INTERACTIONS BETWEEN STEROIDAL ANTI-INFLAMMATORY AGENTS AND COLLAGEN

by

Isadore Kanfer

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PREFACE

Much research has been done on the formation of fibrils from solutions of soluble collagen *in vitro* in order to gain some knowledge of the mechanisms which may occur *in vivo*. The *in vitro* formation of fibres from solutions of collagen has been shown to be extremely sensitive to the nature of the solution environment and the presence of added chemical compounds, and thus constitutes an interesting system for the study of collagen-small molecule interactions.

The present study is concerned with the effects of various corticosteroid drugs, used medicinally as anti-inflammatory agents, on collagen in solution. As these corticosteroids are administered to reduce inflammation in conditions such as rheumatoid arthritis and a host of other pathological conditions in which collagen is implicated, this work has been undertaken in order to establish and characterize any binding mechanisms which may be involved. Furthermore, the corticosteroid drugs available commercially in pure form as the free base or as the water-soluble ester salts offer an interesting range of structural and stereochemical variants for the study of their reaction with a complex and biologically important protein molecule such as collagen.

A great deal of research on drug-protein interactions (Goldstein, 1949; Meyer and Guttman, 1968a) and more specifically, steroid-protein interactions have been reported over the years (Daughaday, 1959; Sandberg *et al*., 1966; Villee and Engel, 1961; Westphal, 1971). Comprehensive reports, however, on steroid-collagen interactions *in vitro* are conspicuously absent from modern scientific literature although relatively superficial accounts have been published (Menczel and Maibach, 1972; Eik-Nes *et al*., 1954). Although work involving the above has appeared relating specifically to the effects of steroids on collagen biosynthesis both *in vivo* and *in vitro* there have been minimal accounts of steroid-collagen interactions tailored to characterize the binding at the molecular level. The effect

of corticosteroids on the metabolism of connective tissue has also received special attention (Asboe-Hansen, 1959; Kivirikko, 1953; Nakagawa and Tsurufuji, 1972). Recently, Uitto et al. (1972) reported the effects of several anti-inflammatory corticosteroids on collagen biosynthesis in vitro, whilst Aalto and Kulonen (1972) reported the effects of several antirheumatic drugs on the synthesis of collagen and other proteins in vitro. The interactions between collagen and certain drugs has also been briefly reviewed (Chvapil, 1967).

Much data also exists on the binding of a wide range of small molecules and ions with serum albumin (Steinhardt and Reynolds, 1969; Scatchard, 1949; Klotz, 1950). Serum albumin, being specialized for a very general transport function and apparently designed for the purpose of combining with a large range of small molecules, has a proportion of possible reactive sites 'buried' within the molecule itself because of its folded conformation. In addition, serum albumin shows a high degree of cooperative binding in contrast to collagen. The latter molecule, with its larger molecular size and weight is specialized for a biologically structural function and has a higher proportion of possible reactive sites which appear relatively more accessible to ligands.

A study of the interactions between corticosteroids and collagen thus provides the opportunity to investigate a protein which is very different from the much studied serum albumin. Because of the limited information available regarding the interaction of steroid drugs and collagen at the molecular level, studies of this nature are relevant to the understanding of the mode of action of steroid compounds which are such an important group of therapeutic substances used in modern medicine.

CHAPTER I

GENERAL INTRODUCTION

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GENERAL INTRODUCTION

A. COLLAGEN

Interest in collagen stems partly from its unusual properties, partly from its likely importance in diseases of connective tissue and partly from its abundance. Collagen is the most abundant protein in mammals and is the major constituent of most connective tissues (Grant and Prockop, 1972a).

During the last twenty-five years or so a great number of reviews on collagen have appeared. Bear (1952) summarized some of the basic information supplied by X-ray diffraction, electron microscopy, and chemical studies and developed a viewpoint regarding the structure of the collagen fibril. Kendrew (1954) reviewed the morphological and physico-chemical properties together with a discussion of the theories of the structure of collagen. Gustavson (1956) in his review, described various aspects of collagen chemistry whilst a series of conferences (Stainsby, 1958; Ramanathan, 1962; and Fitton-Jackson et al., 1964) provided an overall view on the collagen field of study. Harrington and von Hippel (1961) published a review on the structure of collagen and its relation to the collagen-gelatin fold whereas Hall (1963-1965) reviewed various aspects of collagen chemistry. A very comprehensive account of the chemistry and structure of collagen is contained in Volume 1 of the Treatise on Collagen (Ramachandran, 1967) and only recently Traub and Piez (1971) published a review on the chemistry and structure of collagen, concentrating mainly on the developments of the last few years since the abovementioned treatise was published. Volume 2, in two parts, of the Treatise on Collagen (Gould, 1968) appeared a year later and this contained a very comprehensive collection of data on the biology and general biochemistry of collagen.

Many additional reviews have appeared over the last few years but these have specialized on certain aspects of collagen. Gross (1963) discussed the comparative biochemical aspects, Veis (1964) the chemistry and conversion of

collagen to gelatin, Harding (1965) and Piez (1968) the cross-linking and Lowther (1963), Wood (1964) and Cassel (1971) the aggregation phenomena. Bailey (1968), in a more general review, summarized the nature of collagen with respect to the collagen macromolecule whilst Chvapil (1967) in his book, Physiology of Connective Tissue, devoted a section to the physico-chemical structural stability of collagen. Steven (1972) in his very recent review describes the present knowledge of tropocollagen chemistry leading onto the self-assembly of collagen fibrils and their chemical stabilization in the form of polymeric collagen fibrils.

CHEMISTRY

Mammalian collagen molecules have a rod-like conformation and are approximately 3000Å long and 15Å in diameter (Boedtker and Doty, 1955 and 1956; Hodge and Petruska, 1963; Traub and Piez, 1971).

Gross et al. (1954) suggested that there exist molecular units, which they called "tropocollagen" and which they considered to be the building blocks for the collagen fibrils. This tropocollagen unit is a triple polypeptide helical chain in which the three chains are interconnected to one another (via hydrogen bonds and probably other types of linkages), and are also sometimes referred to as the "protofibril" (Ramachandran, 1967; Bear, 1952).

The chains are similar with molecular weights of about 95,000 but each molecule contains at least two different kinds with different amino acid compositions (Piez, 1967). The chains are designated α_1, of which there are two, and a single α_2-chain per individual mammalian tropocollagen molecule (Orekhovitch and Shpikiter, 1957; Steven, 1972). Suggestions that calf-skin collagen contains three different chains can be explained by small differences in length of the α_1 chains caused perhaps by partial hydrolysis or proteolysis occurring either 'in vivo' or during isolation (Müller and Kühn, 1968; Stark and Kühn, 1968; Rauterberg and Kühn, 1971). The three

helical polypeptide chains, each having every third residue as glycine (Gly), are wound around each other to form a three-stranded rope-like structure (Traub, et al., 1969), the individual chains being in the form of left-handed helices. The twist of the three chains has been referred to as a "super-helix". Single tropocollagen molecules have been seen in the electron microscope and the above dimensions confirmed thereby (Hall and Doty, 1958).

Orekhovich et al. (1948 a,b) extracted collagen from rat skin using citrate buffers at pH 3-4.5 and demonstrated that fibrils re-formed on dialysis against water. Orekhovich applied the term "procollagen" to soluble collagen obtained by this citrate buffer extraction to express his belief that it was a precursor of fibrous collagen.

