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1970

DOCTOR OF PHILOSOPHY THESIS

OF

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TITLE ABSTRACT

PROLINE HYDROXYLASE ACTIVITY AND CORTICOSTEROIDS

DEDICATION

THIS THESIS IS DEDICATED TO

MY MOTHER

MY FATHER

AND

ESPECIALLY TO

MY WIFE

FOR ALL THEIR LOVE

ACKNOWLEDGMENTS

I would like to express my deepest appreciation to Dr. George C. Fuller for his guidance throughout this project. I also express my appreciation to the other members of my graduate committee for their continued advice.

I would also like to thank Mr. Donald Costello for his assistance through various phases of this investigation.

I thank Lederle Laboratories of Pearl River, New York for their generous gift of Aristocort triamcinolone diacetate used in this study.

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ABSTRACT

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The biochemical mechanism by which glucocorticoids affect collagen metabolism was investigated. Triamcinolone, hydrocortisone and methylprednisolone significantly decreased liver proline hydroxylase activity in vivo. This was not a manifestation of an anti-anabolic effect on protein. Triamcinolone inhibited liver proline hydroxylase activity in a dose dependent manner in vivo. An observed elevation of liver hydroxylase activity in adrenalectomized animals was decreased to control level by hydrocortisone treatment.

Subdermal implants of three types of sponges (polyurethane, cellulose and polyvinyl) were used to induce granuloma growth as a model system of inflammation. The anti-inflammatory activity of corticosteroids was correlated with inhibition of proline hydroxylase activity in granuloma tissue.

The data indicate that corticosteroids decrease collagen synthesis by inhibiting the rate limiting enzyme. This inhibitory effect on collagen metabolism is correlated with observed anti-inflammatory activity.

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## I. INTRODUCTION

Dramatic effects of glucocorticoid therapy in victims with various collagen diseases (i.e. those disorders firstly characterized by degenerative changes and necrosis and secondly by inflammatory and reparative processes) have been reported (Hench et al., 1949, 1950). These workers showed that large doses of ACTH or cortisone administered to patients suffering from rheumatoid arthritis produced dramatic and prompt improvement.

Although much is known about various metabolic and biochemical changes which occur in connective tissue at intervals after glucocorticoid treatment, little is known about the molecular and biochemical mechanism of action of this group of frequently used drugs on connective tissue metabolism. The ability of this class of drugs to impair the progress of connective tissue diseases is believed to be primarily due to the anti-inflammatory effect of these steroids (Lorenzen, 1969a).

Increased synthesis of collagen occurs as an essential function of the inflammatory process (Gilman, 1968). The effect of glucocorticoids on collagen metabolism is well documented. Collagen content (Houck and Patel, 1965) and collagen synthesis (Fukuhara and Tsurufugi, 1969) are decreased after corticoid administration.

This investigation was conducted to elucidate the biochemical mechanism by which the glucocorticoids affect collagen synthesis and to relate this action to anti-inflammatory activity.

## II. LITERATURE SURVEY

### A. The Inflammatory Process and Proposed Modes of Action of Anti-inflammatory Steroids

Inflammation is the series of biological processes involved in the response of tissues to injury. The two phases of inflammation are the destructive phase and the reparative phase (Houck, 1968). The destructive phase is initiated by localized injury which causes either the formation or extrusion of intracellular materials extracellularly. Histamine, 5-hydroxytryptamine, kinins and proteolytic and collagenase enzymes and other factors promote a response of tissue adjacent to the site of injury (Whitehouse, 1968). Increased vascular permeability results from proteolytic enzyme destruction of the endothelial vasculature. Aggregation of platelets and conversion of fibrinogen to fibrin by an uncharacterized enzyme unique to inflamed connective tissue (Houck et al., 1967) results in thrombus formation and subsequent ischemia of the wounded area. Anoxia and acidosis of the tissue in turn causes necrosis (Houck, 1968).

A drug could produce anti-inflammatory activity by affecting one or more of the biochemical events of the inflammatory process. Various workers have shown that

natural glucocorticoids and synthetic steroids stabilize lysosomes (Weissmann, 1965a & b), possible mediators of inflammation (Weissmann, 1967). Willoughby and Spector (1964) reported that anti-inflammatory drugs are capable of inhibiting both proteases and esterases which attack the microcirculation. Gladner and Houck (1969) showed cortisol mediated the release of at least three proteases, one of which is highly specific for the phenyl-alanine-serine bond in bradykinin. Anti-inflammatory drugs have also been shown to suppress the formation of histamine, 5-hydroxytryptamine and possibly the kinins, all of which are increased during inflammation (Whitehouse, 1968). Mustard et al. (1967) showed that anti-inflammatory drugs inhibit the aggregation of platelets.

The end point of the inflammatory process, repair and restoration of function of the injured tissue, involves proliferation of macrophagic cells, fibroblasts and epithelial cells in the inflamed area. Collagen, a structural protein of connective tissue, is synthesized by the fibroblasts in most tissue. Collagen synthesis occurs as an essential part of the inflammatory process (Gillman, 1968). Delaunay and Bazin (1969) showed that collagen content increased when subacute inflammation was induced by the implantation of polyvinyl sponges.

Since collagen metabolism is an important part of the inflammatory process, it is reasonable to assume that this would constitute a biochemical site of anti-inflammatory activity of glucocorticoids.

B. Collagen and Protocollagen Proline Hydroxylase

Collagen, the most abundant mammalian protein, is synthesized by the fibroblasts as a major structural component of tissue. This protein is composed of three separate polypeptide chains of approximately equal size and has a molecular weight of 300,000 (Hutton et al., 1968). Collagen is composed of approximately one-third glycine, one-third proline and hydroxyproline and one-third other nonsulfur-containing amino acids (Crick and Rich, 1957). It contains no tryptophane and cystine (Udenfriend, 1966). Collagen is the only protein that contains significant amounts of hydroxyproline (Prockop & Kivirikko, 1967).

Collagen biosynthesis involves two processes: (1) polypeptide synthesis and (2) hydroxylation of peptide-bound proline (Juva, 1968). The first step is probably similar to the synthesis of other proteins but the second is unique and might very well prove to be the site of regulation in the collagen biosynthetic pathway. Many workers feel

that procollagen proline hydroxylase is the rate limiting step in collagen synthesis (Udenfriend, 1966).

Procollagen, a soluble protein, serves as the substrate for the hydroxylation step (Prockop & Kivirikko, 1967). When procollagen molecules reach molecular weights considerably greater than 10,000 and contain specified amino acid sequences which are recognized by the hydroxylase enzyme, specific proline residues are hydroxylated (Kivirikko & Prockop, 1967). Bhatmagar et al. (1967) believe that hydroxylation of proline occurs after the completed polypeptides of procollagen are released from the ribosomes. However, Gould (1968) reports that hydroxyproline is associated with polyribosomes. Hydroxylation of free proline does not occur.

The oxygen atom of the hydroxyl group of hydroxyproline is derived from molecular oxygen and not from water (Fujimoto & Tamiya, 1962; Prockop et al., 1963). A direct-displacement hydroxylation by this enzyme was demonstrated by Fujita et al. (1964). These workers using cis and trans-4-monotritio-proline showed that only the tritium atom trans to the carboxyl group was lost during hydroxylation. Later it was shown by Gottlieb et al. (1965) that trans-4-fluoroproline was

incorporated into collagen and converted to hydroxyproline. Thus, the enzyme is classified as a mixed function oxidase. Oxygen and a reducing agent are required cofactors.

The enzyme has been demonstrated in a number of animal tissues (Hutton & Udenfriend, 1968; Takeuchi et al., 1967; Mussini et al., 1967; Fuller and Langner, 1970). The enzyme activity is greatest in tissues actively engaged in collagen synthesis such as embryonic and granuloma tissue (Mussini et al., 1967) and uterine tissue (Halme & Jaaskelainen, 1970).

