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STUDIES ON THE REGULATION OF PROLYL HYDROXYLASE

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by

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ABSTRACT

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The hydroxylation of proline is thought to be one of the critical cellular events necessary for the synthesis and secretion of structural collagen. Using antibody directed against prolyl hydroxylase it has been shown that there is an enzymatically inactive protein related to prolyl hydroxylase in mammalian tissue. This cross-reacting protein is always present in excess relative to active hydroxylase and it is not known whether it is a precursor or a degradation product of prolyl hydroxylase.

The turnover rates of prolyl hydroxylase and immunologically related protein, CRP, were examined using labeled leucine as precursor or by measuring the decay of elevated prolyl hydroxylase and CRP back to basal levels. Prolyl hydroxylase and CRP were purified from neonatal rabbit skin at various times following the administration of ^{3}H-leucine. Prolyl hydroxylase was purified by affinity chromatography. CRP was purified by antibody precipitation from the dialyzed 70% $(NH_4)_2SO_4$ supernatants and subsequent electrophoresis on 10% SDS polyacrylamide slab gels. CRP was shown to migrate similarly to the two prolyl hydroxylase monomers which had molecular weights of 65,000 and 60,000. A smaller

antigenic component (45,000) of CRP was also observed. However, only the higher molecular weight components were used in the turnover studies of CRP. The peak incorporation of label into prolyl hydroxylase was found to be 12 hours while for CRP this occurred within 2 hours. The loss of radioactivity from these protein pools denotes an apparent $T_{\frac{1}{2}}$ for prolyl hydroxylase of 73 hours and a $T_{\frac{1}{2}}$ for CRP of 53 hours. From the specific activity of free skin leucine pools, the effect of reutilization could be corrected and a true $T_{\frac{1}{2}}$ for prolyl hydroxylase of 45 hours was determined.

Prolyl hydroxylase and CRP in the aorta and liver of adult male rabbits were elevated by daily epinephrine-thyroxine treatment for 12 days. The decline of prolyl hydroxylase and CRP with termination of treatment in the aorta denotes $T_{\frac{1}{2}}$ values of 42 hours for both. Calculated enzyme Kd values, by both methods, indicate that breakdown of enzyme does not account for tissue CRP.

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INTRODUCTION

Collagen is the major structural protein in mammalian tissue. The composition of collagen is approximately one-third glycine, one-third proline or hydroxyproline and one-third non-aromatic amino acids. Synthesis occurs by a series of sequential steps starting with the formation of a polypeptide precursor. The lysine and proline residues are subsequently hydroxylated and triple helix formation occurs intracellularly. Following secretion collagen molecules are further processed to form insoluble fibers. The synthesis of collagen is prolific in early development and declines to lower values in adult stages. However, in pathologic conditions such as arteriosclerosis, cirrhotic liver disease and other conditions associated with buildup of connective tissue, there is an increase in the biosynthesis of collagen (Grant and Prockop, 1972). In order to effectively modify this pathological process the control of collagen synthesis must be elucidated.

Prolyl hydroxylase (EC i.14.11.2; proline, 2-oxoglutarate dioxygenase) converts specific prolyl residues in the peptide precursors of collagen to 4-hydroxyproline and is thought to be one of the critical cellular events necessary for the synthesis and secretion of structural collagen (Cardinale and Udenfriend, 1974). Although the importance of prolyl hydroxylase as a controlling factor in collagen synthesis is unclear,

large increases in activity have been reported in a variety of tissues responding to injury induced damage which result in increased collagen synthesis (Grant and Prockop, 1972). This parallelism has resulted in the use of prolyl hydroxylase as a marker for the rate of collagen synthesis, and has helped generate interest in the cellular regulation of this hydroxylase.

McGee et al. (1971), obtained evidence with antibody against prolyl hydroxylase that an enzymatically inactive antigen is present in L-929 fibroblasts which may be a precursor to the active enzyme. Examination of animal tissues using antibody directed against rat (Stassen et al., 1974), rabbit (Fuller et al., 1976) or human prolyl hydroxylase (Tuderman et al., 1975) has confirmed the presence of both forms of antigen (active enzyme and immunologically cross-reacting protein, CRP) in mammalian tissues with CRP always in excess relative to active hydroxylase. However, the demonstration of a precursor product relationship between these two proteins has not been reported.

This study examines the relationship between prolyl hydroxylase and CRP through the determination of the turnover rate of these two protein pools. Prolyl hydroxylase and CRP were purified from neonatal rabbit skin at various time periods following the administration of ^{3}H leucine. To confirm the half-lives observed in the neonates, prolyl hydroxylase and CRP were elevated in the vasculature of rabbits by daily exposure to thyroxine and epinephrine injections (Fuller

and Langner, 1970), and the rate of decay back to basal levels was measured.

LITERATURE SEARCH

Collagen and Prolyl Hydroxylase

Collagen is the major fibrous constituent of skin, tendon, liagment, cartilage, and bone. Its structure is quite unique, having three polypeptide chains linked in a helix formation. At present there are four genetically distinct types of collagen known in mammalian tissue and there are at least five structural genes involved with the synthesis of five different component chains (Miller, 1974). Collagen synthesis occurs by a series of steps starting with the synthesis of polypeptide precursors rich in proline and glycine called procollagen chains (Martin, 1975). While still on the polyribosomal complex susceptible proline and lysine residues are hydroxylated and glycosylation of the resulting hydroxylsyl residues occurs intracellularly. Triple helical aggregates of pro α chains are secreted by the cells and subsequently a segment is cleaved from the non-helical region on both the C and N terminal ends by the action of procollagen peptidases (Fessler et al., 1975; Goldberg, et al., 1975). Further extracellular processing occurs through the action of lysyl oxidase which oxidatively deaminates specific lysine and hydroxylysine residues leaving aldehyde moieties which form extra- and intramolecular cross-links through condensation reactions (Siegel et al., 1970).

It has been suggested that the hydroxylation of proline is one of the rate limiting steps in collagen synthesis (Udenfriend, 1966). Hydroxyproline is important for the structural integrity of collagen. The name prolyl hydroxylase is a descriptive title since it hydroxylates peptidyl bound proline. The enzyme was the first described member of the α-ketoglutarate requiring mixed-function oxygenases. It has been established for some time that molecular oxygen is the source of oxygen for the hydroxyl group, (Prockop, et al., 1962; Fujimoto and Tamiya, 1962). Alpha keto-glutarate is an essential co-factor which is stoichiometrically decarboxylated to succinic acid in relation to the amount of hydroxyproline formed (Rhoado and Udenfriend, 1968; Cardinale, et al., 1971). The "in vitro" reaction also requires a ferrous ion and ascorbic acid reduction-oxidation system. Other reducing agents,such as the tetrahydropteridines or reductones (Hutton et al., 1967) can substitute.

The cellular site at which hydroxylation of proline occurs was debated for some time. Miller and Udenfriend (1971) provided the first evidence that hydroxylation occurs on nascent chains by isolating ribosomes from guinea pig granuloma minces which had been incubated with ^{14}C proline. They showed that the ribosomes contained peptidyl bound ^{14}C proline and ^{14}C hydroxyproline was released from the ribosomes by puromycin treatment. Lazarides and Lukens (1971), confirmed that the site of hydroxylation occurs on nascent chains by

labeling 3T6 fibroblasts with ^{3}H proline and isolating polysomes and showed that they did contain radioactive hydroxyproline. They further showed that when hydroxylation was inhibited, underhydroxylated chains were still released.

The location of prolyl hydroxylase in membrane was suggested by the observation that enzyme activity is increased in homogenates by treatment with detergents (Guzman and Cutroneo, 1973; Harwood et al., 1974). Cutroneo (1974) reported the isolation of a microsomal fraction which contained both the highest specific activity of cellular prolyl hydroxylase and substrate which could be hydroxylated. EM studies using conjugated antibodies to prolyl hydroxylase have confirmed the microsomal localization of the enzyme (Al-Adnani et al., 1974; Olsen et al., 1973). Recently, Peterkofsky and Assad (1976) have shown that low concentrations (0.05%) of detergents such as Triton X-100 or Brij-35 can release prolyl hydroxylase from isolated microsomes. In addition, prolyl hydroxylase, which could subsequently be released from the microsomes by Brij-35, was resistant to trypsin proteolysis at concentrations which removed 40% of the protein from the microsomes. These results suggest that prolyl hydroxylase is located within the cisternae, either bound to the inner membrane or freely soluble.

Prolyl Hydroxylase Substrates

The hydroxylation of proline "in vivo" occurs primarily on nascent procollagen α chains. Thus, most

substrates are fairly large macromolecules but the enzyme will hydroxylate susceptible proline residues of small peptides. McGee, Rhoads, and Udenfriend (1971) studied the vasoactive peptide bradykinin and some of its substituted analogs as substrates for the enzyme. They showed that the minimum sequence required for prolyl hydroxylation was an X-Pro-Gly triplet. Adjacent amino acid residues to this triplet were shown to modify the rate at which hydroxylation occurred. Hutton et al. (1968) showed, using the synthetic peptide (Pro-Gly-Pro)n, that as the molecular weight of the substrate increased from 1,200 to 8,000 its effectiveness as a substrate also increased. This was due to a decrease in K_m while the V_{max} value remained constant.

There has been much work done on determining which susceptible proline residues get hydroxylated. Berg and Prockop (1973a) demonstrated, as others had deduced (Rhoads and Udenfriend, 1968; Fujimoto and Prockop, 1968), that collagen chains can only be hydroxylated when they are in random-coil configuration and not when they are triple helical. Bornstein (1967) showed, using CNBr cleavage fragments of rat α1 chains, that individual proline residues, which are susceptible to hydroxylation, were reproducibly, incompletely hydroxylated. The degree of hydroxylation seems to be tissue specific and is dependent on adjacent amino acids.

Purification of Prolyl Hydroxylase

Prolyl hydroxylase is a uniquitous enzyme being found in all mammalian tissue so far analyzed. The enzyme has been purified to homogeneity from several different sources having

high levels of activity including chick embryos (Berg and Prockop 1973b), newborn rat skin, (Rhoads and Udenfriend, 1970), and human fetal material (Kuutti et al., 1975). The molecular weight of prolyl hydroxylase, obtained from chick and human tissue is 240,000 (Kuutti et al., 1975). The molecular weight of the subunits are 61,000 and 64,000 (obtained by dissociation of the enzyme) suggesting that the enzyme is a tetramer (Kuutti et al., 1975). Amino acid analysis shows that the protein contains a large amount of aspartic and glutamic acid which accounts for its acidic nature.

Several different methods have been used in the purification of the enzyme. Initial purification methods involved $(NH_4)_2SO_4$ precipitation followed by ion-exchange chromatography and gel filtration (Rhoads and Udenfriend, 1970). Subsequently the high affinity of prolyl hydroxylase for its native substrate ($K_m \approx 2nM$ Berg and Prockop 1973a) was utilized to develop an affinity column method for purification of the enzyme (Berg and Prockop, 1973b). This procedure involved affinity chromatography of prolyl hydroxylase on a column containing Ascaris cuticle collagen linked to agarose and the elution of the enzyme from the column using a high concentration of a synthetic substrate, $(Pro\text{-}Gly\text{-}Pro)_n$. Recently, a second affinity column procedure has been developed based on the high affinity of the enzyme for its competitive inhibitor, poly (L-proline) (Tuderman et al., 1975a). The affinity column in this case consists of poly (L-proline), molecular weight 30,000, linked to agarose and the enzyme is eluted with poly (L-proline) which has a molecular weight of 5,700.

As a logical consequence of enzyme purification, antibodies have been developed against rat (Roberts et al., 1973) rabbit (Fuller et al., 1976), chick (Berg et al., 1972), and human (Kuutti et al., 1975) prolyl hydroxylase. Using these antibodies, several assays have been developed to measure enzyme related antigen in tissues. McGee and Udenfriend (1972a) used an antibody to rat prolyl hydroxylase to identify the presence of a protein immunologically related to prolyl hydroxylase (cross-reacting protein, CRP) in L-929 fibroblasts and reported the separation of CRP from enzyme. The main disadvantage of their enzyme immunoassay was that enzymatically inactive cross-reacting proteins could only be measured in extracts which contained little or no enzyme activity. Stassen et al. (1974) modified the original enzyme immunoassay so that it could be used in the presence of large quantities of active prolyl hydroxylase. With the modified enzyme immunoassay, it was shown that tissues of rat and mouse contain large amounts of CRP relative to the amount of active prolyl hydroxylase. McGee and Udenfriend (1972b) were able to isolate and separate these two protein species from early log phase cultures of L-929 fibroblast cells by ion-exchange and gel filtration chromatography. It was found that CRP from the fibroblasts has a molecular weight between 85,000 and 105,000 compared to prolyl hydroxylase which has a molecular weight between 260,000 and 300,000. The relationship of CRP and prolyl hydroxylase is still unclear. Recently, a radioimmunoassay has been developed for human and chick prolyl hydroxylase (Tuderman et al., 1975b).