It has recently been shown that the collagen synthesized and extruded by connective tissue cells is larger than tropocollagen. Preliminary observations indicate that this larger form of collagen, also referred to as "procollagen" (Bellamy and Bornstein, 1971) or "transport form" (Jimenez et al., 1971; Dehm et al., 1972; Bornstein et al., 1972) has different solubility properties from tropocollagen (Layman et al., 1971; Dehm and Prockop, 1971) and does not aggregate into fibres under conditions that induce fibre formation from tropocollagen.

Grant and Prockop (1972b) introduced the term "protocollagen", an additional concept, which they described as being a further polypeptide precursor of collagen. This particular precursor appears to be an un-hydroxylated procollagen, i.e. the appropriate Proline (Pro) and Lysine (Lys) residues being unhydroxylated. The distinction between procollagen and tropocollagen seems to be that the former molecule has intact NH_2-terminal ends prior to leaving the cell during biosynthesis (Jimenez et al., 1971) whereas after extrusion, these NH_2-terminal ends are cleaved enzymatically resulting in the appearance of the latter molecule.

1. Extraction of soluble collagen

Soluble collagen may be extracted from collagenous tissue in two stages. Firstly, the neutral salt-soluble collagen with 0.14-2M NaCl solutions, which also removes soluble proteins and polysaccharide contaminants making the purification of the acid-extracted collagen easier (Piez, 1967), and then the more difficultly soluble collagen with acid buffers, such as citrate (pH 3.7), or dilute acetic acid.

Once extracted and purified, most collagen preparations have very similar solubility properties whether obtained by salt or acid extraction.

2. Primary structure

A consideration of the amino acid composition and sequence describes the primary structure of collagen.

Each of the polypeptide chains in the tropocollagen molecule is coiled into a helix that differs from the α-helix found in most other proteins in that there are no hydrogen bonds between amino acids in the same chain (Ramachandran, 1967). Instead, the helix formed by each chain is stabilized by hydrogen bonds between it and the other two chains in the same tropocollagen molecule. The helix formed by each chain also differs from the α-helix in that the structure is more extended so that the axial distance between one amino acid and the next is 2.91Å instead of the axial distance of 1.5Å found in the α-helix.

The unusual nature of the helix in collagen is largely explained by its unusual amino acid composition. Throughout most of each polypeptide chain, every third amino acid is Gly, and collagen can be considered a polymer of tripeptide units with the formula $(Gly\text{-}X\text{-}Y)_n$ where X and Y denote other amino acids in the two positions between Gly residues. Because of the uniquely high content of the imino acid residues, hydroxyproline (Hypro) and Pro, which have no hydrogen atom on the peptide bonds involving these residues, intramolecularly hydrogen-bond stabilized structures such as in the α-helix

are not found.

Also noteworthy is the fact that collagen contains hydroxylysine (Hlys) and a relatively low content of tyrosine, while the absence of tryptophan and cysteine eliminates the possibility of disulfide cross-links in the molecule. However, an exception to the latter has been reported (Josse and Harrington, 1964).

Since Gly is the smallest amino acid, it can pack tightly into the centre of the triple-stranded tropocollagen molecule and provide HN-groups for hydrogen bonding to O=C-groups in the peptide bonds of the other chains. The amino acid in the "X" position immediately after Gly in the repeating tripeptide structure is frequently Pro and since the nitrogen of Pro is fixed in a ring structure, the presence of Pro limits the rotation of the polypeptide chain. Also, recent evidence indicates that the O=C-group of Pro in this position provides an unusually strong electronegative group and in consequence forms a remarkably stable hydrogen bond with an HN-group on Gly in one of the other two chains (Veis and Nawrot, 1970). The third amino acid in the repeating tripeptide structure is often Hypro, and because it is also an imino acid, rotation of the polypeptide chain is further limited. It was originally suggested that when "Y" in the sequence Gly-X-Y is not Hypro, a second hydrogen bond can be formed with one of the other chains in the triple helix (Ramachandran, 1967). Recent work has shown that there is only one hydrogen bond per triplet, and this bond is always formed between HN-groups of Gly in one chain and an O=C-group of Pro or some other amino acid in the X position of a second chain (Traub, 1969; Traub et al., 1969).

As the collagen molecule matures in the tissue, but still remains soluble, intramolecular and intermolecular covalent bonds are formed. These result in β and γ-components, the former being derived from two α-chains intramolecularly cross-linked, i.e. β_{11} from $\alpha_1\alpha_1$ and β_{12} from $\alpha_1\alpha_2$ etc., whilst the latter arises from three covalently bonded α-subunits, i.e. γ_{111} consists of three α_1-chains while α_{112} consists of two α_1- and one

α_2-chain (Piez, 1967; Veis, 1967).

There is evidence that short, nonhelical peptide chains are appended to the ends of the triple helix. These appendages are referred to as telopeptides and are susceptible to digestion by proteolytic enzymes (Drake et al., 1966). These telopeptides have a limited number of amino acid residues which differ in content and sequence from the typical part of the collagen molecule. The amino acid composition of the telopeptides is rich in polar amino acids and contains little Pro and Hypro. Recent studies with tropocollagen, (Leibovich and Weiss, 1970) in which the C-terminal and N-terminal peptides were selectively removed, have demonstrated the role of the telopeptides in controlling the direction of molecular orientation in fibrillogenesis and also restricting the diameter of the newly formed fibrils.

The side chains of the amino acid residues other than the imino acids ($\pm$ 22%) or Gly ($\pm$ 33%) in mammalian collagen do not appear to participate significantly in intramolecular interactions since steric considerations dictate that these relatively more bulky structures will be directed outwards from the molecule. In general, the content of acidic (aspartic acid and glutamic acid = $\pm$ 12%) side chains can be considered comparable to that of the basic (Lys, histidine and arginine = $\pm$ 8%) side chains when allowance is made for the masking of approximately one-third of the acidic side chains in the amide form in mammalian collagen.

An important consideration regarding the possibility of intermolecular interactions in collagen is the fact that there exists a relatively uniform distribution of segments of polar amino residues which comprise the primary structure along the length of the tropocollagen molecule. The resulting variation of electrical charge between individual molecules is extremely conducive to electrostatic interaction occurring between surface segments when the molecules are aligned in parallel array. Electron microscope studies of collagen fibrils stained with heavy metal atoms that

differentially mark the position of charged side-chain groups reveal a fairly regular alternation of groups of charged and uncharged residues as shown by the pattern of cross-striations along the length of the molecule, with the detailed pattern of distribution slightly asymmetrical in native collagen (Borasky, 1967).

In comparison with the multiplicity of cooperative charge interactions, however, the involvement of polar residues in covalent bond formation appears to be small. Relatively well established cross-links have been described for collagen; these links are either covalent intramolecular cross-links (those within a triple helix) or intermolecular cross-links (those covalently joining peptide chains of one triple-helix to another).

The potential occurrence and available evidence for specific types of cross-linkage have been reviewed comprehensively (Harding, 1965; Grant and Prockop, 1972; Bailey, 1968). The nature of the intramolecular cross-link responsible for joining two α-chains to form a β-chain has been shown to be an α-β unsaturated aldol condensation product formed between two allysine residues (Bornstein and Peiz, 1966; Rojkind, Blumenfeld and Gallop, 1966). The prerequisite for this type of intramolecular crosslink, as for all other crosslinks proposed to date, is the formation of allysine from Lys by lysine oxidase.

The aldelhydic group of the β_{11} and β_{12} type of intramolecular cross-linkage may be involved in further condensation reactions, either intramolecularly to form γ-chains or intermolecularly between two suitably placed tropocollagen molecules to form a dimer system.