The enzyme has been partially purified from foetal rat skin (Rhoads & Udenfriend, 1968) and has been shown to have an absolute requirement for atmospheric oxygen, ascorbate,  $\alpha$ -ketoglutarate and the ferrous ion (Hutton et al., 1967). The importance of ascorbate was shown by Stone and Meister (1962) who reported that hydroxyproline synthesis was disproportionate to proline incorporation in scorbutic tissue. Hutton et al. (1967) suggested that  $\alpha$ -ketoglutarate is an allosteric activator of the enzyme and its cellular concentration regulates the activity of the enzyme and the synthesis of collagen. However, Rhoads and Udenfriend (1968)

demonstrated a stoichiometric decarboxylation of  $\alpha$ -ketoglutarate coupled to the hydroxylation of peptidyl proline. These workers feel that  $\alpha$ -ketoglutarate serves as a specific electron donor, while ascorbate maintains a proper reducing environment, and the ferrous ion acts as an electron carrier.

Since the enzyme involved in the hydroxylation reaction requires oxygen and ferrous ion, unhydroxylated protocollagen peptides may be prepared by incubating embryonic or connective tissue in the absence of oxygen or a chelator of iron, e.g.  $\alpha\alpha$ -dipyridyl (Prockop & Juva, 1965).

The assay of Hutton et al. (1966) is a rapid and specific method to determine collagen proline hydroxylase activity. The method is based on the enzymatic conversion of L-3,4<sup>3</sup>H-peptidyl proline to L-3-<sup>3</sup>H-hydroxyproline and release of a tritium atom from the four position. The released tritium then equilibrates with unlabeled water. Tritiated water is collected and counted. It was shown that equivalent amounts of L-3-<sup>3</sup>H-hydroxyproline and tritiated water were formed.

### C. Glucocorticoids and Collagen Metabolism

Glucocorticoid mediated decrease in collagen content in normal connective tissue (Houck et al., 1967) and

inflamed tissue (Fukuhara & Tsurufuji, 1969; Bavetta et al., 1962) is well documented; however, the mechanism of action is not known. The decrease in collagen content observed after corticoid treatment in animal and man could be explained by a decrease in the rate of collagen synthesis or an increased rate of collagen degradation.

Houck et al. (1967) reported a decrease in cutaneous insoluble collagen in cortisol treated rats. Paralleling this loss in insoluble collagen was the appearance of cutaneous extracellular, free collagenolytic activity. These authors feel that the observed loss in collagen is the result of collagen catabolism through the induction of collagenase activity in skin fibroblasts (Houck & Sharma, 1969). Kivirikko et al. (1965) studied the action of cortisone on the metabolism of collagen by looking at the effect of this drug on the specific activity and total activity of C<sup>14</sup>-hydroxyproline in urine and in skin collagen fractions. These workers showed that the specific activity and total activity of C<sup>14</sup>-hydroxyproline in the soluble collagen fraction of skin were decreased and correlated this, with decreased hydroxyproline excretion. These results suggest that corticoids decrease collagen synthesis.

Furthermore, in a related study these same workers demonstrated that cortisone did not affect the catabolism of insoluble collagen,

The insoluble collagen of rat skin is relatively inert metabolically with a biological half-life of one year (Lindstedt & Prockop, 1966). It is reasonable to assume that the corticoids would change collagen content by altering collagen biosynthesis rather than altering collagen degradation. Further, if the corticoids degraded mature collagen, urinary hydroxyproline levels should have been elevated. Neither Kivirikko et al. (1965) nor Smith (1967) observed catabolic changes in urinary hydroxyproline analyses. Urinary hydroxyproline levels were decreased in both studies. Smith (1967) administered cortisone for at least four days before any change in insoluble skin collagen was observed. Ohno and Tsurufuji (1970) observed no additive acceleration in the release of tyrosine when hydrocortisone was used in combination with the antibiotics, puromycin and cycloheximide in carrageenin granuloma. Lorenzen (1969b) observed no change in the collagen content of the aortic wall with prednisone treatment in rabbits. These results agree with the idea that soluble collagen is the pool that is greatly influenced by corticoids, since the greater part of collagen in the normal aortic wall of adult animal

is probably mature insoluble collagen with a very slow turnover.

D. Granuloma Formation: A Model System of Inflammation to Study Anti-inflammatory Activity of Drugs

Inflamed or diseased connective tissue would be preferable as an experimental model to elucidate the biochemical mechanism of anti-inflammatory activity of steroids rather than normal intact connective tissue. Experimental granulomas induced by artificial means may be used to study the effect of anti-inflammatory drugs on inflamed connective tissue.

Woessner and Boucek (1961) investigated collagen formation produced by implanted polyvinyl sponges in rats. These workers found that collagen content rapidly increased following the seventh day after implantation. Robertson and Schwartz (1953) reported both biochemical and histological evidence of collagen formation in granuloma tissue. The granuloma system provides an excellent model to study chronic in vivo stimulation of the proliferation of fibroblasts, synthesis of collagen, and the biochemical events involved in the regulation of these processes. Compared to other methods of granulation tissue development, e.g. carrageenin, the sponge-induced granuloma has the distinct advantage of being homogeneous and can be separated

from the surrounding tissue (Juva, 1968).

Glucocorticoids are well known inhibitors of granuloma formation (Fukuhara & Tsurufuji, 1969); however, the biochemical mechanism of this anti-granulomatic activity has not yet been clearly elucidated. These workers showed that betamethasone inhibited collagen synthesis in the carageenin induced inflammatory tissue. Juva (1968) showed that changes in protocollagen proline hydroxylase activity paralleled the rate of collagen synthesis in sponge induced granuloma tissue. Since various workers feel that proline hydroxylase is the rate limiting step in collagen biosynthesis (Mussini et al., 1967; Takeuchi et al., 1967; Gribble et al., 1969), the regulation of this enzyme may be the mechanism by which glucocorticoids affect collagen synthesis and granuloma formation.

### III. EXPERIMENTAL

#### A. Animals

Intact (100-150 gm) and 250 gm adrenalectomized male albino Sprague-Dawley rats were supplied by Charles River Breeding Laboratories, Willmington, Massachusetts. Efficacy of the commercial adrenalectomy was determined at autopsy. All animals were housed in rooms maintained at a temperature of 70<sup>o</sup>F with an alternating cycle of 12 hours light.

#### B. Materials

All chemicals used in this investigation were analytical reagent grade or the equivalent. Commercial corticoid preparations used were: methylprednisolone sodium succinate (Solu-Medrol, Upjohn Co., Kalamazoo, Michigan); hydrocortisone acetate (Hydrocortone, Merck, Sharp & Dhome, West Point, Pennsylvania); betamethasone acetate (Celestome Soluspan, Schering Corporation, Bloomfield, New Jersey); and triamcinolone diacetate (Aristocort Forte) which was kindly supplied by Lederle Laboratories, Pearl River, New York.

Saline suspensions of hydrocortisone were prepared by adding hydrocortisone powder (Merck, Sharp & Dome, West Point, Pennsylvania or Upjohn Company, Kalamazoo,

Michigan) to 0.9% (w/v) saline at a concentration of 30 mg/ml and mixing by use of a coaxial homogenizer with a teflon coated pestle until a smooth suspension was obtained. 3-4-<sup>3</sup>H-L-proline (specific activity 5.2 mc/mm) was purchased from New England Nuclear Corporation (Boston, Massachusetts).

C. In Vivo Glucocorticoid Studies on Liver Proline Hydroxylase Activity

These studies were carried out to determine the effect of various glucocorticoids on liver proline hydroxylase activity. The animals received drug (either 100, 150 or 200 mg/kg) intraperitoneally once per day for four days and were sacrificed on day five. In the dose response study with triamcinolone, animals received either distilled water or drug (150 mg/kg, i.p.) daily, and a control group and drug treated group were sacrificed at the times specified. All animals were sacrificed by cervical dislocation followed by decapitation. Livers were perfused with 0.9% (w/x) saline and tissue homogenates (10% w/v) were prepared in 0.25 M sucrose by use of a Polytron ST-10 system (Kinematica GMBH, Luzern, Switzerland). The homogenates were centrifuged for 15 minutes in an International Model B-60 preparative ultracentrifuge at

15,000 xg for 15 minutes at 0°C. The enzyme source, the supernatants, were stored at -15°C until assay.