Regulation of Prolyl Hydroxylase

Activation of collagen synthesis occurs as a response to injury or as a result of rapid growth. The mechanisms for the regulation of collagen synthesis, however, are unclear at the present time. The question of regulation becomes very important in fibrotic diseases where there is an overproduction of connective tissue elements. As a result, prolyl hydroxylase has been investigated as a possible controlling factor in collagen synthesis. It is well established that prolyl hydroxylase activity is increased in tissue when collagen synthesis is stimulated. Siegel (1976) has studied the temporal relationship of the increases in the various enzymatic steps required for collagen biosynthesis in the carbon tetrachloride damaged liver. This data clearly indicates that increased prolyl hydroxylase activity is the first change observed and that this occurs even prior to an increase in collagen chain synthesis. In experimental models for disease states such as epinephrine-thyroxine induced atherosclerosis (Langner and Fuller, 1973), where increases in prolyl hydroxylase occur before there are detectable changes in collagen synthesis, CRP levels are also increased but to a smaller extent than prolyl hydroxylase (Fuller et al., 1976).

It has been proposed that proline hydroxylation is a rate limiting step in collagen biosynthesis (Udenfriend, 1966). This is based on the fact that inhibition of prolyl hydroxylase by α,α' dipyridyl in chick tendon cells causes secretion of procollagen at a very reduced rate (Jiminez et al., 1973).

In a related study, Jiminez et al., (1974) suggests that this is due to the failure of underhydroxylated collagen to form stable triple helices which may be required for secretion. Additional evidence for the necessity of prolyl hydroxylation would include the fact that none of the identified human inheritable connective tissue diseases involves the loss of prolyl hydroxylase activity while there are genetic diseases accredited to each of the other enzymes in the collagen biosynthetic pathway (McKusick, 1972).

Cell culture has been a popular method for studying the regulation of prolyl hydroxylase. Studies with L-929 fibroblasts in culture show that the formation of peptidyl bound hydroxypropline increases toward the end of the logarithmic phase of growth which is accompanied by a sharp increase in prolyl hydroxylase activity (Green and Goldberg, 1963; Gribble et al., 1969). Enzyme activity in early log phase cells can be increased by concentrating the cells to a higher density (Comstock et al., 1970); or by the addition of sodium lactate (Comstock and Udenfriend, 1970), or sodium ascorbate (Stassen et al., 1973) to the culture medium. Administration of protein synthesis inhibitors such as puromycin or cycloheximide do not inhibit these increases in prolyl hydroxylase activity and hydroxyproline formation (Peck et al., 1967; Comstock and Udenfriend, 1970). Further suggesting that protein synthesis is not required for an increase in prolyl hydroxylase activity, McGee et al. (1971) demonstrated taht during cell crowding or lactate treatment the amount of enzyme related antigen remains

constant while enzyme activity increases several fold. On the basis of this data the authors postulated that the CRP present in cells may be a subunit precursor to active enzyme. The work of Stassen et al. (1973) provides additional evidence that these two protein species are functionally related. Using DEAE-Sephadex to separate CRP from active enzyme they were able to show almost quantitative conversion of enzyme to the smaller inactive protein by the treatment of intact L-929 cells with dithiothreitol. When the cells were treated with ascorbate and cycloheximide, enzyme activity could be partially restored. In addition, when cells were incubated for 24 hours in fresh medium, enzyme activity returned to normal.

Kuttan et al. (1975) recently showed that the activation carried out in whole cells could be effected in sonicates of the same cells. The requirements for "in vitro" activation are identical to those needed for the hydroxylation reaction i.e., α-ketoglutarate, ascorbate, ferrous ion and catalase. During activation hydroxyproline is formed (Kuttan, 1976). This would suggest that there is a complex between active enzyme and an underhydroxylated form of collagen. Thus activation occurs when the enzyme is freed due to hydroxylation of the endogenous substrate. It appears that "in vivo" activation in tissue culture is the same as the "in vitro" activation since the same maximum level of enzyme activation is effected by both methods. In addition, once maximum activity is achieved "in vitro" activatible form of the enzyme is different from the

small molecular weight component of CRP since the activatible enzyme, CRP and active enzyme can be separated into three peaks on DEAE-Sephadex (Kuttan et al., 1975). Thus CRP is a heterogeneous pool, but in all probability a portion of the pool is precursor to active enzyme.

In experiments designed to elucidate the mechanisms of fibrosis, McGee et al. (1973) have found a material fractionated from the liver of mice with acute carbon tetrachloride liver injury which stimulates prolyl hydroxylase and collagen synthesis in L-929 fibroblasts. Three collagen stimulating factors were found with an approximate molecular weight of 5,000 which were not found in control livers. It is possible that these factors may control the synthesis of collagen "in vivo".

Protein Turnover

It is now well established that all proteins are continually being turned over. Schimke (1974) has reviewed the subject and has described several common features of protein turnover. First, it appears that most intracellular proteins are degraded intracellularly. Secondly, there is heterogeneity between the rates of degradation of different proteins. In fact, the rate of degradation of a given protein within a cell can change with respect to the metabolic state of that cell.

A multitude of methods has been used to measure protein half-lives but all are based either on time course of

changes in enzyme activity or on the use of isotopic tracers. Rates of synthesis and degradation can be obtained by observing the time course of change in enzyme activity (increased or decreased) after the institution or withdrawal of a stimulus (Segal and Kim, 1963). Any change in enzyme content can be described by the following equation:

$$\frac{dE}{dt} = Ks - KdE$$

E is the concentration of enzyme, Ks is a zero-order rate constant of synthesis and Kd is a first-order rate constant for degradation. At steady state:

$$Ks = KdE$$

If enzyme activity is stimulated to a higher level, $(Eo)'$, and the decay of activity back to basal conditions (Eo) is measured, than at any time t:

$$\ln (E)-(Eo) = \ln (Eo)'-(Eo) - Kdt$$

Thus a plot of ln (E)-(Eo) versus t, as the activity returns to normal steady state levels, allows for the determination of Kd. Assumed in this method is that the observed rate of decay is characteristic of the rate constant of degradation in the basal state.

Although saturation labeling is more precise, the most common method for measuring the rate of degradation using isotopes is the single administration of a radioactive amino acid precursor due to the high cost of label. The loss of

specific activity in the protein is exponential, which allows for the calculation of Kd. The major limitation of this method is reutilization of labeled amino acid. The effect of label reutilization on the measurement of turnover, however, can be calculated (Poole, 1971).

EXPERIMENTAL

Animals

Three-week-pregnant, albino New Zealand rabbits obtained from Gloucester Rabbitry (Gloucester, Rhode Island), were isolated in separate floor pens. Three-day-old rabbit pups were used for label injection and were kept with their mothers until sacrificed. Male albino New Zealand rabbits weighing 1.5 to 2 kilograms were also obtained from Gloucester Rabbitry one week prior to the start of the epinephrine-thyroxine injections. All animals were maintained in rooms at an ambient temperature of 24-27°C, with alternating 12-hour light/dark cycles. All adult animals were offered a commercial laboratory chow and water ad libitum.

Materials

All chemicals used in this investigation were analytical reagent grade. The following radioactive amino acids were purchased from New England Nuclear Corporation, Boston, Massachusetts: L-(4-^{3}H)-proline (25-50 Ci/mM), and L-(4,5-^{3}H)-leucine (35-50 Ci/mM). Antibody directed against rabbit prolyl hydroxylase was prepared in goats in Dr. S. Udenfriend's laboratory at Roche Institute of Molecular Biology, Nutley, New Jersey. Standard prolyl hydroxylase for amino acid analysis, electrophoresis, and immunoassay was prepared by affinity chromatography (Berg and Prockop, 1973). The following specialized reagents were obtained for affinity

chromatography: CNBr-activated Sepharose 4B (Pharmacia), reduced carboxymethylated Ascaris collagen (Applied Science Laboratories) and $(Pro\text{-}Gly\text{-}Pro)_n$ MW 2300 (Miles-Yeda, Ltd).

Purification of Labeled Prolyl Hydroxylase and CRP

Three day old rabbit pups were injected with 4,5-^{3}H-leucine i.p. (42.6 Ci/mM New England Nuclear) and returned to their mother until killed. Animals were killed at various time periods, the skins quickly removed and homogenized 1:5 in cold 0.25 M sucrose containing 10^{-5} M dithiothreitol (DTT) and 10^{-5} M EDTA. A 20,000 x g supernate was prepared and brought to 30% saturation in $(NH_4)_2SO_4$ (via addition of saturated $(NH_4)_2SO_4$ solution). After removal of the pellet by centrifugation, the supernate was brought to 70% $(NH_4)_2SO_4$ saturation and the protein precipitated was harvested by centrifugation at 45,000 x g for 2 hours. This pellet was dissolved in 0.05 M Tris-HCl (pH 7.4) containing 0.2 M glycine, 0.2 M NaCl, and 10^{-5} M in EDTA and DTT and dialyzed against the same buffer to remove remaining $(NH_4)_2SO_4$. After centrifugation at 45,000 x g, this enzyme solution was placed on affinity columns consisting of reduced and carboxymethylated Ascaris collagen coupled to Sepharose 4B as previously described (Berg and Prockop, 1973). Each individual sample was applied to a separate 1.5 x 4 cm column, and enzyme was eluted with 1 ml of buffer containing 10 mg/ml $(Pro\text{-}Gly\text{-}Pro)_n$ (MW 2300). After concentration and washing by ultrafiltration (MINICON®-A-25) enzyme activity was measured. In addition, aliquots of each sample were taken to

measure ^{3}H (determined in 10 ml Aquasol), leucine content and total enzyme related antigen.

CRP was purified from the 70% $(NH_4)_2SO_4$ supernate after dialysis against 0.05 M Tris-HCl (pH 7.4) buffer containing 0.1 M NaCl, 10^{-5} M EDTA and 10^{-5} M DTT, and concentrated in an ultrafiltration cell (Amicon, PM-30 membrane). Each sample was adjusted in volume to a uniform concentration of CRP and the amount of antisera to give maximum precipitation of CRP was determined. Antisera and CRP solution were incubated at 37°C for 30 minutes and then at 4°C for 24 hours. Immunoprecipitates were harvested and washed twice by centrifugation (5,000 x g for 15 minutes). They were then either collected on filters or dissociated and electrophoresed on polyacrylamide gels.

The antibody directed against rabbit prolyl hydroxylase required for these experiments was obtained from an immunized goat using conditions similar to those previously reported for the antiserum directed against rat skin prolyl hydroxylase (Roberts, et al., 1973). The antigen used was rabbit skin prolyl hydroxylase purified by affinity chromatography. Before injection into goats the enzyme was separated from the $(Pro\text{-}Gly\text{-}Pro)_n$ used to elute the enzyme by electrophoresis in 7.5% polyacrylamide at 4°C (Davis, 1966). The tetramer form of the enzyme, which constituted the major band on each gel in these preparations, was identified by its catalytic activity and could be measured in undenatured form as a fluorescent

band in ultraviolet light by staining with anilinonaphthalene sulfonate (Hartman and Udenfriend, 1969). The fluorescent bands corresponding to the enzyme were cut from 10 disc gels, pooled and homogenized in 2 ml of 0.05 M Tris-HCl buffer (pH 7.4) containing 10^{-3} M DTT and 10^{-5} M EDTA. The homogenized gels were emulsified with an equal volume of complete Freund's adjuvant and injected at multiple sites subcutaneously. The goat received approximately 1 mg of the electrophoretically [illegible]zyme protein. Blood was drawn before injection to pro[illegible]ntrol sera. Three months later the goat was given an[illegible] mg of enzyme (after the antibody titers came back to normal). A second booster was given a month later and the antisera taken 2 weeks later. The specificity of the antisera to prolyl hydroxyla[illegible] was demonstrated by immunodiffusion against crude and purified preparations of the enzyme.

Polyacrylamide Electrophoresis

Initial experiments analyzing immunoprecipitates were done on 7.5% acrylamide disc gels (Davis, 1966). This system was also used to determine the purity of enzyme preparations. Immunoprecipitates from the concentrated, dialyzed 70% $(NH_4)_2SO_4$ supernatants and from the resuspended, dialyzed 70% $(NH_4)_2SO_4$ pellets were harvested and washed by centrifugation. The samples were then resuspended in 100 ul of 8 M urea, 1% mercaptoethanol and incubated at 50°C for 2 hours. Enzyme subunits were obtained by denaturing standard enzyme in the same manner. Electrophoresis was carried out at 4 ma. per tube for 2 hours. The gels were stained with Coomassie Brilliant Blue in 12.5% trichloracetic acid (TCA) and destained in 12.5% TCA.

To quantitate the radioactivity present, the gels were sliced and the tritium content determined (in 10 ml Aquasol) following digestion in 0.1 ml H_2O_2.