Excluding the non-polar structural constituents, Gly and Pro, residues carrying various linear and branched hydrocarbon side chains comprising up to four carbon atoms, as well as the aromatic ring structure of the phenylalanine side chain, constitute about eighteen percent of the total residues present in mammalian collagens (Cooper and Russell, 1969). The

inclusion of Pro residues brings the content of non-polar residues located on the molecular surface to about thirty percent of the total, indicating the probability of hydrophobic interactions occurring with similar hydrocarbon structures in the immediate environment.

These hydrocarbon structures are largely immobilized on the polypeptide chains and such multiple interaction between hydrophobic regions in collagen fibril formation thus constitutes a further source of intermolecular association (Russell and Cooper, 1970). The omission of the Gly content may be justified on the basis that its occlusion within the internal structure effectively prevents interaction with the environment.

Residues containing hydroxyl groups in the side chains constitute approximately sixteen percent of the total present when Hypro is included. The main properties conferred by this group would appear to be a capacity for ester-crosslinks to occur involving the carboxyl groups of aspartic and glutamic acid residues as well as providing active sites for hydrogen bond formation with the environment or similar sites on adjacent molecules in collagen fibrils.

The role of the remaining compounds, tyrosine and methionine, remains obscure particularly since the low levels of occurrence in collagen would appear to exclude these constituents from any major structural or functional role.

3. Secondary and tertiary structure

Recent reviews (Ramachandran 1968; Traub and Piez, 1971) have appeared regarding the secondary and tertiary structures of collagen. Many of the earlier structures put forward met some of the requirements but did not fit all the available data on collagen (Bear, 1952; Crick, 1954; Pauling and Corey, 1951; Randall et al., 1952) and it became evident that the structure of collagen could not be accounted for by the usual protein structures, e.g. α-helix.

The secondary structure of collagen is concerned with the individual structure of each of the three polypeptide chains known to be present in the molecule, whilst the tertiary structure involves the arrangement of these relative to each other.

The main stabilization interaction in the superhelix or tertiary structure is derived from the cooperative system of lateral hydrogen bonds between the component polypeptide helices, whereas the secondary structure of the individual chains is maintained by the rigidity conferred by the rotational restrictions which exist at the α-carbon to carbonyl-carbon bonds of the pyrrolidine residues and the partial double-bond character of all the peptide bonds (Harrington and McBride, 1966).

Detailed examination of the X-ray diffraction pattern of collagen indicated that the helix formed by each of the polypeptide chains is a non-integer in the sense that the helix does not make a 360° turn for every Gly-X-Y triplet in the chain (Ramachandran, 1967). In order to reconcile this fact with the evidence that every Gly is packed into the centre of the tropocollagen molecule it is necessary to postulate that the three helical chains are twisted around each other into a superhelix. This superhelix has a repetitive cycle or pitch of about $104\mathring{A}$ whereas the helix formed by each polypeptide chain has a repetitive cycle of about $9.5\mathring{A}$. Since the axial distance between one amino acid and another is $2.91\mathring{A}$, each complete turn of the superhelix contains 36 amino acids ($104\mathring{A}/2.91\mathring{A} = 36$), and each complete turn of the helix within the chain contains an average of 3.3 amino acids ($9.5\mathring{A}/2.91\mathring{A} = 3.3$).

The high percentage of Gly in collagen distinguishes it from all other known proteins with the exception of the closely related fibrous protein elastin (Hall, 1971). The function of Hypro in terms of collagen structure is not understood, but it has been suggested that the introduction of polar hydroxyl groups may modify the hydrophobic-hydrophilic

balance in aqueous media and thereby influence the formation of fibres by the molecule (Gustavson, 1956). Traub and Piez (1971) suggested that contrary to what was once thought, Hypro does not play any special role in stabilizing the molecular conformation. Recently, however, Ramachandran et al. (1973) proposed a hypothesis on the role of Hypro in stabilizing the collagen structure. The role of Hylys has attracted a great deal of attention since it became apparent that the small amount of carbohydrate found in collagen is accounted for by galactose and glucosylgalactose in O-glycosidic linkage to the hydroxyl group of Hylys.

There are a considerable number of known structural features that must be incorporated into a model for collagen. One of the most restrictive being that it must conform to the helical parameters derived from the X-ray pattern. However, it is not readily apparent what the equivalent structural elements of the helix are composed of, nor how they are linked chemically into polypeptide chains.

Another restraint on model building is that all the peptide groups must be planar and conform closely to standard dimensions.

Finally, any satisfactory collagen model must take account of the peculiar amino acid composition and sequence of this protein. Because of the relatively high content of the imino acids Pro and Hypro which have a five-membered ring structure that forms part of the peptide unit, as well as part of the chain on the α-carbon atom, they cannot be accommodated in the α-helix of Pauling and Corey, which is the basic helical structure for such proteins as keratin, myosin, etc. Thus a different basic type of structure is required for collagen to accommodate the steric restrictions of these ring structures. Furthermore, these imino acids cannot form intrachain NH-O hydrogen bonds, which are the basic stabilizing factor in the α-helix. The type of intermolecular hydrogen bonds which these imino acids can form is also significant.

The high glycyl, prolyl and hydroxyprolyl residues in collagen has encouraged the synthesis and study of polypeptides containing these residues as an aid to unravelling the structural and physico-chemical complexities of collagen itself.

Many polytripeptide models for collagen with ordered sequence have been synthesized. These, like collagen, have had Gly as every third residue as well as residues of one or both of the imino acids Pro and Hypro (Kitaoka et al., 1958; Berger and Wolman, 1963; Debabov et al., 1963).

Andreeva et al. (1961, 1963) were able to show that the polymer $(Gly\text{-}Pro\text{-}Hyp)_n$ closely resembles collagen in its optical rotation, infra red spectrum and X-ray powder pattern.

Very soon after the above publications, a large number of papers were published revealing collagen-like X-ray patterns for a number of other polytripeptide sequences viz.:-

$(Gly\text{-}Pro\text{-}Pro)_n$	(Engel et al.,1965; Shitnev et al.,1965; Traub and Yonath, 1965; 1966.)
$(Gly\text{-}Pro\text{-}Ala)_n$	(Traub and Yonath,1965; 1967; Andreeva et al., 1967.)
$(Gly\text{-}Hyp\text{-}Hyp)_n$	(Shibnev et al.,1966; Andreeva et al.,1967.)
$(Gly\text{-}Hyp\text{-}Pro)_n$	(Traub and Yonath,1966; Andreeva et al., 1967.)
$(Gly\text{-}Pro\text{-}Lys)_n$	(Andreeva et al.,1967.)

It appears that all these polymers have helical parameters close to those of collagen, and they presumably have a similar, if not identical, conformation with three polypeptide chains wound about a common axis. This is clearly evident in the case of $(Gly\text{-}Pro\text{-}Pro)_n$ (Traub and Yonath, 1966) and it is now accepted that $(Gly\text{-}Pro\text{-}Pro)_n$ is the most frequently occurring polytripeptide unit occurring in collagen's molecular structure (Traub et al., 1969).

In summary, it is thus seen that each polypeptide chain has a 3-fold left-hand screw axis, such that the screw from one residue to the next has

a rotation of -120° and a translation or displacement in the direction of the axis of 3.12Å. Each of these 3 polypeptide chains is arranged such that there are approximately 3.3 residues per turn of the minor helix and no intra-chain hydrogen bonds. The screw is left-handed to accommodate L-proline. The three helices, with their axes parallel and each about 5Å from the other at the corners of an equilateral triangle, are then coiled around each other to form the triple helix (superhelix) of the collagen molecule. The axes of the three chains twist slowly round each other in a gradual right hand super-helix, having a twist of 30° per three residues. Thus the sense of twist of the superhelix is in the opposite direction to that of the three minor helices. It requires a rotation of -110° and a translation of 2.91Å to go from an α-carbon atom in one chain to the corresponding α-carbon on the next chain. The coiling of the superhelix is such that the α-carbon atom of every third residue comes on the inside of the superhelix, and only Gly with no side chain on its α-carbon atom can sterically fit into this position. It should be noted that if each minor helix had three residues per turn, and not 3.3, then in forming the superhelix by twisting the 3 minor helices, the α-carbon atom of every third residue would not come on the inside of the superhelix.