#### D. Granuloma Studies

Sub-dermal implants of three different types of sponges (polyurethane, cellulose and polyvinyl) were used to induce granulation tissue growth. Sponges were cut into cylinders of 10 mm diameter; 42 mm in length for the polyvinyl type ( $95.4 \pm 4.7$  mg), 38 mm for the polyurethane type ( $67.6 \pm 5.4$  mg) and 22 mm for the cellulose type ( $89.5 \pm 5.2$  mg). The sponges were washed in continuous running water for 48 hours, sterilized at 230°C for 15 minutes in an autoclave, and dried. Prior to implantation the sponges were weighed and stored in sterile saline.

The animals were anesthetized with ether. The dorso-lumbar region was shaved and swabbed with 70% (v/v) ethanol. A trocar was inserted and guided to the dorso-thoracic region. One of each type of sponge was implanted in a certain area: the polyvinyl type on the right, the cellulose type in the middle and the polyurethane on the left. The trocar was withdrawn and the incision was closed with wound clips. The wound clips were removed on the eighth day after sponge implantation.

Daily local injections of steroids administered directly into the sponge were initiated following one day of implantation. One tenth milliliter of commercially prepared steroid preparation was diluted with 0.9 percent (w/v) saline to 1.0 ml. This was injected directly into the sponge at a dose of 0.6 mg of betamethasone per rat per day and 4.0 mg of triamcinolone per rat per day. Treatment was continued daily for the next four days and all animals were sacrificed on day six after sponge implantation.

The animals were killed by either cervical dislocation and guillotining or by anesthetizing with ether and decapitation. The granuloma tissue was removed from the sponge. Tissue homogenates 10 percent (w/v) were prepared either by freezing the tissue in liquid nitrogen, pulverizing and homogenizing in 0.25 M sucrose in a coaxial homogenizer with a teflon coated pestle or, by using a Polytron ST-10 system. The homogenates were centrifuged as described previously.

#### E. Substrate Preparation

3'4'-tritium labeled proline rich hydroxyproline deficient peptide substrate was prepared from chick by the method of Hutton et al. (1966). Ten day minced

decapitated chick embryos, approximately 6 gm, were incubated aerobically for 90 minutes at 37°C in 8.5 ml of modified Krebs-Ringer buffer<sup>a</sup> and 500 uc of 3-4-<sup>3</sup>H-L-proline and 1 mM  $\alpha$ - $\alpha$ 'dipyridyl. Following incubation the contents of twelve incubation beakers (60 gm tissue) were pooled. The pooled material was centrifuged at 105,000 xg for 90 minutes by use of the International model B-60 preparative ultracentrifuge. The resulting pellet was extracted at 0°C with 0.5 N acetic acid (2 ml/gm chick embryo) with continuous stirring for 90 minutes. The extraction was continued overnight without stirring. The material was centrifuged at 105,000 xg for 90 minutes, and the supernatant containing the substrate was dialyzed against two changes of distilled water and one change of 0.5 M tris (hydroxymethyl) aminomethane-hydrochloride (pH 7.5) buffer.

F. Protocollagen Proline Hydroxylase Assay

Proline hydroxylase activity in the 15,000 xg supernatant was measured by the method of Hutton et al. (1966). This procedure is based on the stoichiometric formation

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<sup>a</sup> 1.285 gm NaCl, 0.224 gm KCl, 0.144 gm MgSO<sub>4</sub>, 0.144 gm CaCl<sub>2</sub>, 0.0544 gm KH<sub>2</sub> PO<sub>4</sub>, 2.10 gm NaHCO<sub>3</sub>, 1.80 gm dextrose Q.S. to one liter with distilled water.

of tritiated water and tritiated hydroxyproline when the proline rich polypeptide substrate is incubated with enzyme and cofactors. Each incubation mixture contained 0.5 ml labeled substrate, 0.45  $\mu$ M ferrous ammonium sulfate, 7.5  $\mu$ M ascorbic acid, 0.9  $\mu$ M  $\alpha$ -ketoglutarate, 0.1 to 0.2 ml 15,000 xg supernatant Q.S. to an incubation volume of 3.0 ml with 0.5 M tris-HCl buffer.

The samples were incubated aerobically at 30°C for 30 minutes. The reaction was terminated by the addition of 50% (w/v) trichloroacetic acid while the samples were on ice. The resulting tritiated water was distilled and a 1.5 ml aliquot was counted in 15 ml of BBS-3 cocktail<sup>a</sup> at approximately 20% efficiency. Correction for efficiency was determined through automatic external standardization. The samples were counted for a period of time sufficient to reduce the standard deviation of the counting rate to 5% or less. All samples were corrected for background and non-enzymatic hydroxylation of the substrate.

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<sup>a</sup> The scintillation cocktail was prepared as follows: 0.05 gm PCPOP plus 1.0 gm PPO Q.S. to 100 ml with toluene and to this was added 30 ml BBS3 (Beckman, Fullerton, California).

### G. Protein Determination

Protein concentration in the 15,000 xg supernatant was determined by the method of Lowry et al. (1951), using bovine plasma albumin (Calbiochem, Los Angeles, California) as standard. Enzyme samples (0.2 ml) were diluted ten fold with 0.5 N NaOH in screw capped culture tubes. A reagent blank was prepared by taking 0.2 ml of 0.25 M sucrose and carrying this sample through the entire procedure. The samples were placed in a hot water bath for 1 hour until protein dissolution was completed. The samples were next allowed to stand at room temperature to cool. A 0.3 ml aliquot of each sample was added to 1.0 ml of 0.5 N NaOH and 5.0 ml of Reagent A<sup>a</sup>, mixed and allowed to stand for 20 minutes. Next, 0.5 ml of Reagent B<sup>b</sup> was added to each sample, mixed and allowed to stand at room temperature for 40 minutes for color development. The absorbance at 500 mu was read on a Beckman DB spectrophotometer.

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<sup>a</sup> Reagent A: 1.0 ml of a 1% (w/v)  $\text{CuSO}_4 \cdot \text{H}_2\text{O}$  solution and 1.0 ml of 2.7% (w/v) potassium tartarate solution added to 100 ml of 2% (w/v)  $\text{Na}_2\text{CO}_3$  solution.

<sup>b</sup> Reagent B: Commercial folin-phenol reagent diluted to 1 N with distilled water.

## H. Statistical Methods

The Student's-t-Test (Snedecor, 1956) was used to test for differences between means throughout this investigation. The formula employed as the basis for the computer programs used is as follows:

$$t = \frac{\bar{x}_1 - \bar{x}_2}{s^* \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

where:

$$s^* = \sqrt{\frac{(n_1 - 1) s_1^2 + (n_2 - 1) s_2^2}{n_1 + n_2 - 2}}$$

df taken as:  $\frac{(n_2 \text{ variance } x + n_1 \text{ variance } y)^2 (n_1 n_2)}{n_2^3 \text{ variance } x^2 + n_1^3 \text{ variance } y^2} - 2$

where: variance x =  $\frac{\{(x^2) - \frac{(\sum x)^2}{n_1}\}}{n_1 - 1}$

variance y =  $\frac{\{(y^2) - \frac{(\sum y)^2}{n_2}\}}{n_2 - 1}$

#### IV. RESULTS AND DISCUSSION

Triamcinolone, hydrocortisone and methylprednisolone (150 mg/kg) produced a significant decrease in specific activity of proline hydroxylase in liver (Table 1). This decrease was significant when enzyme activity was expressed per gram tissue equivalent or per liver. Triamcinolone proved to be the most potent inhibitor of liver proline hydroxylase activity in vivo. Neither hydrocortisone, methylprednisolone nor triamcinolone when added directly into the incubation medium inhibited enzyme activity. Thus intact tissue is required for these drugs to produce enzyme inhibition. Attempts to demonstrate the presence of an inhibitory substance in the supernatants from the steroid treated animals were not successful. A mixing experiment of control supernatant and triamcinolone supernatant gave greater than additive enzyme activity.

Steroid induced changes of distinct metabolic activities in connective tissue may result from a general inhibition of protein synthesis (Davidson, 1963) or from an unspecific depression of a basic metabolic process (Nocenti, et al., 1964). It is unlikely that the inhibitory effect on proline hydroxylase activity is a

Table 1: Glucocorticoid Induced Decrease of Rat Liver Proline Hydroxylase Activity.