The final system for studying the incorporation and decay of label in CRP was the 10% SDS-polyacrylamide slab system (70 cm x 10 cm x 2.5 mm) described by Laemmli and Faure (1973). Washed immunoprecipitates were brought up in 25 ul of 6 M urea, 2% mercaptoethanol, 2% SDS and 10% glycerol, heated at 50°C for 2 hours, and 20 ul of the dissociated samples were loaded into separate wells of the slab gel apparatus (Pharmacia). Standard enzyme was also denatured in a similar manner as well as RNA-polymerase, carboxymethylated bovine serum albumin, ovalbumin and chymotrypsinogen A for molecular weight dermination. Electrophoresis was carried out at 60 V until the samples entered into the 6% stacking gel and then 120 V (constant voltage) for 2 hours. Slabs were stained and fixed with 0.05% Coomassie Brilliant Blue (w/v) in 10% aqueous acetic acid. The gels were scanned at 590 nm on a Gilford 250 spectrophotometer with a grid slit plate of 2.36 x 0.05 mm and a slit width of 0.2 mm. After scanning the gels were fractionated and the tritium content determined.

Determination of Free Leucine Specific Radioactivity in Homogenates

From each of the 20,000 x g supernatants 0.4 ml aliquot was taken and the protein precipitated with 50 ul of 50% TCA.

After 30 minutes on ice the samples were centrifuged and the supernates harvested. Each sample was placed on a Dowex 50W-8X column (5 ml bed volume) and was washed with 15 ml distilled water. The columns were eluted with 2 ml of 10N NH_4OH and 2 ml fractions were collected. The radioactive fractions were pooled, lyophilized and then resuspended in 100 ul of 0.15N lithium citrate buffer (pH 2.2). Tritium content was determined in 10 ml Aquasol and leucine content determined by amino acid analysis on a Durrum D-500 analyzer (Lee, 1974).

Amino Acid Analysis of Purified Enzyme Samples

The leucine content of labeled enzyme samples as well as standard prolyl hydroxylase pools were determined by amino acid analysis after hydrolysis. The standard enzyme pools were purified in large batches according to the previously described affinity method (Berg and Prockop, 1973), using a 1.5 x 30 cm column. After the enzyme was eluted from the column, with 10 ml of $(Pro\text{-}Gly\text{-}Pro)_n$ (10 mg/ml), it was concentrated 10-fold in an ultrafiltration chamber with a membrane having a 30,000 MW cut-off. For amino acid analysis and electrophoresis enzyme was separated from $(Pro\text{-}Gly\text{-}Pro)_n$ using a Sephadex G-200 or G-150 column (0.9 x 30 cm). Enzyme pools were dialyzed exhaustively against distilled water before hydrolysis.

The dialyzed standard pools and the labeled enzyme sample were hydrolyzed at 110°C in 6N HCl, 0.5% phenol for 20 hours in tubes sealed under a 25 millitore vacuum. The

samples were then evaporated to dryness, brought up in lithium citrate buffer and analysis was carried out on the Durrum D-500 analyzer (Lee, 1974).

Epinephrine-Thyroxine Induced Arteriosclerosis

Prolyl hydroxylase and CRP were elevated in the vasculature of male New Zealand rabbits by daily injections of epinephrine and thyroxine for 12 days (Fuller and Langner, 1970). Epinephrine (0.025 mg/kg for the first 5 days; 0.050 mg/kg thereafter) was injected via the marginal ear vein with an infusion pump (Harvard Apparatus Company Inc., Model 940, Dover, Massachusetts). Thyroxine (0.050 mg/kg), freshly prepared, was injected intraperitoneally. Animals were killed by cervical dislocation 1, 3, 5, and 8 days after the last injection. The aorta and liver were quickly removed and chilled. Freshly dissected samples of thoracic aorta and liver tissues were homogenized in a Polytron system at 4°C in a buffer consisting of 0.25 M sucrose, 10^{-5} M EDTA, 10^{-5} M DTT and 0.1% Triton X-100 (aorta 1:10 w/v and liver 1:20 w/v). Aliquots of the 15,000 x g supernates were taken for prolyl hydroxylase, CRP, and protein assays.

Prolyl Hydroxylase Activity

Prolyl hydroxylase activity was measured by the methods based on the stoichiometric formation of tritiated H_2O and hydroxyproline when a substrate consisting of a polypeptide rich in 4-^{3}H proline is incubated with enzyme and cofactors,

as described by Hutton et al. (1966). The complete hydroxylating system (1 ml total volume) contained approximately 600,000 cpm of labeled substrate (0.1 ml), 0.5 mM ascorbic acid, 0.1 mM α-ketoglutarate, 0.1 mM ferrous ammonium sulfate, Tris-HCl buffer 0.05 M (pH 7.5), 2% bovine serum albumin and catalase (0.02 ml), which are experimentally determined optimum conditions. Incubations were ended after 30 minutes by the addition of 0.1 ml of 50% trichloroacetic acid and the tritiated water formed was collected by vacuum distillation. The distillate (0.8 ml) was added to 10 ml of Aquasol (New England Nuclear) and the radioactivity determined.

Preparation of Substrate for Prolyl Hydroxylase Assay

The tritium labeled substrate was prepared using the method of Hutton et al. (1966). Five hundred 7 to 8 day chick embryos were removed, decapitated and the bodies placed in ice-cold Krebs-Ringer buffer[a] (Stone and Meister, 1962). The intact embryos were washed twice with ice-cold Krebs and a mince was made in the presence of a small amount of ice-cold Krebs. After the tissue was washed in buffer and drained, 5 to 6 gram aliquots were placed in 50 ml beakers followed immediately by sufficient Krebs buffer to bring volume to 20 ml. After adding 0.5-1.0 mCi 4-^{3}H-proline and 20 ul of 1 M α,α′-dipyridyl, the

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[a]NaCl, 1.285 g; KCl, 0.224 g; $MgSO_4$, 0.144; $CaCl_2$, 0.144 g; KH_2PO_4, 0.0544 g; $NaHCO_3$, 2.10 g; D-glucose, 1.80 g and distilled water to 1 liter.

mixture was incubated at 37°C for 2 hours in a metabolic shaker under O_2-CO_2 (5%-95%). At the end of 2 hours, the aliquots were centrifuged at 90,000 x g for 90 minutes and the supernates discarded. The newly formed collagen was extracted from the pellet by 0.5 M acetic acid (2 ml/gm tissue), with stirring, overnight. The supernatant containing the newly formed prolyl hydroxylase substrate was then centrifuged at 90,000 x g for 90 minutes and dialyzed against 10 volumes of 0.01 M Tris buffer (pH 7.4) with at least 4 changes. After dialysis, with the pH above 7.0, the substrate was autoclaved for 15 minutes and redialyzed against 3 changes of 10 volumes of Tris buffer. The redialyzed substrate was calibrated with standard rabbit enzyme and frozen in 10 ml aliquots.

Enzyme-Immunoassay for CRP

The enzyme immunoassay is based on the fact that prolyl hydroxylase is inhibited by its antibody and that enzymatically inactive but immunologically reactive protein (CRP) can compete with enzyme for antibody binding sites. Thus when cross-reacting protein is preincubated with a standard amount of antiserum, the expected inhibition of prolyl hydroxylase activity is reduced. The extent to which the enzyme is displaced from the antibody is related to the amount of antigen added to the antiserum. Heat in activated enzyme is used as standard. This relationship is used to express total prolyl hydroxylase related antigen and CRP as enzyme equivalents.

Protein Determination

Protein was measured by the method of Lowry *et al.* (1951). The homogenizing buffer was used as a blank and treated as the other samples. Bovine serum albumin was used as a standard. The intensity of the blue color that develops was read at 750 nm on a Gilford 250 spectrophotometer.

Statistical Methods

a.) Mean: $X = \frac{1}{n} \sum X_i$

where n = sample size

X_i = sum of values for each sample

b.) Standard deviation: $S^2 = \frac{1}{n} \sum (X_1 - \bar{X})^2$

c.) Student's "t" Test: $$T = \frac{\bar{X}_1 - \bar{X}_2}{\sigma \sqrt{\frac{1}{N_1} + \frac{1}{N_2}}}$$

where

$$\sigma = \sqrt{\frac{N_1 S_1^2 + N_2 S_2^2}{N_1 + N_2 - 2}}$$

d.) Linear Regression: $Y = b_o + b_1 X$

where $b_1 = \frac{(X - \bar{X})(Y - \bar{Y})}{(X - \bar{X})}$

$$B_O = \bar{Y} - b_1 \bar{X}$$

e.) The influence of reutilization of leucine on the apparent turnover of prolyl hydroxylase was calculated by the method of Poole (1971). Stepwise integration of the differential equation was obtained using a nonlinear regression program developed by Metzler et al. (1974).

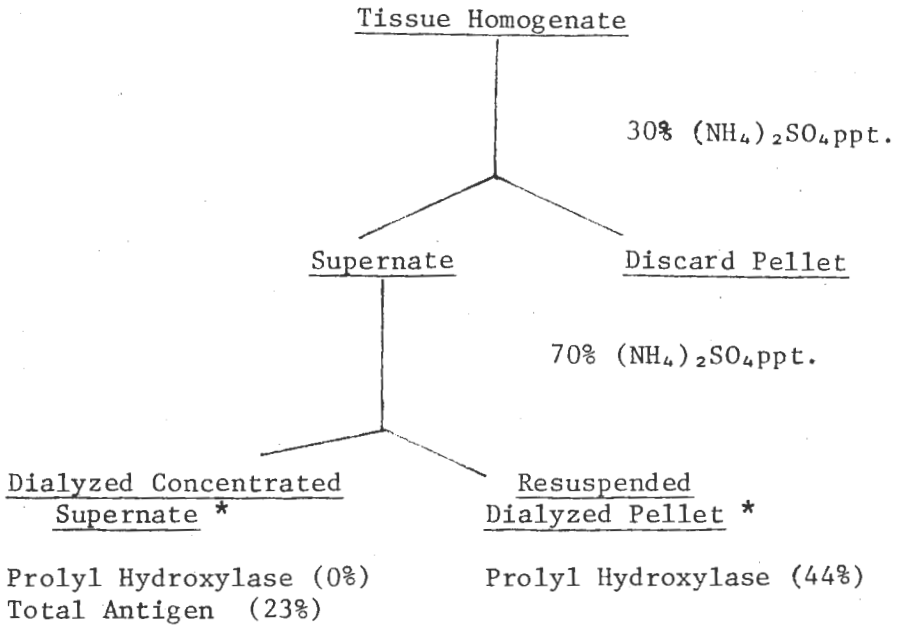
RESULTS

This investigation examined the turnover relationship of prolyl hydroxylase to an enzymatically inactive immunologically cross-reacting protein (CRP) found in all tissue. Prolyl hydroxylase and CRP were purified from the skin of 3 to 8 day rabbits. It was found that the prolyl hydroxylase activity of neonatal skin did not change significantly over this time period. Hydroxylase activity was determined to be 81.1 ± 7.3 cpm/ug protein ($\pm$ S.E., N=13) in the 20,000 x g homogenate supernates. CRP, as assayed in the same enzyme units, was 320.4 ± 2.19 cpm/ug protein while total antigen was 412.2 ± 20.9 cpm/ug protein. Thus, in this study, as in earlier reports (Stassen et al., 1974; Tuderman et al., 1975), CRP levels were much higher than prolyl hydroxylase levels.

The recovery of total antigen and prolyl hydroxylase activity during purification of enzyme and CRP are shown in Table I. After 30% $(NH_4)_2SO_4$ precipitation, some antigenic material was lost while total enzyme activity increased. The loss of antigenic material could be explained by the salting out of enzyme substrate complexes which recently have been identified in tissue extracts (Kuttan et al., 1975) while the increase in enzyme activity may be the result of the removal of a competing endogenous substrate and/or removal of endogenous inhibitor. The 70% $(NH_4)_2SO_4$ precipitation step completely removed prolyl hydroxylase activity from the super-

TABLE 1

PERCENT RECOVERY OF PROLYL HYDROXYLASE AND TOTAL ANTIGEN DURING PURIFICATION



*These fractions were processed as described in the text.

natant so that during antibody precipitation a CRP fraction that was devoid of active enzyme was obtained. The pellet from the 70% precipitation did contain some CRP, however, this does not bind to the affinity column and thus would not contaminate the final enzyme preparations.

Enzyme was purified from the resuspended, dialyzed 70% pellets by affinity chromatography. The purity of the enzyme preparations was examined by disc electrophoresis on polyacrylamide gels (Davis, 1966). In each of the enzyme preparations, only a single band was seen which contained the enzyme activity. After incubation with urea and mercaptoethanol, two smaller molecular weight bands were obtained which correspond to the two monomeric subunits of prolyl hydroxylase (Berg and Prockop, 1973). Immunoassay of enzyme preparations before and after dissociation into subunits showed no change in immuno reactivity. For amino acid analysis, the affinity purified enzyme preparations were freed from contaminating (Pro-Gly-Pro)n by Sephadex chromatography. The amino acid composition of rabbit prolyl hydroxylase is very similar to that of human prolyl hydroxylase. Both contain a high proportion of acidic amino acids (Table II).