4. Quaternary structure

The collagen fibre represents an end product in a series of interrelated steps. The quaternary structure is thus involved with the problem of how the collagen molecules align themselves to produce native fibrils having a repeat period between 640 and 710Å. Furthermore, any theory proposed must also account for the other known natural or synthetic types of collagen fibril structure. Schmitt, Hall and Jakus (1942), in their original examination of collagenous fibres by electron microscopy, proposed a terminology for the various structural hierarchies which can be discerned.

Accordingly, the term "fibre" is used for microscopically visible aggregates of variable thickness, ranging in diameter in various preparations from 20 to 200 μ. Smaller aggregates are called "primitive fibres", and these may range from 2 to 10 μ in diameter. Further subdivision by mechanical or mild chemical treatment gives the still smaller units termed "fibrils", of diameters from 0.5 μ down to approximately 100 to 200Å. In general, the fibril may be considered as the smallest unit in which the characteristic structural pattern may be observed in the electron microscope.

i) Electron microscopy

Staining in electron microscopy is used primarily to increase contrast. Positive staining involves the use of ionic electron-dense compounds such as phosphotungstic acid believed to indicate the position of polar groups in collagen molecules (Hodge, 1960). The negative staining method aims to surround or embed the specimen in electron-dense material. Potassium phosphotungstate at pH 6.8-7.4 is widely used as a negative stain (Brenner and Horne, 1959) and has been found particularly useful for high resolution electron microscopy. Negative staining enhances the visualization of fine structure. It has been shown that the light cross-striations seen in negatively stained collagen preparations are due to lateral bonding of the tropocollagen molecules. It appears that both negative and positive staining of collagen for electron microscopy are complimentary to each other in the interpretation of the electron micrographs under consideration (Cox and Grant, 1969).

Characteristic bands or striations of fibrils have been recognised on electron micrographs since 1942 (Schmitt, Hall and Jakus, 1942). These patterns were caused by the alternation in a regular manner, of dark and light bands along the fibril length.

With improvements of electron microscopy techniques, it was found that a much more detailed band pattern is observable in many instances within the

major axial period (i.e. the distance from the edge of one dark band to the similar edge of the next dark band) - the so-called "intra period fine structure" of the fibril. By 1955 Nemetschek, Grassman and Hofmann, had succeeded in resolving thirteen intraperiod bands in certain collagen fibrils. The view held by Schmitt and Gross (1948), regarding the fine structure is that its occurrence arises from the lateral alignment of the characteristic chemical features of the protofibrils, and that the extent to which this is resolved depends largely on various factors operative during the preparative procedure.

As a result of studies with positively stained native collagen fibrils the idea was put forward that the banding in native collagen fibrils resulted from a regular packing of tropocollagen molecules such that each tropocollagen molecule was displaced by a quarter of the molecular length with respect to its immediate neighbours (Schmitt et al., 1955; Hodge and Schmitt, 1960; Schmitt and Hodge, 1960), thus giving rise to the "Quarter-stagger" theory. According to this theory the macromolecules are linked end-to-end by covalent bonds of the telopeptides to form linear polymers (Gross et al., 1954; Hodge et al., 1960; Hodge and Schmitt, 1958; Hodge and Schmitt, 1960; Schmitt and Hodge, 1960) (Fig. 1).

The quarter-stagger of the heads of the macromolecules corresponds to a repeating axial period of either 640Å (Cox and Grant, 1969) or 670Å (Bouteille and Pease, 1971; Segrest and Cunningham, 1971; Miller and Parry, 1973) the former measurement being obtained from electron microscope studies whilst the latter was obtained from X-ray diffraction patterns of collagen fibrils. Diffraction methods have the advantage that the structure of collagen fibrils can be examined while the tissue is in its "native" state, whereas electron microscopy requires fixative and staining procedures, which can perturb the structure of collagen fibrils (Bouteille and Pease, 1971). They have, on the other hand, the fundamental limitation that the amount, quality and to a

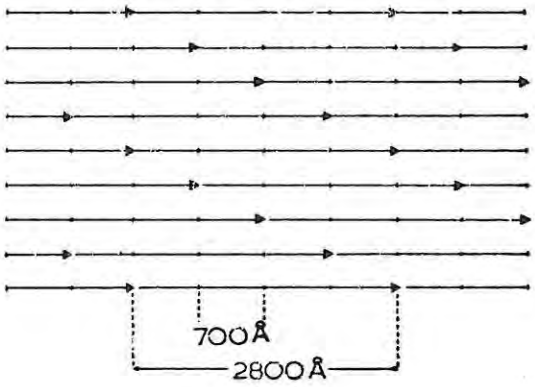


Fig. 1. The original quarter-stagger concept of fibril packing with collagen macromolecules linked end-to-end and with a displacement of one-quarter of the macromolecular length.

Reproduced from Cooper, D.R. and Russell, A.E. (1969).

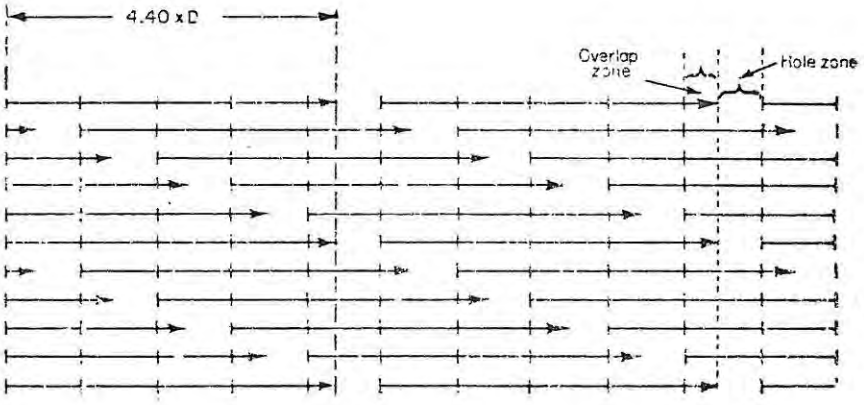


Fig. 2. Illustration of proposed packing in native type fibrils of collagen molecules in staggered array with hole zones of 0.6D and overlap zones of 0.4D.

Reproduced from Hodge, A.J. et al., (1965)

certain extent, the nature of the X-ray data is affected by the relative orientation and the diameters of the fibrils themselves in the tissue under consideration. In the native form of collagen each cross striation is considered to arise from 4 macromolecules in adjacent protofibrils with a corresponding accurate transverse alignment of 4 different but "equivalent loci".

Application of negative staining methods showed that the previous measurements of the lengths of a segment long-spacing particle (SLS - vide infra) were about 10 percent too short making it difficult to account for 4 molecular units of 640Å each being accommodated within the proposed length of 2800Å.

Olsen (1963 a,b) and Hodge and Petruska (1963) suggested that linear polymerization of tropocollagen molecules was by end overlap of the terminal 10 percent of the molecules. This brought about modification of the quarter-stagger hypothesis (Hodge et al., 1965). It was thus considered that the tropocollagen molecules aggregated into protofibrils by end overlap of the terminal 10 percent of the whole macromolecule and that these protofibrils were staggered by the length of a complete repeat period or multiples thereof to produce native fibres (Fig. 2).