	<u>N</u>	<u>DPM <sup>3</sup>H<sub>2</sub>O</u> <u>MG PROTEIN</u>	<u>MG SUPERNATANT PROTEIN</u> <u>GM TISSUE</u>
CONTROL	5	700±98 <sup>a</sup>	71.0±1.6
HYDROCORTISONE <sup>b</sup>	4	294±63 <sup>c</sup>	53.7±4.8 <sup>c</sup>
TRIAMCINOLONE	5	132±25 <sup>c</sup>	47.3±1.6 <sup>c</sup>
METHYLPREDNISOLONE	5	448±58 <sup>c</sup>	67.3±6.0

- a. Enzyme activity is expressed as the mean (+ S.E.) of the amount (dpm) of (<sup>3</sup>H)H<sub>2</sub>O formed from (3,4-<sup>3</sup>H) proline per milligram of protein per 30 minutes.
- b. Animals received 150 mg/kg of drug i.p. for four consecutive days and were sacrificed 18 hours after last injection.
- c. Significantly different from control at P < .05.

manifestation of an anti-anabolic effect on supernatant protein since protein concentration could be further decreased when proline hydroxylase inhibition by hydrocortisone was maximal (Table 2). This argument is further strengthened by the observation that triamcinolone decreased proline hydroxylase activity by 70 percent in the absence of an effect on supernatant protein (Table 3).

Triamcinolone inhibition of liver proline hydroxylase activity is linearly related to the number of daily doses (Figure 1). Significant inhibition of enzyme activity was observed 24 hours after drug treatment. This dose dependent relationship suggests that the hydroxylase enzyme is the biochemical target for glucocorticoid induced alteration of collagen metabolism. Smith (1967) administered cortisone for at least 4 days before any change in insoluble skin collagen was observed. Thus, the anti-anabolic effect is seen much earlier than a catabolic effect on the insoluble pool as proposed by Houck et al. (1967).

Liver proline hydroxylase activity was elevated in adrenalectomized animals and was decreased to the control level by hydrocortisone treatment for four consecutive

Table 2: The Effect of Hydrocortisone on Liver Proline Hydroxylase Activity and Protein in the 15,000 xg Supernatant.

	<u>N</u>	<u>DPM <sup>3</sup>H<sub>2</sub>O</u> <u>MG PROTEIN</u>	<u>MG SUPERNATANT PROTEIN</u> <u>GM TISSUE</u>
CONTROL	5	1345±30 <sup>a</sup>	75.2±3.1
HYDROCORTISONE <sup>b</sup>			
100 MG/KG	6	899±64 <sup>c</sup>	65.2±1.9 <sup>c</sup>
200 MG/KG	6	892±48 <sup>c</sup>	57.3±1.7 <sup>c</sup>

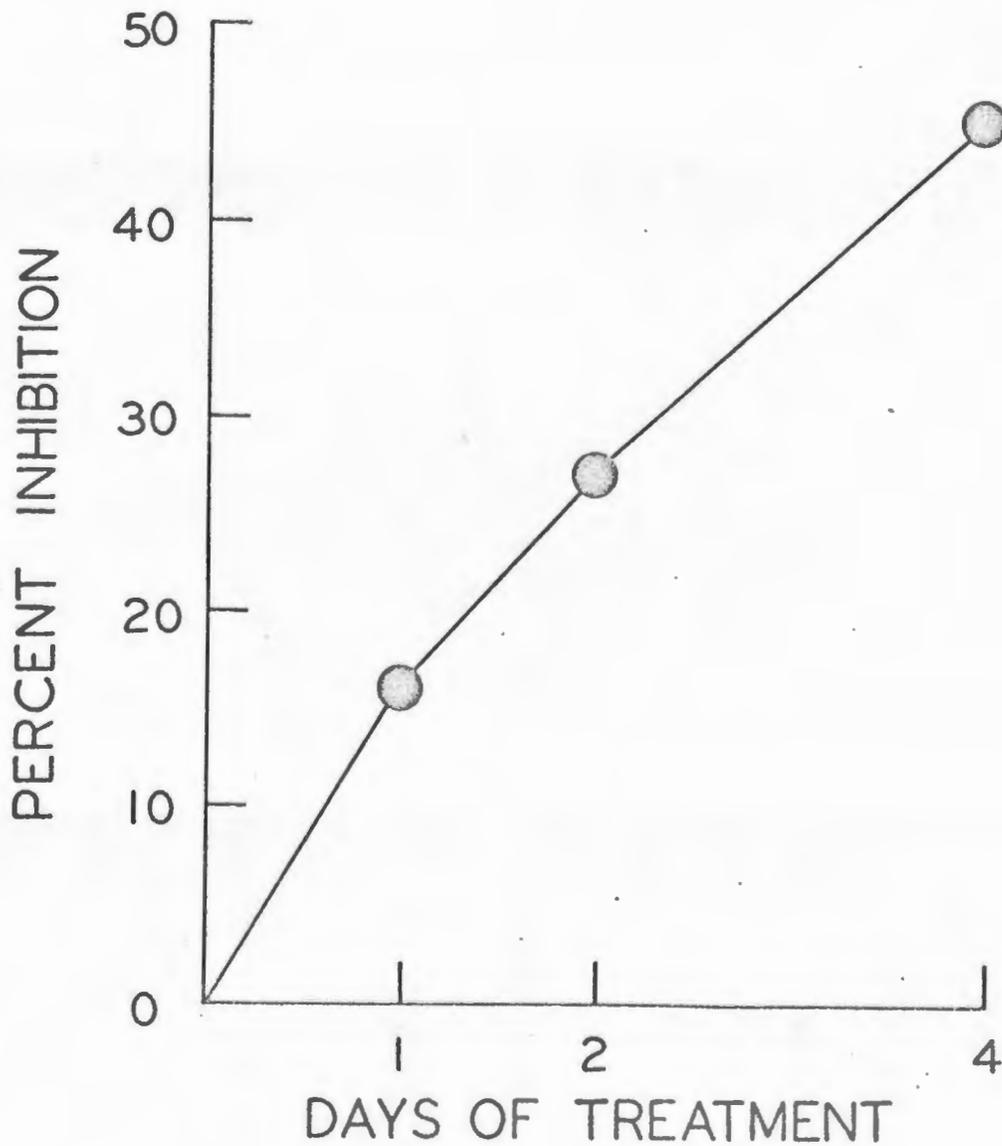
- Enzyme activity is expressed as the mean ( $\pm$  S.E.) of the amount (dpm) of (<sup>3</sup>H)H<sub>2</sub>O formed from (3,4-<sup>3</sup>H) proline per milligram of protein per 30 minutes.
- Animals received 150 mg/kg of drug i.p. for four consecutive days and were sacrificed 18 hours after last injection.
- Significantly different from control at P<.05.

Table 3: Effect of Triamcinolone and Betamethasone on Granuloma Body Weight Ratio and Granuloma Proline Hydroxylase Activity in the Rat.

	<u>N</u>	<u>GM GRANULOMA</u> <u>100 GM</u> <u>BODY WEIGHT</u>	<u>DPM <sup>3</sup>H<sub>2</sub>O</u> <u>MG PROTEIN</u>	<u>MG SUPERNATANT PROTEIN</u> <u>GM TISSUE</u>
CONTROL	8	0.231±0.02	3153±173 <sup>b</sup>	45.7±2.8
TRIAMCINOLONE <sup>a</sup>	8	0.128±0.02 <sup>c</sup>	1335±262 <sup>c</sup>	43.7±4.0
(4.0 MG/RAT)				
BETAMETHASONE	8	0.145±0.01 <sup>c</sup>	786±105 <sup>c</sup>	35.3±3.0 <sup>c</sup>
(0.6 MG/RAT)				

- a. One polyvinyl sponge was implanted subcutaneously in the dorso-thoracic region of each male rat on day 1. Drug was injected directly into the sponge daily for the next 4 days and all animals were sacrificed on day 6.
- b. Enzyme activity is expressed as the mean (+ S.E.) of the amount (dpm) of (<sup>3</sup>H)H<sub>2</sub>O formed from (3,4-<sup>3</sup>H) proline per milligram of protein per 30 minutes.
- c. Significantly different from control at P<.05.