In preliminary experiments which attempted to determine the relative incorporation of ^{3}H-leucine into prolyl hydroxylase and CRP, immunoprecipitates were made from both the 70% supernatant and pellet fractions. A neonatal rabbit pup was injected with 2mCi of ^{3}H-leucine and killed after 2 hours. The skin

TABLE II

AMINO ACID COMPOSITION OF PROLYL HYDROXYLASE FROM RABBIT SKIN

Amino Acid	Rabbit enzyme[1] (residues/1000)	Human enzyme[2] (residues/1000)
Aspartic acid	109 ± 2	122 ± 2
Threonine	50 ± 1	51 ± 2
Serine	64 ± 12	45 ± 1
Glutamic acid	144 ± 2	142 ± 6
Proline	54 ± 6	50 ± 0
Glycine	84 ± 8	72 ± 1
Alanine	87 ± 1	89 ± 3
Valine	54 ± 2	58 ± 1
Methionine	10 ± 1	- -
Isoleucine	37 ± 2	45 ± 0
Leucine	92 ± 4	98 ± 0
Tyrosine	31 ± 1	22 ± 2
Phenylalanine	50 ± 2	52 ± 1
Lysine	73 ± 7	93 ± 4
Histidine	22 ± 1	19 ± 1
Arginine	40 ± 2	42 ± 1

Values were not corrected for losses during hydrolysis.

[1]Expressed as mean ± S.E. for the analysis of three separate enzyme pools.

[2]From Kuutti, Tuderman and Kivirikko, 1975.

was removed, fractionated, and antibody was added to the dialyzed 70% supernatant (10 mls) and to the resuspended, dialyzed 70% pellet (10 mls). The resulting immunoprecipitates were harvested, dissociated with urea and mercaptoethanol, and electrophoresed on 7.5% polyacrylamide disc gels. In both preparations 2 bands were seen of equal intensity that corresponded to the 2 subunits of prolyl hydroxylase. The gels were then sliced and the radioactivity determined. As can be seen in Figure I, the monomers derived from the 70% supernatant appear to be of much higher specific radioactivity than those from the 70% pellet. Similar experiments using short labeling periods (<2 hours) substantiated these results.

A more extensive study was undertaken to determine whether the differences in incorporation of label into these two proteins could be explained by differences in turnover. The turnover rates of both CRP and prolyl hydroxylase were measured by the decay of radioactivity from these protein species. Prolyl hydroxylase and CRP were purified from neonatal rabbit skin at various time periods following the injection of 5 mCi/60g of ^{3}H-leucine. Figure II shows the amount of ^{3}H-leucine in prolyl hydroxylase as a function of time after a single administration. Prolyl hydroxylase was purified by affinity chromatography from the 70% $(NH_4)_2SO_4$ pellet and the specific activity of ^{3}H-leucine in the purified enzyme is expressed as CPM incorporated per unit of hydroxylase related antigen. From the figure it would appear that maximum label

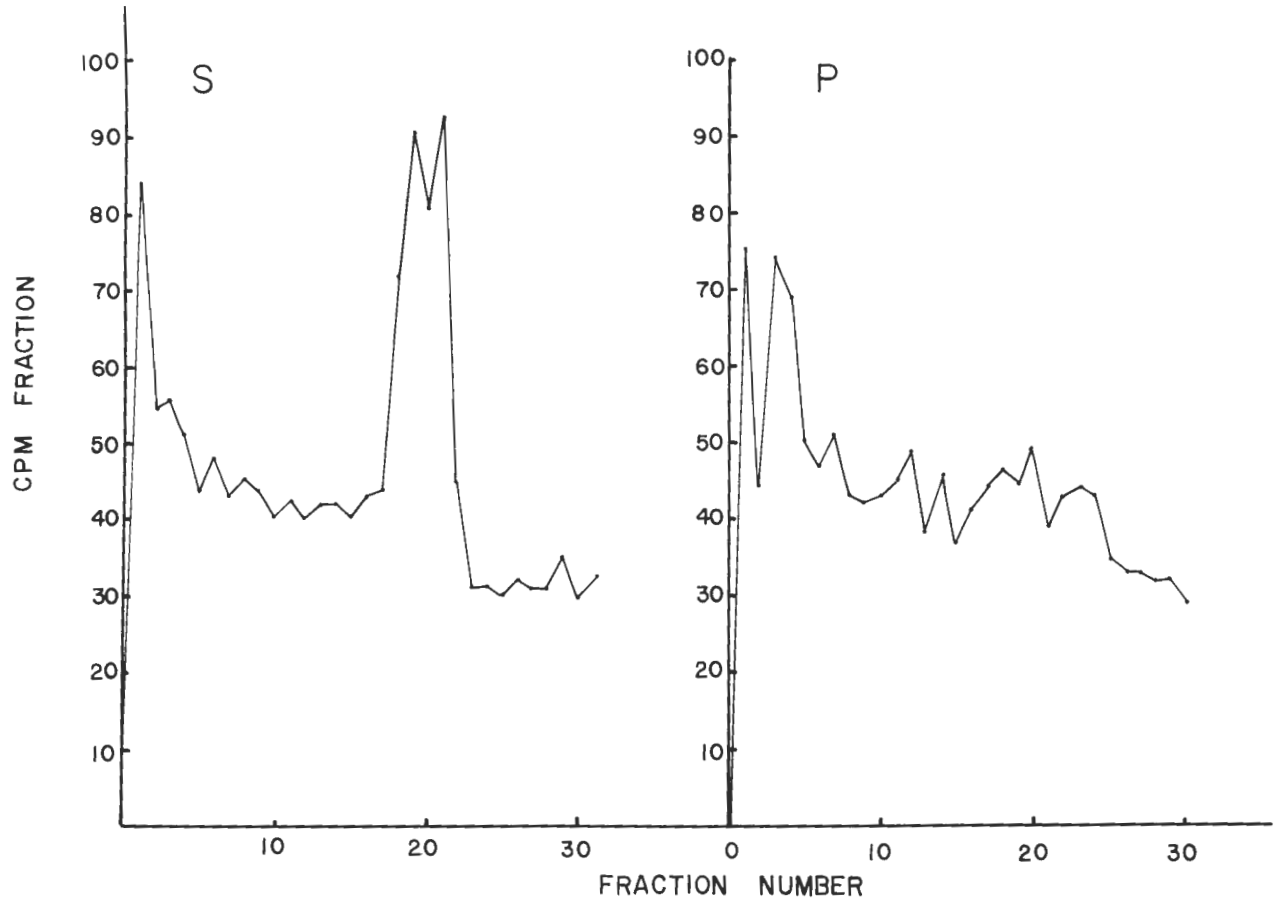


Figure I - Immunoprecipitates from the 70%$(NH_4)_2SO_4$ supernatant (S) and from the 70%$(NH_4)_2SO_4$ pellet (P) of neonatal rabbit skin 2 hours post injection with 2mCi ^3H-leucine. Immunoprecipitates were prepared as described in the text, dissociated and electrophoresed on 7.5% polyacrylamide gels. The gels were then sliced and the radioactivity determined.

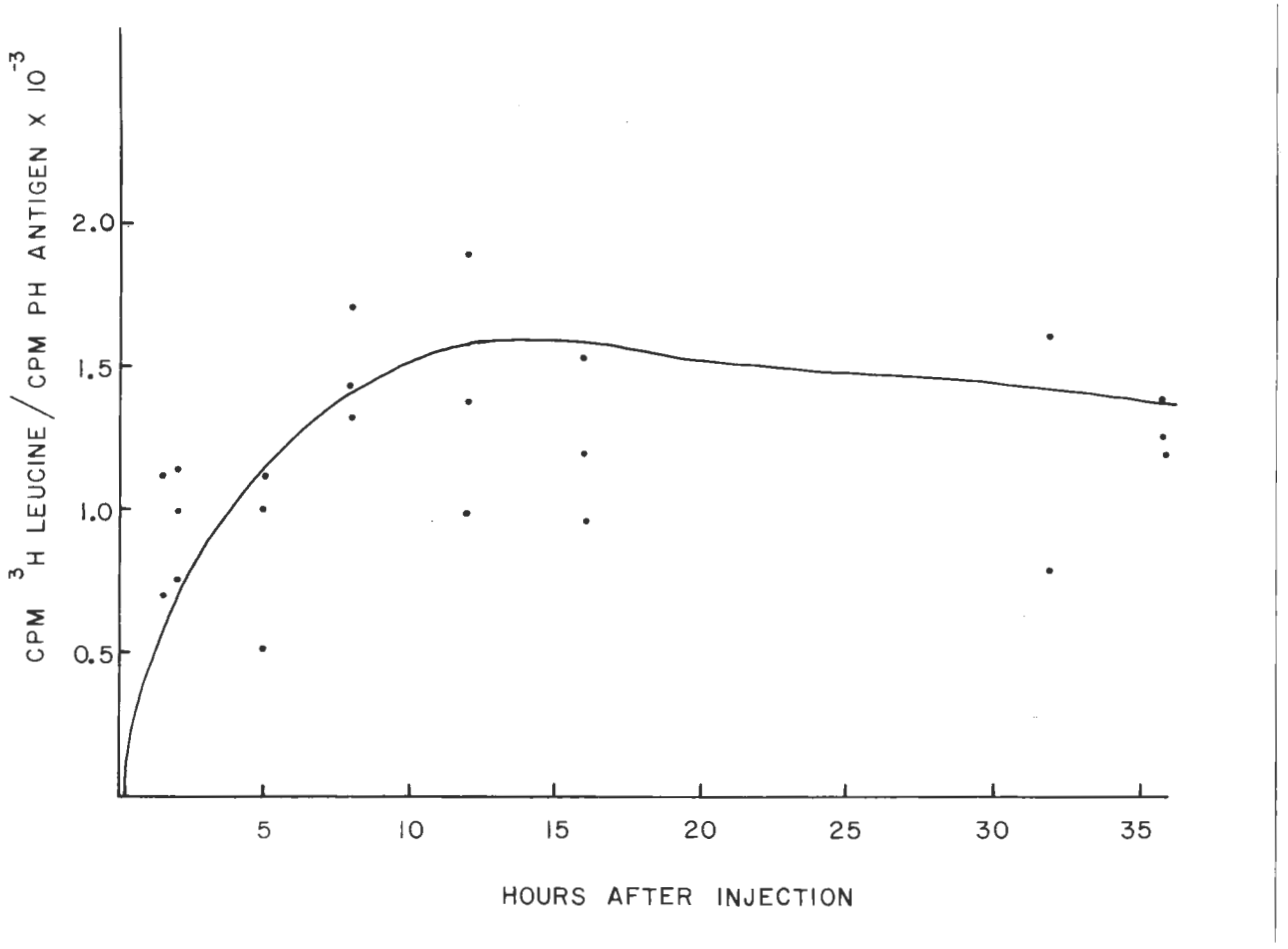


Figure II - Specific radioactivity of leucine in skin prolyl hydroxylase as a function of time following the administration of ^{3}H-leucine. Neonatal rabbits were injected with 5mCi/60g of ^{3}H-leucine and killed at the times indicated. The skins were removed, fractionated and prolyl hydroxylase was purified by affinity chromatography. Specific activity is expressed as cpm incorporated per unit of hydroxylase related antigen.

incorporation occurs approximately 12 hours following injection.

The decay of labeled leucine from skin prolyl hydroxylase is shown in Figure III. Animals were again injected with 5mCi/60g of ^{3}H-leucine and killed at the times indicated. The skins were removed and fractionated, and prolyl hydroxylase was purified by affinity chromatography. Leucine specific activity is expressed as CPM incorporated per nanomole of leucine in the purified enzyme samples. Because of the low amount of protein in these hydroxylase samples, it was not possible to obtain a direct determination of leucine concentration for every sample. Instead, a correlation was obtained between prolyl hydroxylase activity of purified enzyme samples and leucine content. Many of the purified enzyme samples from this experiment plus a few additional samples were hydrolyzed (N=14) and their leucine concentration determined by amino acid analysis. A highly significant correlation coefficient was obtained (r=0.996) between hydroxylase activity and leucine concentration: $y=0.742x10^{-5}x + 0.06168$ where y = nanomoles of leucine and x = CPM of prolyl hydroxylase activity. Applying this equation to the hydroxylase activities of the purified enzyme samples enabled us to express the specific radioactivity in these samples in terms of leucine concentration.