Cox et al. (1967) and Grant et al. (1965), criticized this modified quarter-stagger theory and proposed an alternative theory. This hypothesis considered that the tropocollagen molecule involved 5A bands and 4B bands (Fig. 3). A three-dimensional model constructed on the basis of Fig. 3 is illustrated in Plate 1. The 5 main A zones are responsible for intermolecular bonding, and are therefore termed the "bonding zones" whilst the 4B zones are termed the "non-bonding zones". The A zones are each approximately 280Å and the B zones are each 360Å long. Thus, the length of one A plus one B zone corresponds to the repeat distance of 640Å. This hypothesis also considers that the bonding zones contain amino acid residues arranged in a manner so that, when 2 bonding zones approach one another

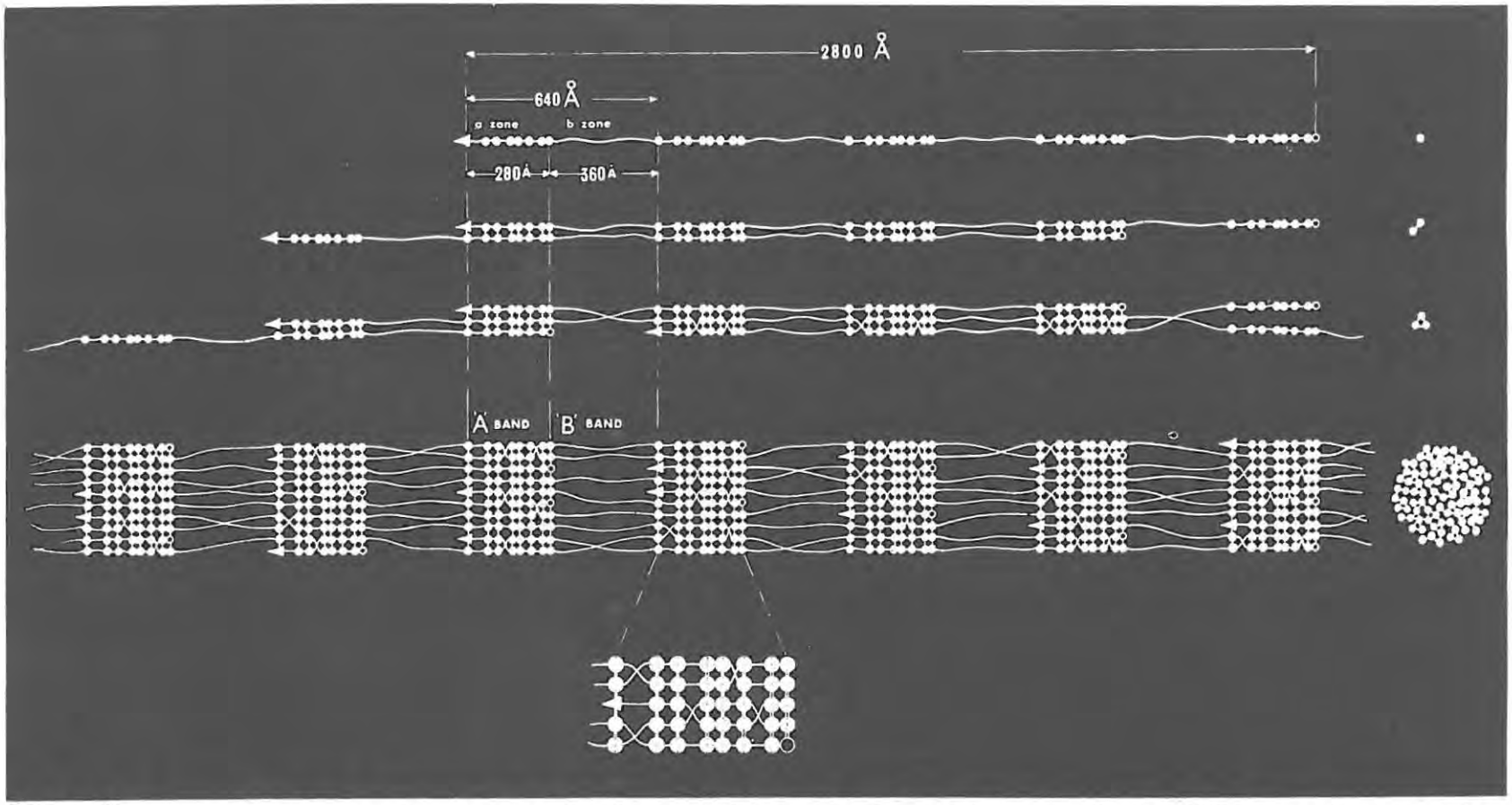


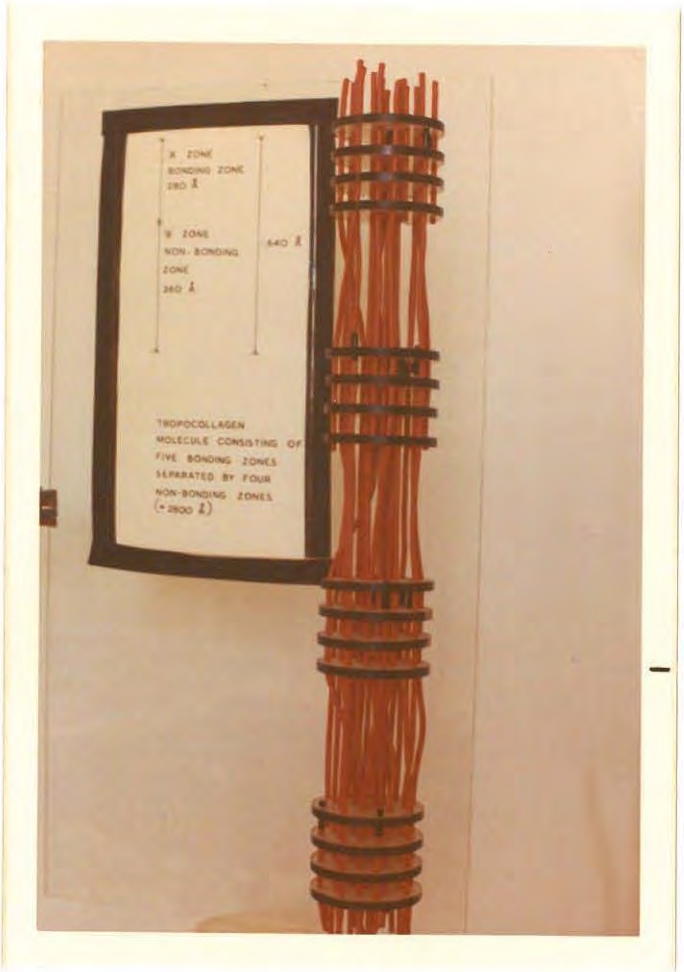
Fig. 3 Formation of a native collagen fibril with 640 A periodicity from tropocollagen macromolecules 2800 A long. The tropocollagen macromolecule is divided into 5 main bonding or *a* zones (approximately 280 A) separated by 4 main non-bonding or *b* zones (approximately 360 A). Some of the bonding sites within the main bonding zones of the macromolecules are represented by asymmetrically arranged white dots. By virtue of this asymmetry the macromolecules are polarized. White arrows and black dots at the ends of the macromolecules are inserted to emphasize this polarity. For clarity, minor bonding sites within the 4 main non-bonding zones are omitted.