FIGURE 1: DOSE RESPONSE OF TRIAMCINOLONE ON LIVER PROLINE HYDROXYLASE ACTIVITY. ANIMALS WERE TREATED WITH DISTILLED WATER OR TRIAMCINOLONE (150 mg/kg, i.p.) DAILY AND A CONTROL GROUP AND TREATED GROUP WERE SACRIFICED AT THE TIMES SPECIFIED. ALL DRUG TREATED GROUPS WERE SIGNIFICANTLY DIFFERENT FROM CONTROL VALUES.



days at a dose of 150 mg/kg, i.p. (Table 4). These results explain the reported increase in the amount of collagen in carrageenin induced granulomas in adrenalectomized guinea pigs (Robertson and Sanborn, 1958) and the potentiated granuloma development in adrenalectomized rats (Atkinson, et al., 1962; Ashford & Penn, 1965), as compared to intact controls.

The pharmacological importance of the inhibitory effect of these steroids on proline hydroxylase activity as a possible biochemical mechanism of anti-inflammatory activity depends upon the effect of these compounds on hydroxylase activity in inflamed or diseased connective tissue. The induction of experimental granulomas by artificial means offers an excellent model system to study anti-inflammatory activity. Sub-dermal implants of three different types of sponges (polyurethane, cellulose and polyvinyl) were used to induce granuloma growth. Supernatant protein decreased from day 0 to day 15 of granuloma growth ( $P < .05$ ), increased from day 15 to 25 and remained at 35-40 mg/gm until day 70 (Figure 2). This pattern of total protein was common to all three sponges. Proline hydroxylase activity is of paramount importance in the overall regulation of collagen

Table 4: The Effect of Hydrocortisone on Liver Proline Hydroxylase Activity in Adrenalectomized Rats.

	<u>N</u>	<u>DPM <sup>3</sup>H<sub>2</sub>O</u> <u>MG PROTEIN</u>	<u>MG SUPERNATANT PROTEIN</u> <u>GM TISSUE</u>
CONTROL	6	902+81 <sup>a</sup> 2,3	74.7+2.8 <sup>2,4</sup>
HYDROCORTISONE <sup>b</sup>	6	511+50 <sup>1,3,4</sup>	58.5+3.3 <sup>1,3</sup>
ADRENALECTOMIZED	8	1248+34 <sup>1,2,4</sup>	75.0+3.9 <sup>2,4</sup>
ADRENALECTOMIZED AND HYDROCORTISONE	5	834+106 <sup>2,3</sup>	62.5+2.2 <sup>1,3</sup>

a. Enzyme activity is expressed as the mean (+ S.E.) of the amount (dpm) of (<sup>3</sup>H)H<sub>2</sub>O formed from (3,4-<sup>3</sup>H) proline per milligram of protein per 30 minutes.

b. Animals received 150 mg/kg of drug i.p. for four consecutive days and were sacrificed 18 hours after last injection.

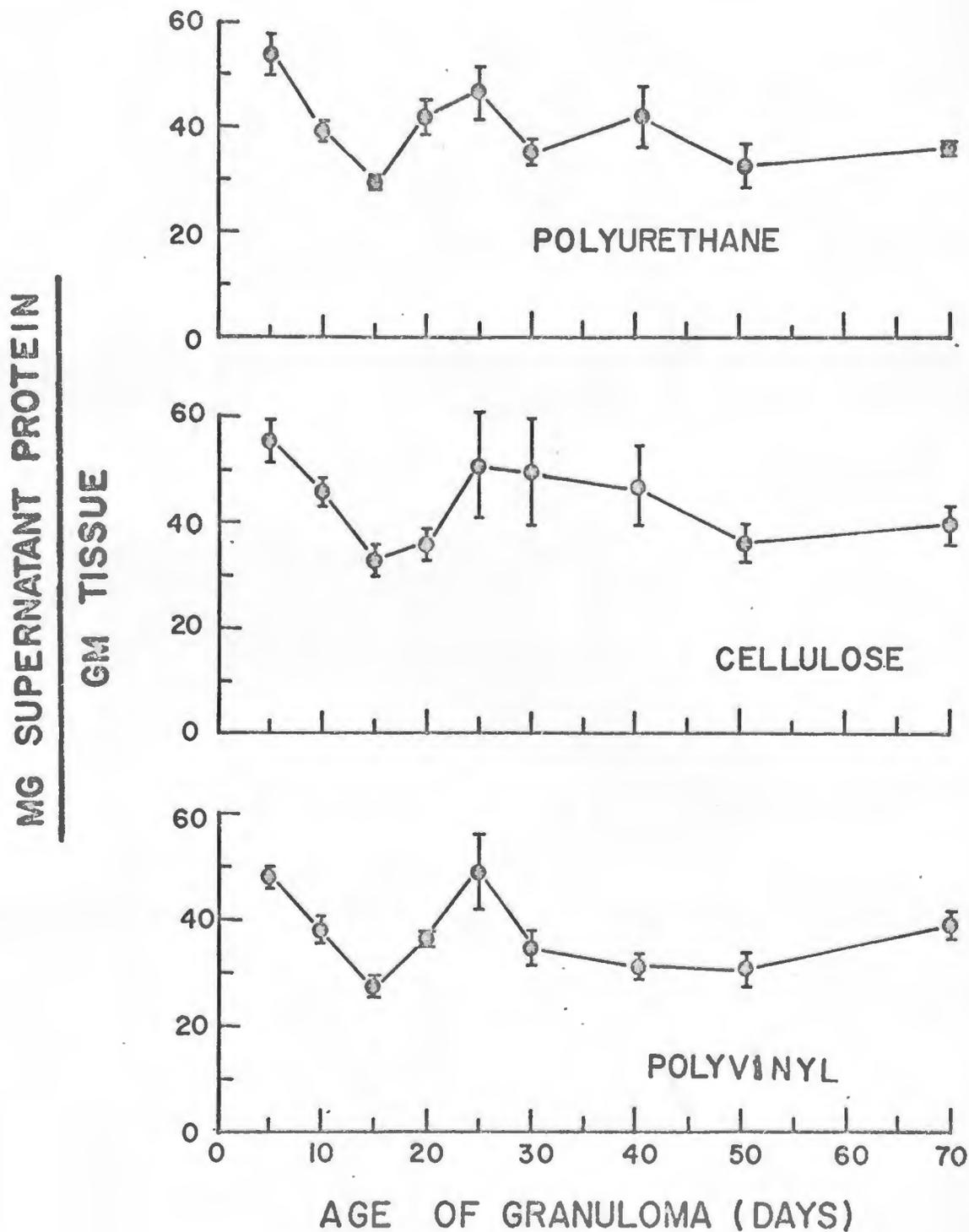
<sup>1</sup>Significantly different from control at P<.05.

<sup>2</sup>Significantly different from hydrocortisone at P<.05.

<sup>3</sup>Significantly different from adrenalectomized at P<.05.

<sup>4</sup>Significantly different from adrenalectomized and hydrocortisone at P<.05.

FIGURE 2: SUPERNATANT PROTEIN OF GRANULOMA TISSUE.  
 EACH POINT REPRESENTS THE MEAN + STANDARD  
 ERROR OF 3-9 GRANULOMAS OF THE SAME AGE.



biosynthesis. Increases in proline hydroxylase activity (Figures 3, 4, 5 and 6) were observed well before literature reports of the appearance of collagen fiber deposition in growing granuloma tissue (Viljanto, 1964; Viljanto and Kulonen, 1962).

Proline hydroxylase activity in granuloma tissue induced by the polyurethane sponge and polyvinyl sponge was highest on day 5 of granuloma growth. The increase in specific activity (DPM <sup>3</sup>HHO/mg protein) in the granuloma tissue induced by the polyurethane sponge (Figure 3) could be accounted for by the decrease in protein as shown in Figure 5. Proline hydroxylase activity was lowest at day 70 after sponge implantation in all cases (Figures 3, 4 & 6). Peak proline hydroxylase activity was observed on day 10 of growth using cellulose sponge (Figure 6). Less variation in enzyme activity was observed with the cellulose sponge than was observed with the polyvinyl and polyurethane types. However, the cellulose sponge was extremely difficult to separate from the granuloma tissue. Since enzyme activity in the granuloma induced by the cellulose sponge increased (Figures 5 & 6), whether enzyme activity was expressed per gram tissue equivalent, per milligram protein, or per

FIGURE 3: PROLINE HYDROXYLASE ACTIVITY IN THE 15,000 xg SUPERNATANT OF GRANULOMA TISSUE PRODUCED BY SUBCUTANEOUS IMPLANTATION OF POLYURETHANE SPONGES. EACH POINT REPRESENTS THE MEAN ENZYME ACTIVITY + STANDARD ERROR OF 4-8 GRANULOMAS OF THE SAME AGE.