To determine the rate of turnover, the decay of radioactivity in a given protein is assumed to be first order and can be expressed by $\frac{dP(t)}{dt} = -kP(t)$ where P(t) is the specific

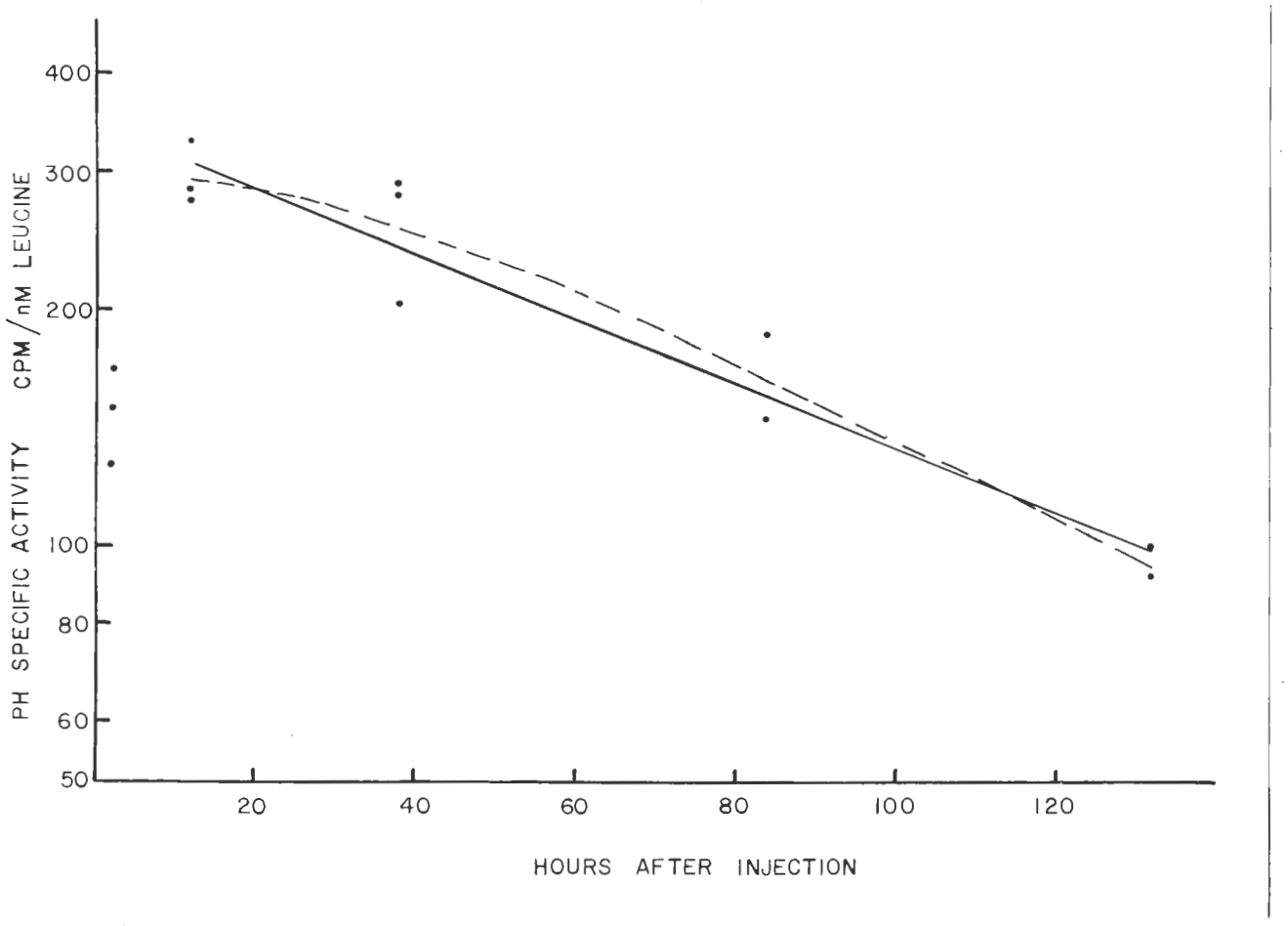


Figure III - Specific radioactivity of leucine in skin prolyl hydroxylase as a function of time following the administration of ^{3}H-leucine. Neonatal rabbits were injected with 5mCi/60g of ^{3}H-leucine and killed at the times indicated. The skins were removed, fractionated and prolyl hydroxylase was purified by affinity chromatography. The solid line represents the best fit line from a computer regression program. The dotted line represents the solution to the equation which corrects for reutilization.

radioactivity in the protein and k is its rate of destruction. Assuming this equation to be accurate, the points in Figure III were regressed by computer from 12 to 132 hours and the solid line, representing a $T_{\frac{1}{2}}$ of 73 hours, was plotted. This was significant at the $P < .005$ level. Poole (1971), however, has suggested that in these types of labeling experiments there is a significant amount of reutilization of precursor and that the appropriate differential equation is $\frac{dP(t)}{dt} = k \left/ \overline{F}(t) - P(t) \right/$ where P(t) represents the specific radioactivity of precursor in the protein and F(t) represents the specific radioactivity for the pool of the precursor.

In order to correct for reutilization, free leucine activity was measured in the same experiment. Figure IV shows the decay of radioactivity in free skin leucine after injection. Similar to other studies utilizing radioactive amino acids, there is a rapid decay phase during the first few hours after injection. Regression by computer of the points representing the slow decay phase resulted in the equation $F(t)=269.92e^{-0.01946t}$ which fits the data reasonably well ($r=0.961$). To correct for reutilization the equation $\frac{dB(t)}{d(t)} = k \left/ \overline{F}(t) - P(t) \right/$ was integrated stepwise with $F(t)=269.92e^{-0.01946t}$ utilizing a non-linear regression program. The best fit with the data in Figure IV was obtained with a $k=0.01539\ hr^{-1}$. As shown by the dotted line on Figure III this fits the data very well and corresponds to a true $T_{\frac{1}{2}}$ of 45 hours.

Immunoprecipitation was used to determine the turnover of CRP. Figure V shows an antibody precipitation curve where

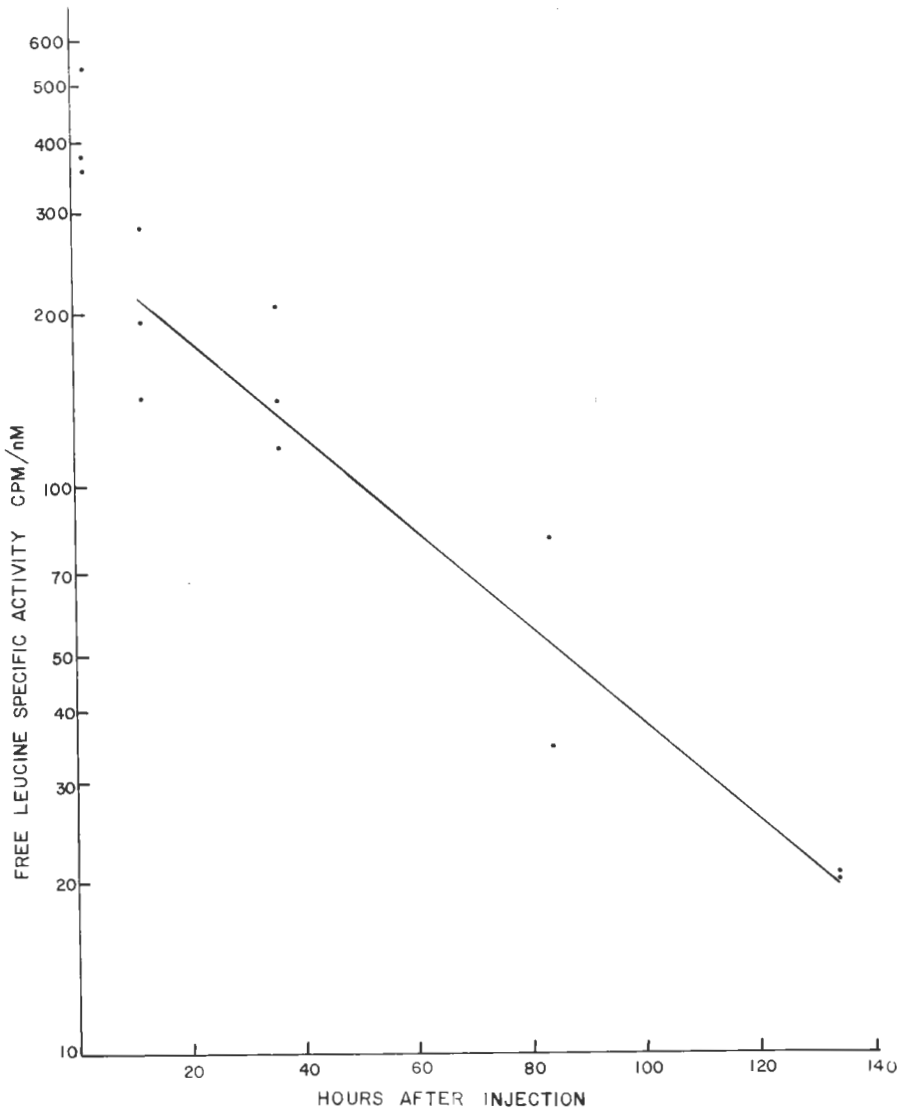


Figure IV. Specific radioactivity of free skin leucine as a function of time following the administration of ^{3}H-leucine (5mCi/60g). Aliquots (0.4ml) were taken from the 20,000 x g rabbit skin homogenate supernatants and the protein precipitated with 50% TCA. The samples were centrifuged and the supernatants harvested. Each sample was placed on a Dowex 50W-8X column and subsequently eluted with 10N NH_4OH. The radioactive fractions were pooled, lyophilized and resuspended in lithium citrate buffer. Tritium content was measured and leucine content was determined by amino acid analysis.

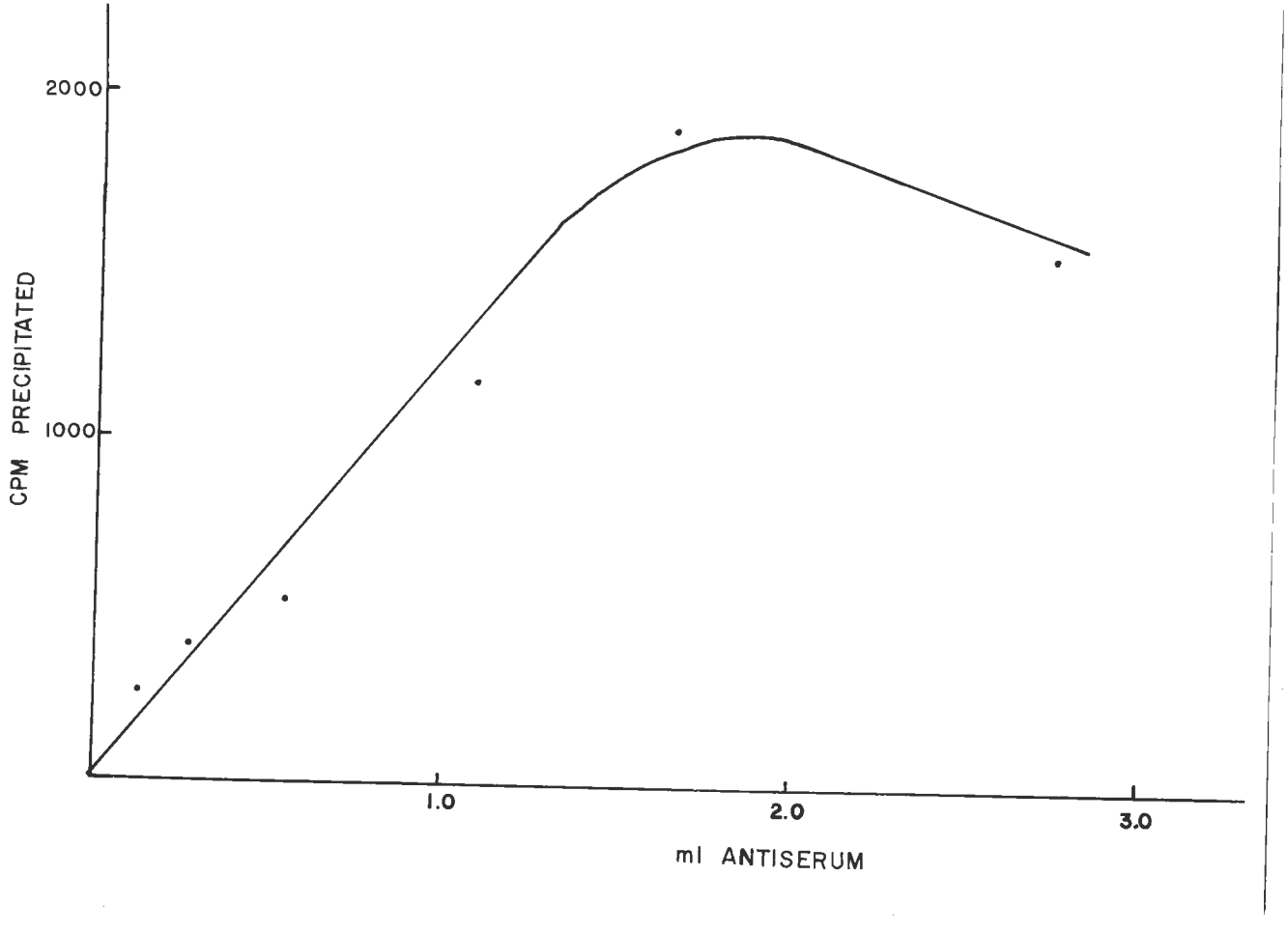


Figure V. Antibody precipitation curve where increasing amounts of antisera were added to a constant amount of CRP solution. Each point represents the mean of duplicate samples. Neonatal rabbits labeled with 5mCi of ^{3}H-leucine were killed after 2 hr and 70% supernatants prepared from the skin. The supernatants were then pooled, dialyzed, and concentrated to a uniform concentration of CRP ($5x10^{5}$ CPM of enzyme related antigen/ml). Five ml aliquots were taken and antibody added. The resulting precipitates were collected and washed by centrifugation, collected on filters and the tritium content determined.

increasing amounts of goat antiserum were added to a constant amount of CRP solution. Neonatal rabbits labeled with 5mCi of ^{3}H-leucine were killed after 2 hours and the 70% supernatants prepared from their skin. The supernatants were then pooled, dialyzed, and concentrated to a uniform concentration of CRP (5×10^{5} CMP of enzyme related antigen/ml). Equal aliquots were taken (5.0 mls) and antibody was added. The resulting precipitates were collected and washed by centrifugation, dissolved in 1N NaOH and collected on filters (Whatman GF/A) in the presence of 10% trichloroacetic acid containing 10^{-3}M leucine. The filters were then washed, digested with Protosol® (New England Nuclear) in scintillation vials and the ^{3}H content determined.