When such tropocollagen macromolecules are assembled so that any main bonding zone on one macromolecule has an equal probability of initial lateral crosslinking, in a structurally complementary manner, with any main bonding zone on another macromolecule the various stages depicted in the formation of the native fibril occur.

The morphologic flexibility of the tropocollagen macromolecules is emphasized and their ability to cross one another in both the **A** and **B** bands is illustrated. The ratio of the number of macromolecules in the **B** band to the number of macromolecules in the adjacent **A** band approaches 4:5. The cross-sectional packing on the right has no particular significance as the suggested method of assembly is limited to the longitudinal arrangement of the macromolecules and does not restrict the transverse arrangement.

Reproduced from Cox, R.W. and Grant, R.A. (1969)

PLATE NO. 1



Three-dimensional model of native collagen fibril constructed on the basis of Fig. 3 (N.B. Only 4 A zones and 3 B zones are shown in this photograph).

close enough, intermolecular electrostatic and hydrogen bonds are formed. The 5 bonding zones per macromolecule may not be identical in amino acid residue content or sequence, but are sufficiently similar for an equal probability to exist for any bonding zone on one macromolecule to crosslink with any bonding zone on another macromolecule. The basic idea is that the macromolecules associate in a random way to form fibrils. Therefore any overlap region of adjacent macromolecules in the fibril can involve 1 to 5 bonding zones. The non-bonding zones, on the other hand, may be regarded as regions capable of forming only a few intermolecular bonds compared with the main bonding zones. Also, in view of the evident morphologic flexibility of tropocollagen molecules as seen in the electron microscope when using negative staining, it is not necessary to assume that the macromolecules combine in strictly parallel array, as has usually been assumed in the past, but that they may cross one another at small angles in both A and B bands. Therefore, the assembly of collagen molecules in this theory occurs by a random process, in contrast to the rigid alignment of molecules required by the quarter-stagger theory. According to this theory, intermolecular cross-links consist of electrostatic, hydrogen or hydrophobic bonds, while in the quarter-stagger theory it has been suggested that covalent bonds of the telopeptides may be involved in the assembly of macromolecules.

Further refinements have appeared in the literature recently. These have been concerned with the alignment of the tropocollagen molecules in 3 dimensions with retention of the quarter-stagger relationship between every nearest neighbour molecule. This is readily achieved in 2 dimensional models, but when this idea is extended to packing in a cylindrical form it becomes apparent that the quarter-stagger theory cannot be preserved for all neighbouring molecules. In order to achieve the correct packing, a number of theories have recently been proposed.

Veis et al. (1967) have based their concepts of the molecular packing in the collagen fibril on the presence of a fundamental packing unit

consisting of 4 macromolecules with a quarter-stagger lying parallel in a helix. These form a right-handed helical arrangement of 4 macromolecules displaced successively from the origin by 0,1,2 and 3 fundamental repeat distances (D) along the axis and 90° about the axis (Fig. 4). The fibril is built up with tetramers fitting into each other end-to-end, leaving a unit 'hole' (0.6D) and having a regular overlap (0.4D), and forming microfibrils of indefinite length. The microfibrils are then packed in register, side by side, to give a fibril.

This hypothesis has been criticized by Cox and Grant (1969) on the basis that the choice of a tetramer from which to construct the microfibrils leads to a repeating pattern along each microfibril whereby 0.6D holes occur regularly within each period for 3.6D of microfibril length, to be followed by a length of 1.4D where no such 0.6D spaces occur. The effect of packing this type of microfibril in register, side by side, is to produce a final fibril having repeating lengths of 1.4D that did not include a 0.6D hole resulting in a defect appearing in the final collagen fibril.

Smith (1968) has introduced a further theory in which the fundamental unit consists of 5 tropocollagen macromolecules as compared with the 4 of Veis et al. (1967). According to Smith's idea of molecular packing, the collagen molecules are arranged end-to-end with a 0.6D hole between successive macromolecules, with a quarter-stagger between adjacent molecules, and with 5 molecular strands arranged in the form of a cylinder (Fig. 5), thus preserving the quarter-stagger for all adjacent molecules. These cylindrical units or filaments can then aggregate to form fibrils. These filaments possess 1D periods, therefore the secondary stage of aggregation of filaments to form fibrils need not involve any further exact 1D stagger. The only requirement is for the periods of all aggregating filaments to be in register, and this could be achieved by a stagger between adjacent macromolecules in different filaments of an integral number of the period D. This model thus utilized the strict 1D stagger and the random type of aggregation (Cox et al., 1967 and Grant et al., 1965).

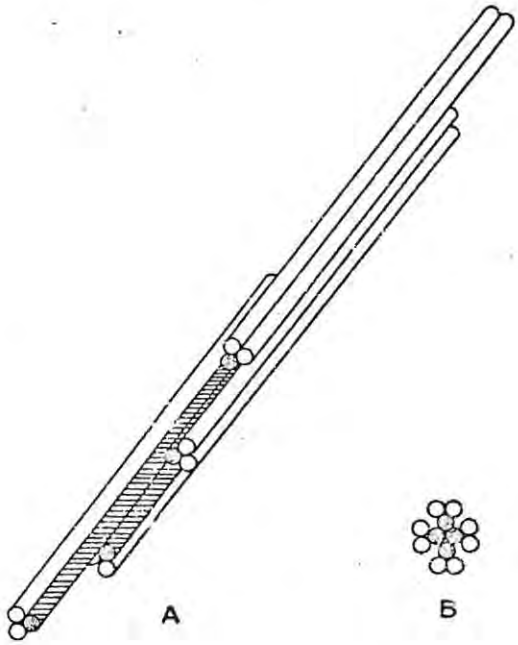


Fig. 4. A and B. Schematic view of the phasing of collagen macromolecules with respect to each other in the fundamental tetrad model of collagen monomer units, so that the α_2-chains (shaded) meet with other α_2-chains in a specific arrangement. A, dimensional view showing the α_2-core in a quarter-stagger arrangement; B, end view. The α_1-chains are unshaded. (After Veis, A. et al., 1967).

Reproduced from Cooper, D.R. and Russell, A.E. (1969).

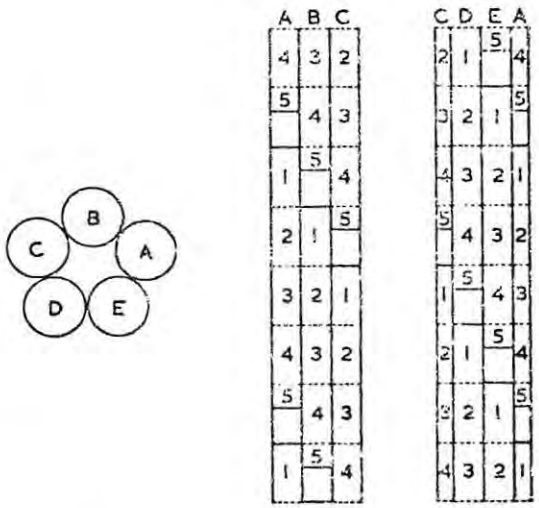


Fig. 5. Diagrams of end view and longitudinal cross-section of 5-strand filament model of collagen macromolecules with 1D periods (length of macromolecules = 4.4D) and hole zones (0.6D). (After Smith, J.W., 1968).

Reproduced from Cooper, D.R. and Russell, A.E. (1969).