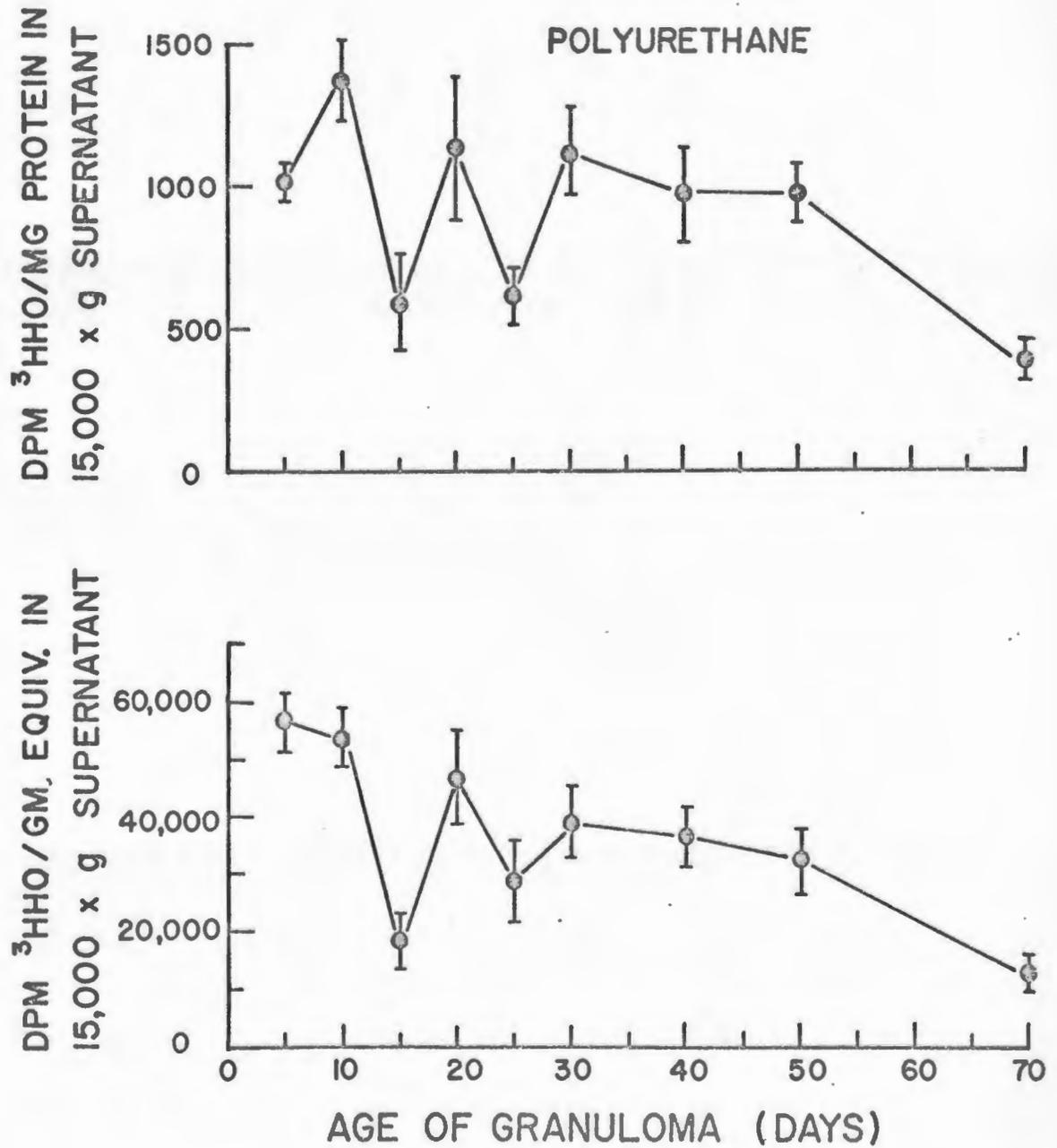
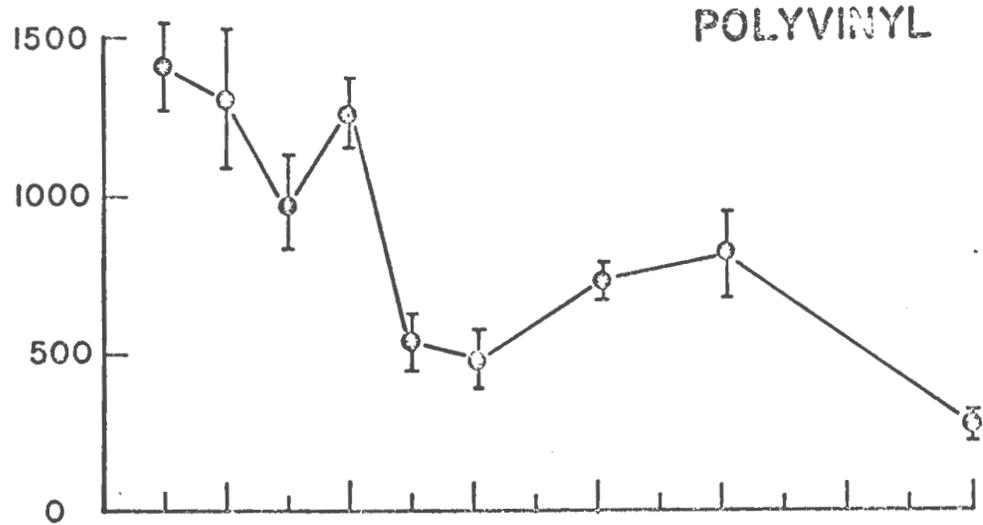
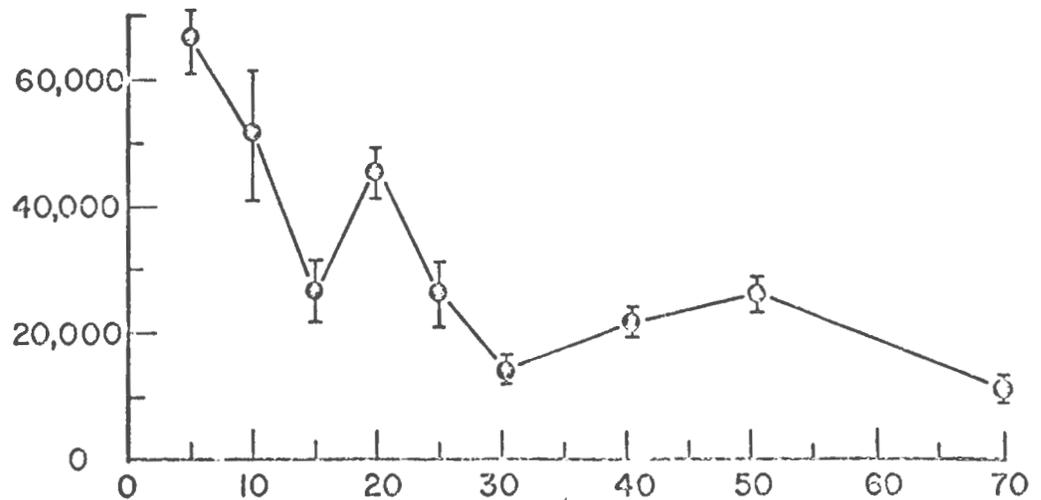


FIGURE 4: PROLINE HYDROXYLASE ACTIVITY IN THE 15,000 xg SUPERNATANT OF GRANULOMA TISSUE PRODUCED BY SUBCUTANEOUS IMPLANTATION OF POLYVINYL SPONGES. EACH POINT REPRESENTS THE MEAN ENZYME ACTIVITY + STANDARD ERROR OF 4-9 GRANULOMAS OF THE SAME AGE.