Figure VI shows the decay of radioactivity in CRP after the injection of ^{3}H-leucine. Immunoprecipitates were prepared from the 70% supernatant fractions. Each sample was adjusted to a uniform concentration of CRP ($5x10^{5}$CMP of enzyme related antigen/ml) and 1 ml of antibody was added to 2.5 ml of CRP solution to give maximum precipitation. The immunoprecipitates were harvested, washed, and collected on filters and the radioactivity determined. From the regression line, a $T_{\frac{1}{2}}$ of 47 hours was obtained for CRP. This is substantially shorter than that found for prolyl hydroxylase without correcting for reutilization. There was no detectable delay in the amount of time needed to reach maximum labeling (peak occurred within 2 hours).

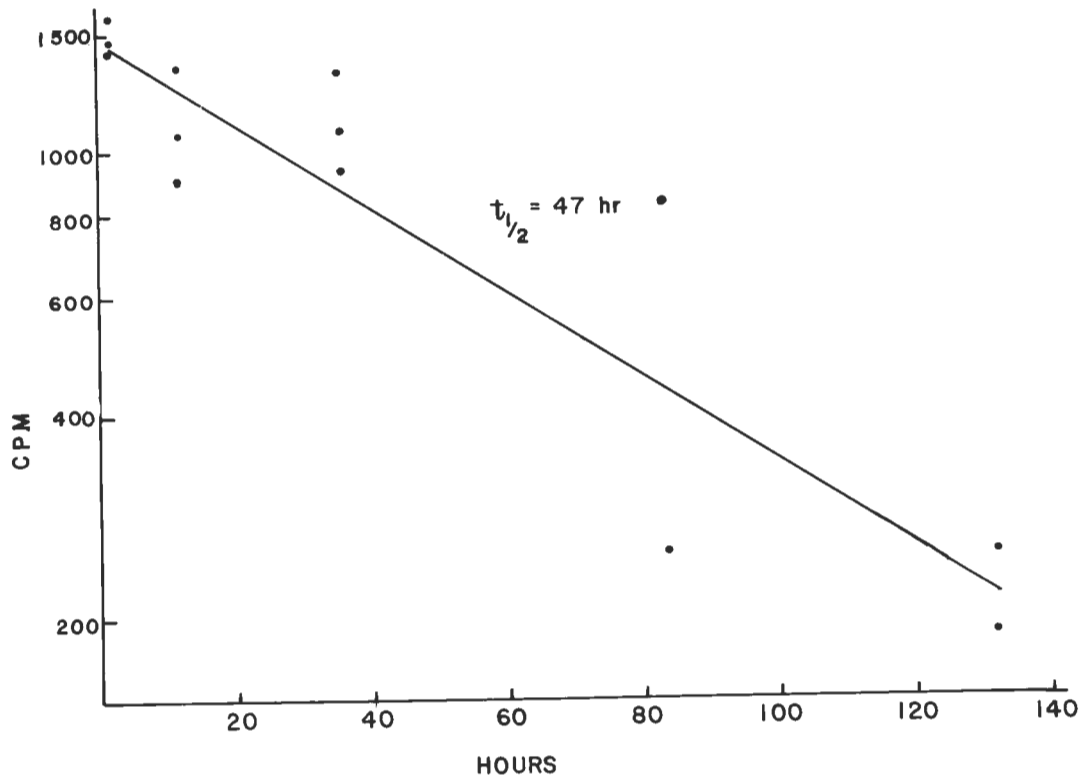


Figure VI - Loss of ^{3}H-leucine from CRP as a function of time following a single administration (5mCi/60g). Immunoprecipitates of CRP were prepared from concentrated, dialyzed 70% $(NH_4)_2SO_4$ fractions derived from the skins of neonatal rabbits. The immunoprecipitates were washed, collected on filters and the radioactivity determined. (Best fit line from computer regression program.)

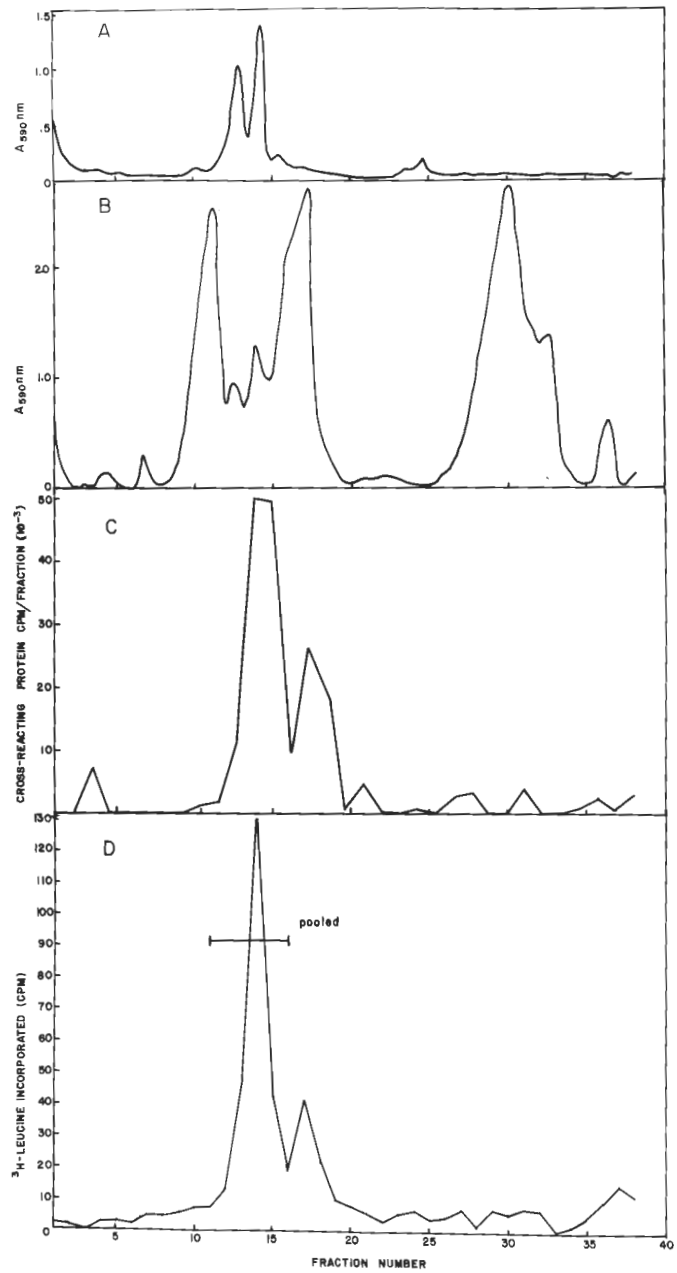


Figure VII. SDS-acrylamide electrophoresis of immunoprecipitated CRP and purified prolyl hydroxylase. A. Electrophoretic pattern of denatured affinity enzyme after staining. B. Electrophoretic pattern of a dissociated immunoprecipitate of CRP after staining. C. Antigenicity of the proteins eluted from a duplicate immunoprecipitate. D. Radioactivity contained in the same immunoprecipitate as in B.

The immunoprecipitates of CRP were dissociated and analyzed on 10% SDS slab gels and typical results are seen in Figure VIIB. A comparison with the electrophoretic pattern of purified enzyme (Figure VIIA) shows that the radioactivity present in the dissociated precipitate migrates similarly to the two prolyl hydroxylase monomers. Upon elution of the proteins present in the dissociated precipitate from the fractionated gel, it was demonstrated that these radioactive protein species were antigenically similar to prolyl hydroxylase (Figure VIIC) in the immunoassay, using active enzyme as standard. Thus, it was concluded that the dissociated precipitate of CRP from the 70% $(NH_4)_2SO_4$ supernatant contained protein that corresponded to the prolyl hydroxylase monomers. In addition, a third radioactive (Figure VIID), antigenic peak (Figure VIIC) was noted that was of smaller molecular weight. The position of this band was partially obscured by one of the antibody subunits when the gels were scanned for optical density (Figure VIIB). In other gels not shown, this band was more distinct. This protein did not correspond to any of the subunits of prolyl hydroxylase but it was antigenic in the enzyme immunoassay. The amount of radioactivity present in this peak was much less than the two major peaks.

The SDS gels were calibrated using known molecular weight standards. The relative migration of the three antigenic protein species plus the standards are plotted in

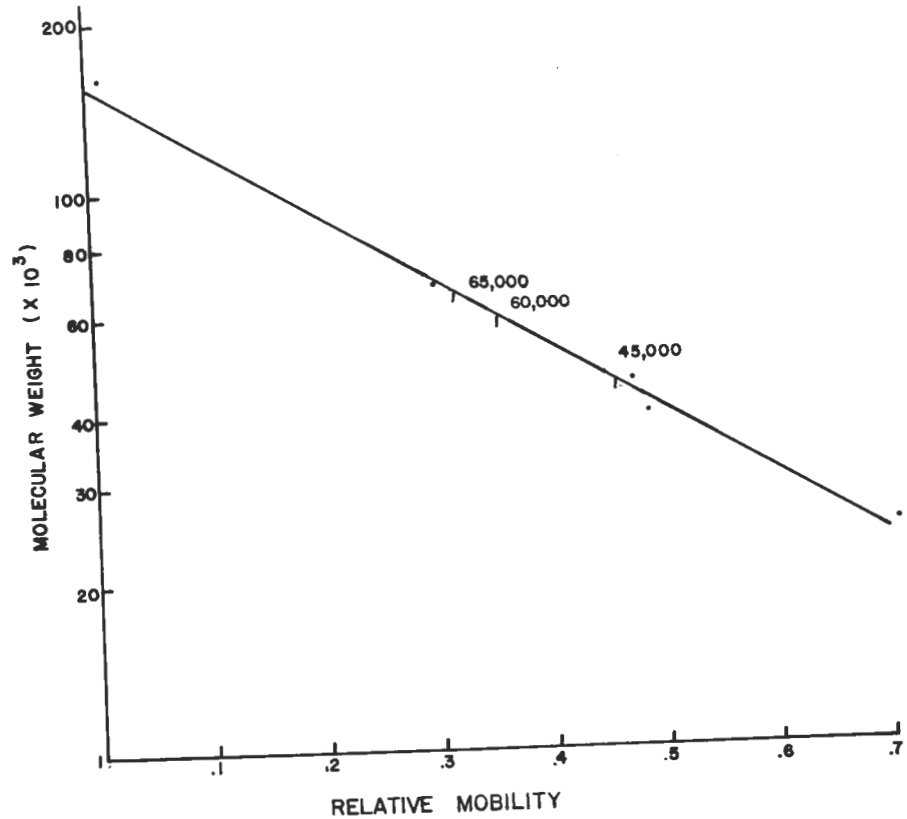


Figure VIII. Determination of the molecular weights of the subunits of rabbit prolyl hydroxylase and CRP by SDS-acrylamide electrophoresis. Vertical lines represent the three subunits of CRP. Standards: β and α subunits of RNA-polymerase, carboxymethylated bovine serum albumin, ovalbumin and chymotrypsinogen A.

Figure VIII against the logarithm of their molecular weights. The two major subunits of CRP and prolyl hydroxylase migrated with a mobility corresponding to molecular weights of 65,000 and 60,000. These values are close to what has been reported previously for the monomers of human and chick prolyl hydroxylases (Berg and Prockop, 1973; Tuderman et al., 1975). The third antigenic component of CRP was shown to have a molecular weight of 45,000.