X-ray diffraction evidence for the 5-stranded model has also been presented by Miller and Wray (1971), who, in addition have interpreted their X-ray data to indicate a laterally packed arrangement of five-stranded "coiled coiled coils", each of which runs through the fibril parallel to the axis, with only a small departure from the alignment of their respective gap/overlap patterns in axial register. Yuan and Veis (1973) in their studies of fibre formation using solutions of native acid-soluble- and pronase-treated acid-soluble collagen, showed that the molecular end-regions of collagen molecules played a very important role in molecular registration within the unit assemblies. Their conclusions also supported the type of molecular models proposed by Veis et al., (1967 and 1970) and by Smith (1968) in which fibres are formed from limiting microfibrils themselves comprised of unit assemblies of four or five collagen molecules. Further evidence from X-ray data (Segrest and Cunningham, 1973; Miller and Parry, 1973) provides additional evidence which favours the five-stranded model.

In addition to the native fibrils, Schmitt et al., (1953); Randall et al., (1953 and 1955); Jackson and Randall, (1953), found that collagen can be precipitated in forms other than fibrils with 640Å periodicity striations by alteration of pH and ionic strength or by adding certain non-specific agents, such as adenosine triphosphate (ATP) and chondroitin sulphate (Highberger et al., 1951; Schmitt et al., 1955). They distinguished three forms which when stained with phosphotungstic acid, had a characteristic appearance in the electron microscope, in addition to the non-striated fibrils and native-type fibrils:

a) Striated fibrils with a repeating pattern of about 210Å,

b) striated fibrils with nonpolarized repeating pattern of about 2600Å (fibrous-long spacing, FLS), and

c) a segmented form about 2600Å long having a polarized pattern (segment-long-spacing, SLS).

All these forms were found to be inconvertible (Fig. 6).

Kühn et al., (1957, 1958) showed that the collagen fibrils stain with phosphotungstic acid by virtue of interaction with positively charged Lys and, more particularly, arginine residues. Staining with uranyl acetate, or chromium salts which also show up a fine structure, is thought to be due to uptake of uranyl ions by acidic chains (Kühn and Gebhardt, 1960).

Since all the different ordered forms of precipitated collagen are associated with the apposition of polar regions of different molecules and the mode of precipitation depends strongly on ionic strength, pH, and the presence of traces of highly charged molecules such as ATP, the intermolecular forces which determine the mode of precipitation are thought to be predominantly electrostatic in nature. The nature of some of the polar groups which are important in this respect was investigated by Martin et al. (1961), who implicated the imidazole groups, by Kühn et al. (1959) and Bensusan et al. (1962), who showed the importance of the ε-NH_2 of Lys and Hylys; and by Bensusan and Scanu (1960), who postulated that ionization of the phenolic group of tyrosyl residues is an important step in fibril formation.

ii) Fibril kinetics

It has been shown (Bahr, 1950; Vanamee and Porter, 1951) that native-type fibrils form in collagen solutions over a restricted range of pH and ionic strength. The conditions required for the aggregation of tropocollagen molecules 'in vitro' have been investigated by a large number of research groups (Randall et al., 1952, 1955; Gross and Kirk, 1958; Schmitt et al., 1955; Schmitt, 1959; Hodge and Schmitt, 1960; Wood and Keech, 1960; Bensusan and Hoyt, 1958; Bensusan and Scanu, 1960; Wood, 1960 a,b,; Wood, 1962; Cassel et al., 1962; Bianchi et al., 1966).

The study of the kinetics of fibril formation has been facilitated by the form of the collagen precipitate which, over a wide range of experimental conditions, is a uniform gel whose optical density (E) can be easily measured and is proportional to the amount of collagen precipitated to a good

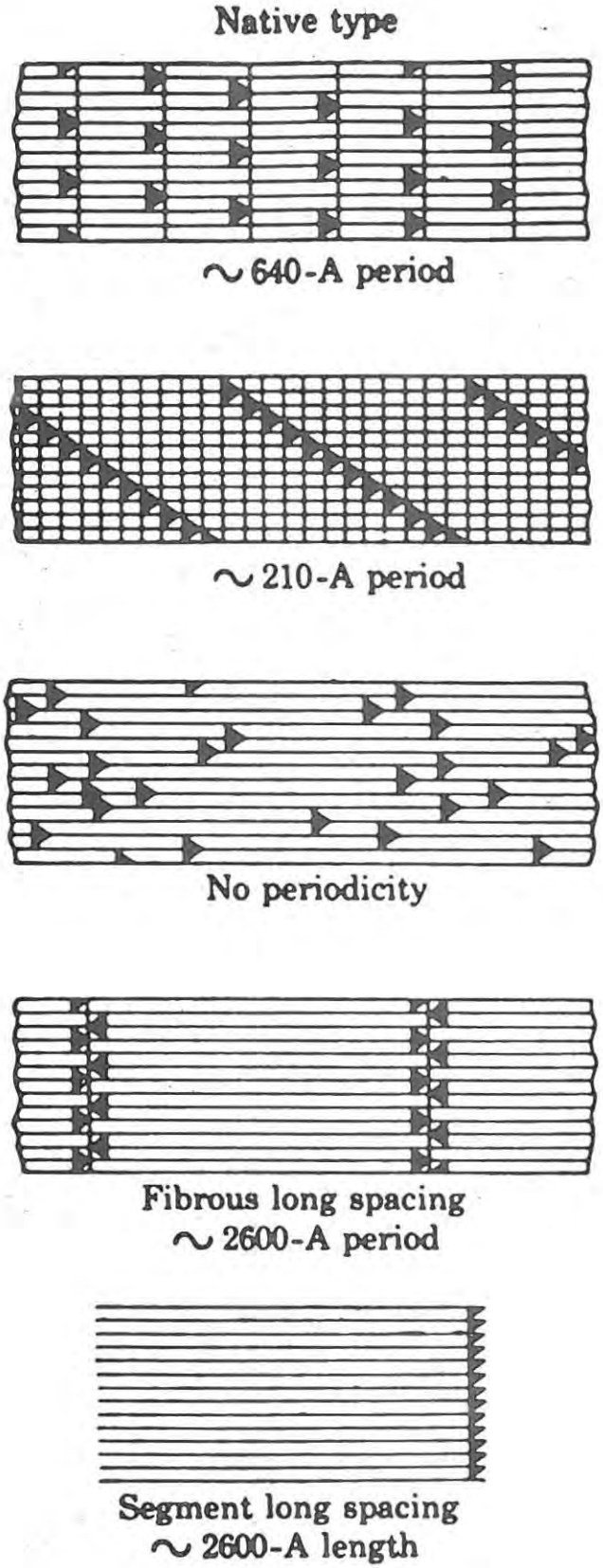


Fig. 6. Schematic two-dimensional view of various modes of aggregation of collagen molecules 'in vitro', illustrating the generation of the various periodicities.

Reproduced from Schmitt, F.O. (1956).

approximation (Gross and Kirk, 1958; Wood and Keech, 1960). Wood and Keech (1960) have reported that slight variations in the pre-treatment of collagen solutions and in experimental procedure have a marked effect on the kinetics and that it is difficult, therefore, to compare the results of different research groups. In spite of these difficulties, a number of common features can be observed.