DPM <sup>3</sup>H HO/MG PROTEIN IN  
15,000 x g SUPERNATANT



DPM <sup>3</sup>H HO/GM EQUIV. IN  
15,000 x g SUPERNATANT



AGE OF GRANULOMA (DAYS)

FIGURE 5: PROLINE HYDROXYLASE ACTIVITY PER GRANULOMA. EACH POINT REPRESENTS THE MEAN ENZYME ACTIVITY + STANDARD ERROR OF 3-9 GRANULOMAS OF THE SAME AGE.

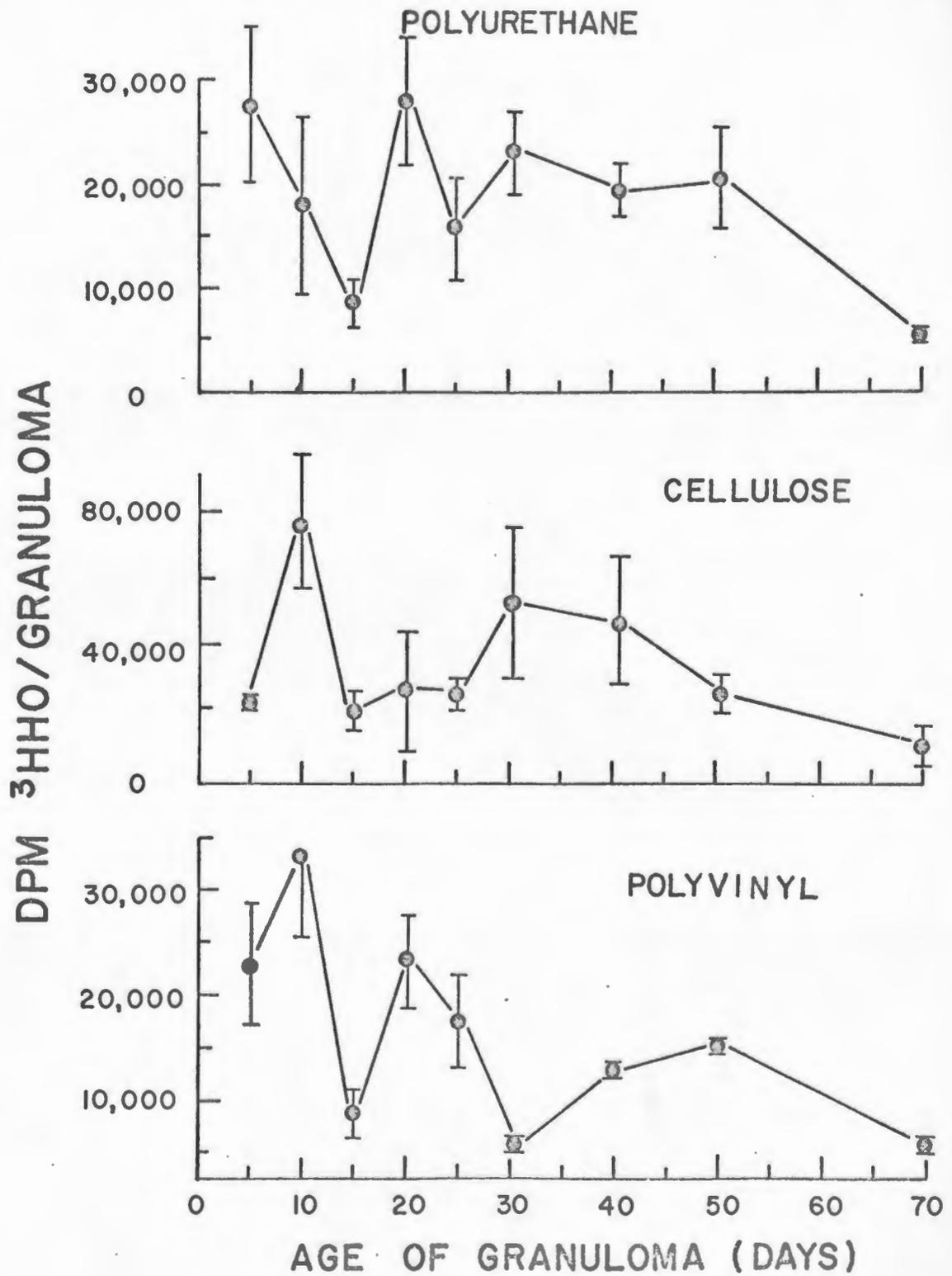
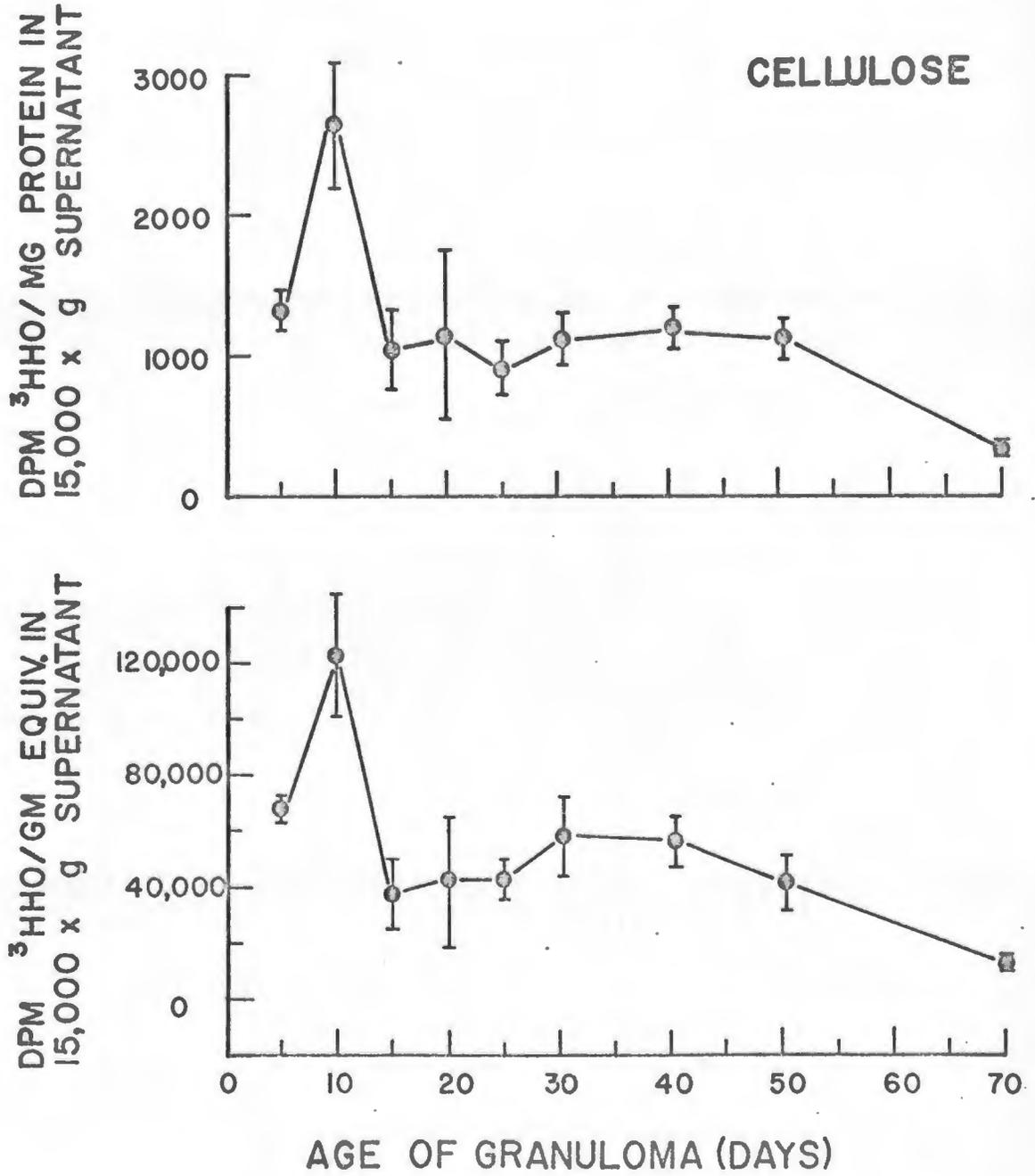


FIGURE 6: PROLINE HYDROXYLASE ACTIVITY IN THE 15,000 xg SUPERNATANT OF GRANULOMA TISSUE PRODUCED BY SUBCUTANEOUS IMPLANTATION OF CELLULOSE SPONGES. EACH POINT REPRESENTS THE MEAN ENZYME ACTIVITY + STANDARD ERROR OF 3-8 GRANULOMAS OF THE SAME AGE.



granuloma at the same time that general protein metabolism decreased (Figure 2), a synthesis de novo of proline hydroxylase is suggested. In vitro mixing experiments using the 15,000 xg supernatants of day 10 and day 70 produced no more than additive enzyme activity. Thus, the mechanism of increased proline hydroxylase activity in this in vivo model system in which fibroblast proliferation and collagen synthesis occurs appears not to be an activation process as is thought to be the case in growing fibroblast cells in culture (Gribble et al., 1969). The possibility of an activation process requiring the intact tissue, such as a closely coupled system (e.g., an enzyme or biochemical substance) which functions to bring the rate limiting enzyme and proline rich polypeptide together to initiate collagen biosynthesis, cannot be ruled out.