Radioactivity from the dissociated immunoprecipitate which migrated with molecular weight hydroxylase was used to determine the turnover of CRP. The concentrated dialyzed 70% $(NH_4)_2SO_4$ supernatants were derived from the same skins from which prolyl hydroxylase was purified (see Figure III). Each sample was adjusted to a uniform concentration of CRP ($5x10^5$ CPM of enzyme related antigen/ml) and 2 mls of antibody was added to 5 mls of CRP solution to give maximum precipitation of CRP-antibody complex. This precipitate was harvested and washed twice by centrifugation. The samples were then dissociated and electrophoresed on the 10% SDS gels. For turnover analysis, the radioactivity in the higher molecular weight bands of CRP were pooled (see Figure VIID) and background counts were subtracted. Figure IX shows the presence of ^3H-leucine into CRP as a function of time following a single administration as determined on the SDS gels. The $T_{\frac{1}{2}}$ for the major subunits of CRP was found to be 53 hours without correction for reutilization. Al-

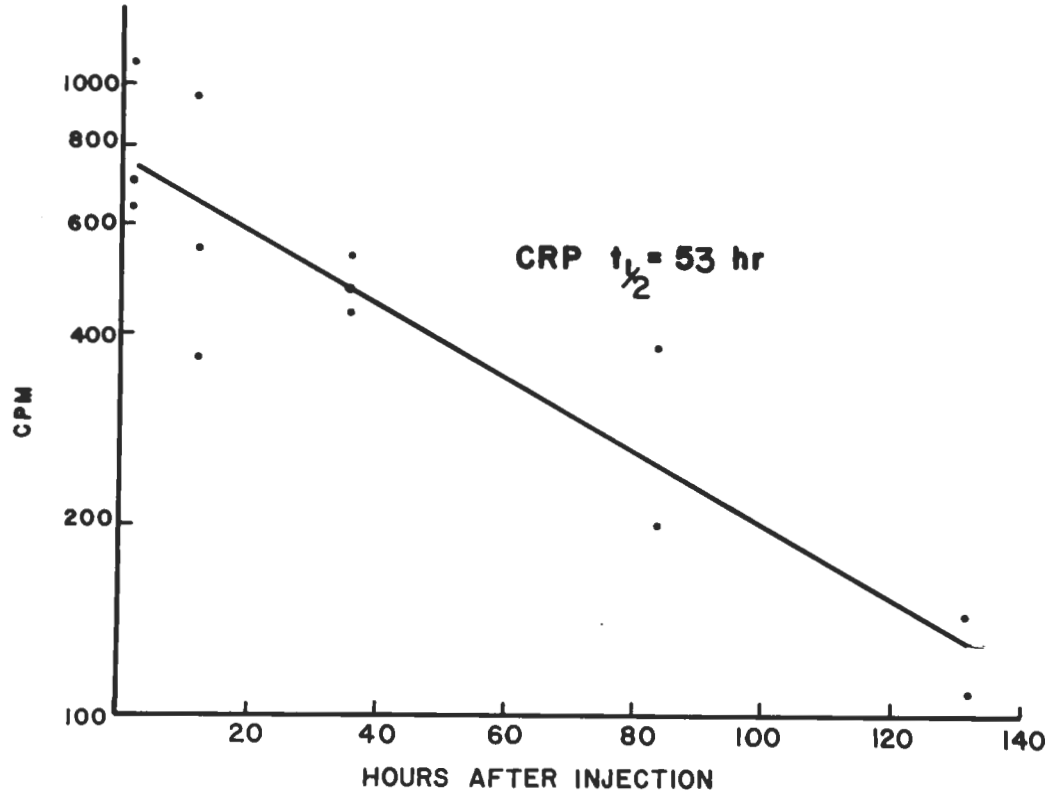


Figure IX. Loss of ^{3}H-leucine from CRP as a function of time following a single administration (5mCi/60g). Immunoprecipitates of CRP were prepared from concentrated, dialyzed 70% ammonium sulfate fractions derived from the skins of neonatal rabbits. The immunoprecipitates were dissociated and electrophor sed on 10% SDS-acrylamide gels. The gels were then sliced and the radioactivity determined. Counts in the two major band of CRP were pooled and background substracted. (Best fit line from computer regression program.)

though this is slightly longer than that determined with the use of filters, it is still significantly shorter than that found for prolyl hydroxylase without correction for reutilization. Again, no detectable delay in incorporation of ^{3}H-leucine in CRP was seen in this and other experiments utilizing radioactive immunoprecipitates analyzed on SDS polyacrylamide gels.

The validities of the $T_{\frac{1}{2}}$s obtained by measuring the loss of label from neonatal rabbit skins were tested in an experiment where prolyl hydroxylase and CRP were elevated in vasculature and liver of adult rabbits. From the decay back to basal levels, $T_{\frac{1}{2}}$s could be calculated for both prolyl hydroxylase and CRP. Adult male rabbits were subjected to an arteriogenic protocol of daily thyroxine-epinephrine injections for 12 days. Both CRP and hydroxylase were increased in the aorta and liver as seen in Table III. Prolyl hydroxylase in the aorta was increased approximately four-fold while in the liver it was increased 2.5 fold. These values compared favorably to those reported by Fuller and Langner (1970) and Fuller et al. (1976), using the same experimental model. In the aorta CRP levels were much higher than those of hydroxylase and epinephrine-thyroxine treatment significantly increased CRP levels in the aorta.

The fall of prolyl hydroxylase activity in the aorta and liver after the removal of hormonal treatment is shown in Figure X. Enzyme levels return to normal in a little over one week in the aorta and in a little less time in the liver.

TABLE III

PROLYL HYDROXYLASE (PH) AND CROSS-REACTING PROTEIN (CRP) VALUES (CPM/ug PROTEIN) IN ARTERIOSCLEROTIC[1] RABBIT TISSUE

	AORTA		LIVER
	PH	CRP	PH
CONTROL	6.0 ± 0.9	310 ± 55	3.8 ± 0.4
ARTERIOSCLEROTIC	23.7 ± 0.9	753 ± 116	9.4 ± 2.1

Values represent mean ± S.E. of at least 4 animals.

[1]Injection of thyroxine (0.050 mg/kg for 12 days, i.p.) and epinephrine (0.025 mg/kg for the first 5 days, and 0.050 mg/kg thereafter, i.v.).

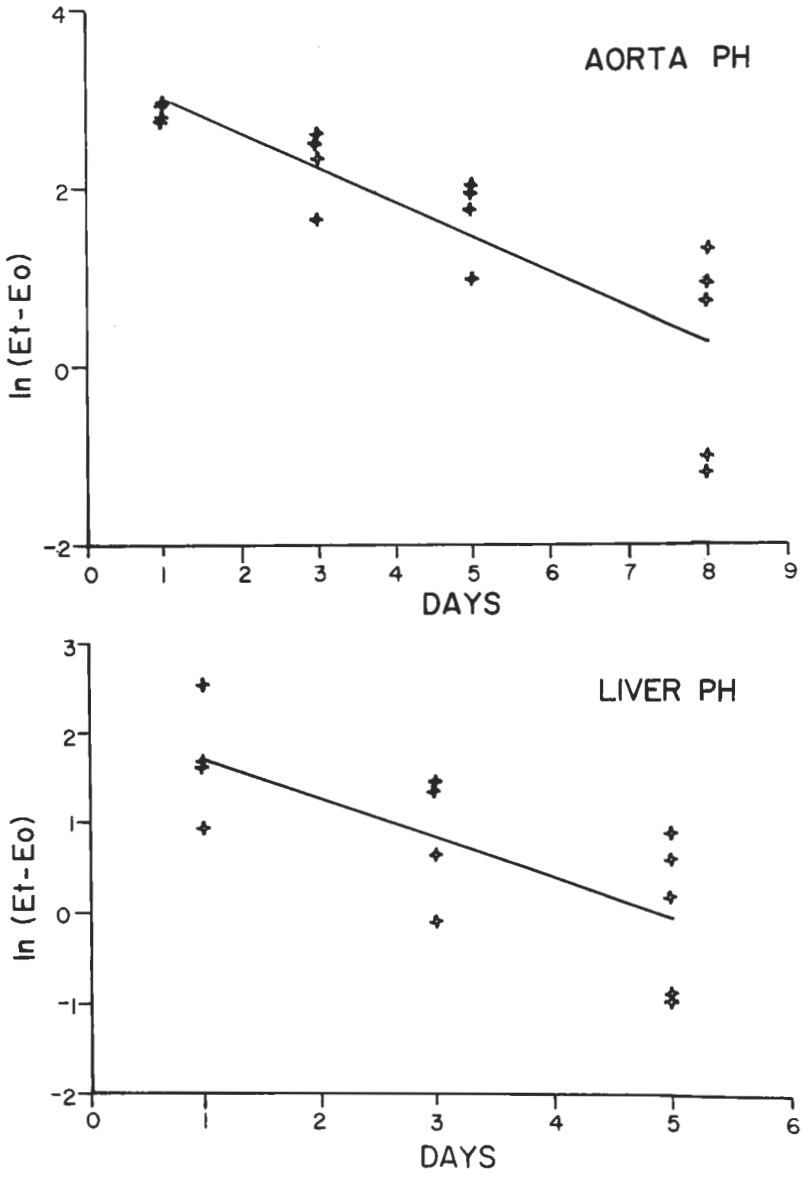


Figure X. Fall of prolyl hydroxylase activity in the aorta and liver after removal of treatment. Prolyl hydroxylase was elevated by daily administration of thyroxine (0.05 mg/kg, i.p.) and epinephrine (0.025 mg/kg, i.v. for 5 days followed by 0.05 mg/kg i.v. for 7 days). (Et - Eo) is plotted logarithmically vs. time. (Best fit lines from computer regression program.)

The logarithm return of elevated enzyme activity to normal levels (Et-Eo), is plotted against time. From the regression lines, which were highly significant, the $T_{\frac{1}{2}}$ of prolyl hydroxylase in the liver was found to be 38 hours and in the aorta, 42 hours. These values are very close to what was obtained in the labeling experiment after correction for reutilization.

The fall of elevated CRP in the aorta after cessation of treatment is shown in Figure XI. Similar to hydroxylase activities, CRP levels return to normal in a little over a week. From the regression of ln (Ct-Co) versus time after treatment, where Ct = CRP level at time t and Co = CRP level under basal conditions, a Kd of 0.0125 hr^{-1} was obtained and a $T_{\frac{1}{2}}$ of 55 hours calculated. This is very close to the $T_{\frac{1}{2}}$ obtained for prolyl hydroxylase in the aorta, and is in contrast to what was found in the labeling experiment where CRP in the 70% supernatant had a shorter $T_{\frac{1}{2}}$ than did active hydroxylase.

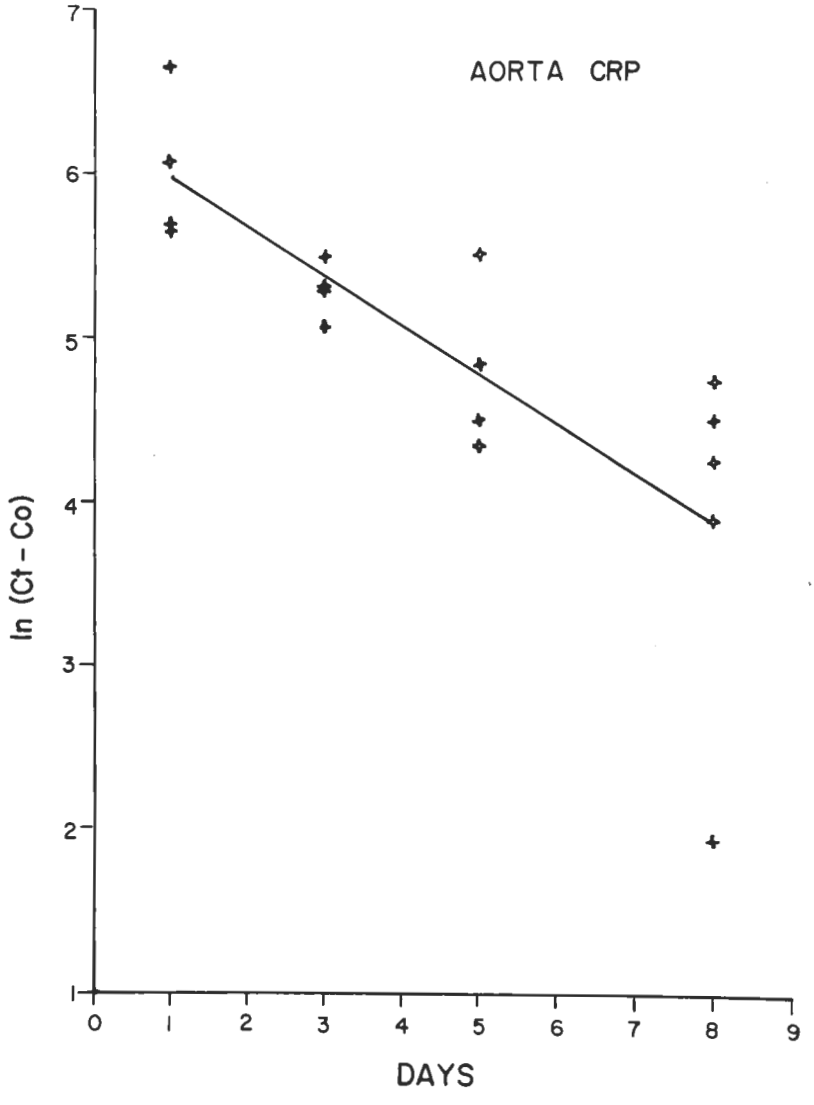


Figure XI. Fall of CRP in the aorta after removal of treatment. CRP was elevated by daily administration of thyroxine (0.05 mg/kg, i.p.) and peinephrine (0.025 mg/kg, i.v. for five days followed by 0.05 mg/kg i.v. for 7 days). Ln (Ct-Co) is plotted vs. time. (Best fit from computer regression program.)