Mussini et al. (1967) observed proline hydroxylase activity on day 2 and peak activity on day 5 in carageenin induced granuloma tissue. Enzyme activity in their study was reported as specific activity (CPM/mg protein). Fluctuations in the amount of total protein during granuloma growth were observed in the present investigation (Figure 2). Thus, the observed increase in enzyme activity

reported by Mussini et al. (1967) may have been relative to the amount of protein present in the 15,000 xg supernatant.

These data further support the hypothesis that proline hydroxylase activity is a deciding factor in the formation and deposition of collagen fibers in granuloma tissue as is the case in embryonic tissue, foetal tissue, wound healing (Mussini et al., 1967), CCl<sub>4</sub> toxicity (Takeuchi et al., 1967) and the arteriosclerotic process in rabbits (Fuller and Langner, 1970). Hydroxyproline residues start accumulating after day 5 and reaches its peak at day 25 in granuloma tissue induced by sub-dermal implants of viscose cellulose sponges (Viljanto, 1964; Viljanto & Kulonen, 1962) and polyvinyl sponges (Gould, 1958). With both sponges little hydroxyproline is found up to day 5 and 6 and a few new collagen fibers are noted histologically (Viljanto, 1964; Gould, 1958). From day 7 onward accompanying the accumulation of hydroxyproline was noted fiber formation with maximum accumulation of collagen fibers taking place between day 14 and day 28.

The temporal increase of proline hydroxylase activity in the cellulose sponge induced granuloma tissue could explain the early increase in plasma hyproprotein (a

collagen-like hydroxyproline containing compound) during the first week of cellulose sponge implantation observed by Kumento and Kulonen (1967). These workers reported a two-fold increase of protein-bound hydroxyproline during the accumulation of collagen in cellulose sponge induced granulomas in rats.

Since the polyvinyl sponge posed the least problem in separating the granuloma tissue from the sponge, and the variability of enzyme activity was relatively low, this sponge type was used to screen for anti-inflammatory activity and steroidal effects on proline hydroxylase activity. Triamcinolone and betamethasone treatment inhibited granuloma growth and decreased proline hydroxylase activity (Table 3). The data show the biochemical mechanism by which betamethasone inhibited collagen synthesis in carageenin induced inflammatory tissue (Fukuhara & Tsurufuji, 1969). Some workers (Nocenti et al., 1964) have used the argument that since steroids do not decrease total hydroxyproline but do reduce collagen synthesis as determined by labeled proline incorporation, the primary steroidal effect may be to depress a basic cellular metabolic process. However,

steroid induced alteration of collagen resulting from decreased collagen synthesis will only be manifested when the tissue collagen has had time to turn over.

Although some workers feel that the inhibitory effect of steroids on collagen may be explained by stabilization of lysosomes (Weissmann, 1965a & b) or by the increased activity of collagenase (Houck & Sharma, 1969), the results of the present investigation indicate that these drugs decrease collagen synthesis by inhibiting the rate limiting enzyme. After administration of cortisol, Houck et al. (1967) found that the loss of cutaneous collagen is at least ten-fold greater than normal loss of all tissue collagen from the whole rat. This author concluded that inhibition of collagen anabolism alone cannot explain the observed decrease in collagen concentration. However, in the overall synthesis of collagen proposed by Prockop and Kivirikko (1967), the conversion of the insoluble to one of the more soluble pools resulting from a depletion of this latter pool is quite feasible. There is evidence of the close dependence of collagen pools on one another. The

proportion of acid soluble collagen increased in subacute inflammation produced by polyvinyl sponge implants only after the level of insoluble collagen remained constant (Delaunay and Bazin, 1969). Houck and Patel (1965) observed a 22 percent decrease in the insoluble pool of collagen and a three-fold increase in hydroxyproline concentration of soluble collagen. The specific activity of hydroxyproline after a pulse label of radioactive proline decreased by five fold. Dilution of the soluble pool by unlabeled hydroxyproline from the insoluble pool alone does not account for the decrease in hydroxyproline specific activity observed in the soluble pool. The effect of steroids on proteolytic and collagenolytic activities (Houck and Sharma, 1969) may be the mechanism for rendering the insoluble pool more soluble. However, the ability of these corticosteroids to inhibit proline hydroxylase probably accounts for the induced decrease in collagen content produced by these drugs.

My results are in agreement with those of Kivirikko et al. (1965) and Smith (1967) that the corticoids decrease collagen through a depressant effect on the synthesis of soluble collagen. My data indicate that the mechanism by which corticoids accomplish this decrease in soluble

collagen is the inhibition of proline hydroxylase, the rate limiting enzyme in the collagen biosynthetic pathway.

## V. SUMMARY AND CONCLUSIONS

(1) Triamcinolone, hydrocortisone and methylprednisolone significantly decreased liver proline hydroxylase activity. A dose dependent relationship of triamcinolone on liver proline hydroxylase activity was observed.

(2) Liver proline hydroxylase activity was significantly elevated in adrenalectomized rats and was decreased to control level by hydrocortisone treatment.

(3) The temporal relationships for proline hydroxylase activity and protein content were determined during granuloma growth induced by sub-dermal implants of polyurethane, cellulose and polyvinyl sponges. The sponge-induced granuloma affords an excellent in vivo model to study the regulation of the proposed rate limiting enzyme of the collagen biosynthetic process. Studying the biochemical events temporally related to increased or decreased enzyme activity and/or the amount of enzyme in this model system might lead to further elucidation of the biochemical regulators of this rate limiting enzyme.

(4) The polyvinyl sponge was used to study the effects of steroids on the growth of granuloma tissue and proline hydroxylase activity. Granuloma body weight

ratio was used as a parameter of anti-inflammatory activity. Local treatment with triamcinolone and betamethasone decreased granuloma growth and inhibited proline hydroxylase activity.

(5) These data support the hypothesis that the decrease in collagen content observed after glucocorticoid treatment is mediated through inhibition of proline hydroxylase activity.

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VII. VITA

Kenneth Robert Cutroneo was born to Mr. and Mrs. Santo J. Cutroneo on October 9, 1943 in Providence, Rhode Island. Mr. Cutroneo obtained his elementary education in Johnston and North Providence, Rhode Island. He obtained his secondary education in North Providence, Rhode Island. In 1962 Mr. Cutroneo enrolled at Providence College and received the Bachelor of Arts degree in biology in June, 1966. Mr. Cutroneo entered the graduate school of the University of Rhode Island in September, 1966 where he completed the requirements for the Master of Science degree in pharmacology in August, 1968 and the Doctor of Philosophy degree in pharmaceutical science in September, 1970. He is a member of the Alpha Epsilon Delta and Delta Epsilon Sigma fraternities and the Rho Chi, Phi Sigma and Sigma Xi societies.

Mr. Cutroneo is married to the former Carole Frances Del Giudice of Providence, Rhode Island. They are the proud parents of a son, Kenneth Robert Cutroneo II, born on August 21, 1970.

Mr. Cutroneo has accepted a post-doctoral position under Dr. Edward Bresnick in the Department of Pharmacology at Baylor College of Medicine, Houston, Texas.