DISCUSSION

In this investigation, ^{3}H-leucine labeled prolyl hydroxylase was purified from neonatal rabbit skin by affinity chromatography and was found homogenous by polyacrylamide gel electrophoresis. Upon urea and mercaptoethanol dissociation and subsequent SDS-electrophoresis, two subunits were obtained which migrated with mobilities corresponding to molecular weights of 65,000 and 60,000. These values are in agreement with the previously reported values for the monomers of human and chick prolyl hydroxylases (Kuutti et al., 1975; Berg and Prockop, 1973). Amino acid analysis of purified rabbit hydroxylase also demonstrated a close homology to chick and human hydroxylase (Kuutti et al., 1975; Tuderman et al., 1975a).

The CRP levels in the neonatal rabbit skin were found to be four times that of active enzyme. Stassen et al. (1974) showed that this same ratio of CRP to active enzyme was present in neonatal mouse skin. In newborn rat skin the ratio was approximately 20 to 1. In the immunoassay used in this investigation, no change in total immunoreactive protein was observed with enzyme before or after dissociation by mercaptoethanol. The high levels of CRP present in tissue as compared to active enzyme is not the result of increased immunogenicity upon breakdown of the tetrameric form of the enzyme.

During the purification of prolyl hydroxylase a large proportion of the total CRP pool was found in the 70% $(NH_4)_2SO_4$

supernatant while all active enzyme was precipitated. It should be noted that some CRP is also found in the 70% pellet (Stassen et al., 1974). Upon immunoprecipitation, dissociation and subsequent electrophoresis, CRP was shown to migrate similarly to the two prolyl hydroxylase monomers. In addition, a third labeled antigenic component of CRP was found that had a molecular weight of approximately 45,000. Mouse skin CRP has been shown to have a molecular weight of 80,000 - 100,000 as determined by gel chromatography (Stassen et al., 1974). Thus native CRP may be of slightly higher molecular weight than either of the two prolyl hydroxylase monomers found after dissociation of enzyme. The 45,000 molecular weight component of CRP found in this investigation may be attached to either one of the two hydroxylase monomers to form a 110,000 molecular weight CRP molecule. It cannot be excluded, however, that this 45,000 molecular weight protein may be a breakdown product of the native form of CRP or of the tetrameric enzyme which has a reported molecular weight of 240,000 (Tuderman et al., 1975a).

A list of the Kds and $T_{\frac{1}{2}}$s obtained in this investigation is shown in Table IV. A $T_{\frac{1}{2}}$ of 45 hours after correction for leucine reutilization was obtained for rabbit skin prolyl hydroxylase as determined from the rate of decay of incorporated ^{3}H-leucine. The $T_{\frac{1}{2}}$ obtained for CRP could not be corrected for the reutilization of leucine because an insufficient amount of CRP was available from the gels for amino acid analysis. Reutilization error is highest at early time points,

TABLE IV

SUMMARY OF RABBIT PROLYL HYDROXYLASE (PH) AND CRP TURNOVER DATA

	$T_{\frac{1}{2}}$(hr)	Kd(hr^{-1})	Tissue Level (CPM/ug)	Ks[1] (CPM/ug-hr)
Adult liver PH[2]	38.45	0.0180	3.8	0.0684
Adult aorta PH[2]	42.40	0.0163	6.0	0.0978
Adult aorta CRP[2]	53.37	0.0125	310	3.875
Neonatal skin PH[3] (uncorrected)	73.23	0.00946	81	0.766
Neonatal skin PH[3] (corrected)	45.03	0.0154	81	1.247
Neonatal skin CRP[3] (uncorrected, determined on gels)	52.98	0.0131	320	4.192
Neonatal skin CRP[3] (uncorrected, determined on filters)	47.47	0.0146	320	4.672

[1] Ks = Kd x Tissue level

[2] Determined by the fall of elevated levels

[3] Determined by the loss of ^{3}H-leucine

therefore the apparent CRP turnover in this experiment is greatly overestimated. Inferences about the relationship between prolyl hydroxylase and CRP utilizing apparent $T_{\frac{1}{2}}$'s or Kd's remain valid since both decay curves are subject to reutilization from the same amino acid pools. Multiplying the apparent Kd's of CRP and prolyl hydroxylase by the levels of each in the neonatal rabbit skin we can estimate the proportion of prolyl hydroxylase and CRP being broken down per unit time. These calculations indicate that the levels of CRP found cannot be accounted for only by the breakdown of enzyme. Thus, these data would support the hypothesis that a proportion of the CRP in cells is precursor to active enzyme. This is also supported by the rapid appearance of label in CRP as compared to prolyl hydroxylase.

The Kd's obtained from the fall of elevated prolyl hydroxylase activity in adult rabbit aorta and liver are similar to the corrected values obtained in the labeling experiment using neonatal rabbit skin (see Table IV). The turnover rates determined for prolyl hydroxylase in these experiments were 1.7-1.9 days which are similar to those determined for the proteins of the endoplasmic reticulum as a whole (Arias et al., 1969). Thus, prolyl hydroxylase appears to turn over at the same rate at which the membrane is being replaced. However, the Kd for CRP in the adult rabbit aorta was found to be similar to that of prolyl hydroxylase. Again, in this tissue, the total amount of CRP determined cannot be accounted for by the

breakdown of active enzyme. The fact that the $T_{\frac{1}{2}}$s for both CRP and prolyl hydroxylase were the same is significant. The discrepancy in CRP $T_{\frac{1}{2}}$ observed between the two biological systems may be related to the fact that in the arteriosclerotic animals we measured total CRP in the homogenates, not just the 70% supernatant CRP, as in the labeling experiment. The possibility exists that a portion of the CRP pool is degradation production of prolyl hydroxylase. Stassen et al. (1974) were able to separate both CRP and prolyl hydroxylase from neonatal skin by gel filtration and ion-exchange chromatography, but the CRP peaks obtained all had shoulders. In the present study, several molecular weight species were identified in the CRP pool. Only the higher molecular weight subunits are found in active enzyme. Thus, the CRP pool as obtained by immunoprecipitation in this study contains unidentified heterogeneity.

It is possible that the difference between CRP and prolyl hydroxylase in the time required for maximum label incorporation, is a reflection of the difference in the turnover rates of these two proteins. Poole (1971) has shown that proteins with long $T_{\frac{1}{2}}$s require a longer period of time to reach maximum label incorporation than proteins which turn over at a more rapid rate. A rapid initial decrease of the incorporated radioactivity from liver microsomes has been reported (Arias, 1969). Negishi and Omura (1972) demonstrated that this biphasic decay is a property of the microsomal membrane proteins themselves. They have suggested that the rapid loss of

a considerable proportion of newly synthesized proteins from the microsomes, after their association with membrane, reflects the mechanism by which proteins are inserted in the membrane. Individual proteins of the microsomal membrane such as NADPH-cytochrome-c-reductase (Negishi and Omura, 1972) and cytochrome P-450 (Levin and Kuntzman, 1969) have also been shown to have this biphasic decay curve after administration of radioactive precursors.

Prolyl hydroxylase is a microsomal bound enzyme (Harwoood et al., 1975). Subcellular fractionation studies, utilizing chick embryo liver, have demonstrated that prolyl hydroxylase is concentrated in the microsomal fraction together with various microsomal marker enzymes, such as NADP-cytochrome-c-reductase and glucose-6-phosphatase (Helfre, et al., 1976). The observation that prolyl hydroxylase, being a microsomal protein, does not exhibit a fast initial decline in specific radioactivity during the first few hours after label administration, and no evidence of biphasic decay, may have functional importance. The rapid turnover of CRP present in the 70% $(NH_4)_2SO_4$ supernatant may, however, be analogous to the fast decay phase of other microsomal proteins. This observation, when considered with the lack of delay in incorporation of label into CRP, may thus be relevant to the mechanism by which newly synthesized CRP or prolyl hydroxylase tetramers are incorporated into or onto the microsomal membrane as functional enzyme units. If CRP exists in cells as a cytoplasmic protein

its turnover, compared to enzyme, could be predicted to be more rapid if membrane or ribosomal protein are degraded primarily as free cytoplasmic protein, as suggested by Dice and Schimke (1972).

Epinephrine-thyroxine treatment raised prolyl hydroxylase activity in both the aorta and the liver. Increased prolyl hydroxylase activity can be seen after five days of this treatment, with a redoubling after each subsequent 5 days of treatment, which is prior to the appearance of fibrous plaques (Fuller and Langner, 1970). CRP was also significantly elevated in the aorta. This finding corresponds to results previously reported (Fuller et al., 1976), and supports the hypothesis that increases in prolyl hydroxylase activity "in vivo" are the result of new synthesis, with facilitiation of the conversion of CRP into prolyl hydroxylase as the active enzyme is inserted onto the microsomal membrane. Comparison of enzyme decay curves with those for increased activity "in vivo" also suggest that active enzyme levels may be elevated by inhibition of prolyl hydroxylase degradation. Large increases in prolyl hydroxylase activity have been reported in a variety of tissues responding to injury-induced damage (Hussain et al., 1976; Langner and Fuller, 1970; Mussini et al., 1967). These increases in enzyme activity occur over a period of days and are compatible with the slow turnover of prolyl hydroxylase. This stabilization of prolyl hydroxylase within the microsomes, leading to elevated enzyme

levels, could not be the same as ascorbate activiation observed in tissue culture, which is maximal within hours after administration (Stassen et al., 1973) and appears to be the result of the dissociation of enzyme-substrate complexes (Kuttan et al., 1975).

CONCLUSIONS

1) Rabbit skin prolyl hydroxylase was purified to homogeneity by affinity chromatography as judged by polyacrylamide electrophoresis. Molecular weight determination and amino acid analysis showed close homology to other vertebrate prolyl hydroxylases.

2) The turnover rates of prolyl hydroxylase and CRP were measured and the values obtained for active enzyme were very similar in all tissues examined. The $T_{\frac{1}{2}}$ for prolyl hydroxylase was found to be 38 hours in the adult rabbit liver and 42 hours in the adult rabbit aorta. Using neonatal skin, a corrected half-life of 45 hours was determined for the enzyme. In comparison, the apparent CRP $T_{\frac{1}{2}}$ was significantly shorter than the apparent $T_{\frac{1}{2}}$ of prolyl hydroxylase in the neonatal skin. No difference was seen between CRP and prolyl hydroxylase turnover in the adult rabbit aorta.

3) The rates of synthesis of CRP in the adult aorta and neonatal skin were very close. In the aorta, CRP synthesis was 40 times that of active enzyme synthesis. Using uncorrected data, the rate of synthesis of CRP in neonatal skin was approximately 6 times that of prolyl hydroxylase. Thus, the total amount of CRP in tissue cannot be accounted

for by breakdown of the enzyme. The data supports the idea that a proportion of the CRP pool is precursor to active enzyme.

4) In neonatal skin, maximum label incorporation into CRP was observed and occurred in a much shorter period of time compared to prolyl hydroxylase. Although this difference can be explained by the difference in turnover rates of these two proteins, the early incorporation of label into CRP supports the hypothesis that part of the CRP pool is precursor to enzyme. Further, this early incorporation of label into CRP may reflect the mechanism by which CRP or active enzyme is attached to the microsomal membrane.

5) Injury-induced arteriosclerosis, by epinephrine-thyroxine treatment significantly raised both prolyl hydroxylase and CRP levels in the aorta. Increases in enzyme activity occur over a period of days and are compatible with the slow turnover of prolyl hydroxylase. Increases in enzyme activity "in vivo" could be the result of increased synthesis or inhibition of degradation and do not appear to be the result of cofactor activation.

6) In all tissues examined, CRP levels were much higher than those of prolyl hydroxylase. These high levels of CRP Present in tissue are not the result of increased immunogenicity upon breakdown of the enzyme.

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