Graphs of the extent of precipitation against time all show a sigmoid portion preceded by a more or less marked lag phase during which no precipitation is observed (Gross and Kirk, 1958; Bensusan and Hoyt, 1958; Wood, 1958; Wood and Keech, 1960). Both phases of the process are accelerated by increasing the temperature or collagen concentration, but are retarded by adding increasing concentrations of inorganic salts. The magnitude of the latter effect is dependant upon the nature of the inorganic ions. At relatively low ionic strength (0.09-0.135) in tris buffer (pH 8.3), the retarding effect of added anions increased in the order SCN^-, I^-, Br^-, Cl^-, $SO_4^=$, $S_2O_3^=$ (Bensusan and Hoyt, 1958). The difference between cations was smaller, although divalent ions (Ba^{++}, Ca^{++}, Mg^{++}) had a greater effect than K^+, Na^+, and Li^+. The effect of phosphate appears to be intermediate between those of I^- and Cl^-, but it seems likely that phosphate has other specific effects attributable to the binding of phosphate ions to sites on the protein (Bensusan and Hoyt, 1958; Bensusan, 1960; Gilbert, 1960). This fact emerged when Bensusan and Hoyt (1958), found that SCN^- ions retarded precipitation in tris buffer (pH 8.3) but when phosphate buffer was used (Gross and Kirk, 1958; Bensusan, 1960) precipitation was accelerated. It has also been shown that variation of the kinetics with pH is buffer dependent. Bensusan and Hoyt found that with tris buffers precipitation occurred at a maximum rate at pH 7.3 - 8.2, depending only slightly on ionic strength. With phosphate buffers, on the other hand, precipitation occurred at a minimum rate at pH 7.1 - 7.5 (Wood and Keech, 1960). A similar effect of pH with phosphate buffers was observed at higher temperature (37^oC) when the reaction was slowed

down to a measurable rate by 0.24M urea (Gross and Kirk, 1958).

The importance of electrostatic interactions was shown by Bensusan (1960) during his systematic analysis of the effect of a series of alcohols on the rate of fibril formation. At alcohol concentrations greater than about 0.6M precipitation was accelerated and the magnitude of this effect correlated with the decrease in dielectric constant of the medium. Lower alcohol concentrations accelerated precipitation, an effect attributed to interaction between the aliphatic portions of the alcohol molecules and the non-polar side chains of the protein.

It has also been shown that change of pH, ionic strength, etc., affects the size of the fibrils produced (Vanamee and Porter, 1951). Wood and Keech (1960) have attempted to relate fibril width to the kinetics of fibril formation. The fibril widths correlated well with the optical density of the collagen gels but it was evident that no simple relationship existed between fibril width and the rate of fibril formation.

The appearance of two well-defined phases during fibril precipitation - the lag and growth phases respectively - implicates two separate consecutive reactions. Wood and Keech (1960) showed that both phases were accelerated by increasing the temperature (20-37^{o}C) at which the whole reaction took place. At the same time it was found that fibril width decreased. However, if the lag phases of a number of precipitations were allowed to occur at the same temperature and only their growth phases conducted at various temperatures, the width of the fibrils produced was unaltered in spite of the fact that the rate of the second phase varied with temperature. This suggested that the same number of fibrils was formed in each experiment and that the formation of these fibrils was initiated during the lag phase. Bard and Chapman (1973) also showed that nucleation of new fibrils become less probable as growth proceeds, with the later-aggregating collagen adding to pre-existing structures. Their findings further suggested that accretion of this later aggregating collagen occurs predominantly at the smaller-diameter end regions of fibrils

and that lateral growth slows down and eventually ceases once the diameter has reached a value somewhere in the range of 600 - 2,400Å.

Mathematical analyses of the rate of fibril formation were given by Wood (1960) and Cassel et al. (1962) whilst thermodynamic studies of the assembly of native collagen fibrils 'in vitro' were reported by Cooper (1970).

Cassel et al. (1962) investigated the kinetics of native-type fibril formation over a wide range of collagen concentrations (0.001 - 0.40%) at temperatures of 16 - 40°C. They showed that although the rate of precipitation was also influenced by protein concentration, the rate was highly temperature dependent.

Both Wood (1960) and Cassel et al. (1962) were in agreement that nuclei are not constant in number during the lag phase but increase with time. Although studies of the kinetics of fibril formation have given much information regarding the growth phase, details of nucleation are rather vague.

The work on neutral-salt-soluble collagen (Wood, 1962) confirmed the general idea of the two-phase nature of fibril formation, but has also shown that a rather more complex situation exists.

Wood (1962) also showed that acid-soluble collagen nucleates more readily than neutral-salt-soluble collagen and suggested that differences in the aggregation behaviour of the various collagens and collagen fractions might be related to the degree of cross-linking.

ii) Effect of polyanions on fibrillogenesis

Because of the abundant occurrence of mucopolysaccharides in developing connective tissue at sites of collagen formation, investigations were undertaken to establish the effects of these mucopolysaccharides on fibrillogenesis. Highberger et al. 1951, and Randall et al. 1952, showed that mucopolysaccharides cause precipitation of collagen from tissue extracts over a pH range of 3 - 4. In many instances, however, the fibrils precipitated under

these conditions were either devoid of structure or showed spacings other than the 640Å periodicity associated with native fibres.

The observations that fibril formation took place in the absence of added mucopolysaccharide suggested that these compounds do not play a major role in fibril formation (Jackson, 1958) although the possibility that 'in vivo' they play a part in stabilizing the fibril after its formation was not discarded. Lowther (1963) suggested an indirect role through the formation of an extracellular viscous gel which may play the role of confining collagen molecules near a cell surface and, thus, by a concentration effect, assist in their aggregation to fibrils.

The first observations of the effect of polyanions such as chondroitin sulphate, hyaluronic acid, keratin sulphate and heparin sulphate in a neutral pH range indicated little or no effect on the rate of native-type collagen fibril formation. Wood (1960b) showed that very low concentrations of chondroitin sulphates A and C and kerato-sulphate accelerated the nucleation phase and rate of fibril development, whereas heparin, deoxy-ribonucleic acid, and a series of dextran sulphates of different molecular weights and degree of sulphation had an opposing effect. Such effects were eliminated by prior dialysis of the collagen solution against phosphate buffer. On the other hand, chondroitin sulphate B and hyaluronic acid were without effect on fibril formation. Keech (1961) from his electron micro-scope studies reported that precipitation accelerators produced thinner fibrils, whereas the presence of inhibitors provided thicker fibrils.

The high sensitivity of the structural features of compounds on fibrillogenesis 'in vitro' is shown in a number of examples, viz. :- Chondroitin sulphates A and B have different effects in spite of their almost identical structural similarity. Caygill (1969) showed that ascorbic acid delays or prevents native-type fibril formation whilst dehydro-ascorbic acid is without effect. Trnavska et al. (1966) showed differences between homogentistic and gentistic acid effects on the lag phase.

Toole and Lowther (1968) described the precipitation of acid-soluble collagen by chondroitin sulphate-protein. Instantaneous precipitation occurred at physiological pH and ionic strength at 40°C and involved about two thirds of the total collagen. Electron microscopic examination indicated that native fibrils were precipitated. These authors suggested that aggregates of chondroitin sulphate-protein and tropocollagen form instantaneously and that these act as sites for the second stage of precipitation of fibrils. On the basis of their findings 'in vitro' they proposed a sequence of events for collagen fibrogenesis 'in vivo'.

In a further investigation, Wood (1963a) confirmed a previous hypothesis that lathyrogens act by altering the aggregation stage of collagen fibrillogenesis, possibly by inhibiting the cross-linking mechanism (Martin et al., 1963). Convy and Wynn (1967) extended this work by studying the action of semicarbazide (a lathyrogen) on fibril formation 'in vitro'. They suggested that semicarbazide binds covalently to ester bonds and that the extent of binding is both pH and temperature dependent. In a later paper, Ayad and Wynn (1970) concluded that semicarbazide was bound to an αβ-unsaturated aldehyde believed to be the main intramolecular cross-link of the β-unit (Bornstein et al., 1966) and that this binding affected the nature (final fibril width) and the stability of fibrils reconstituted from solution.

Mathews and Decker (1968) studied the effects of a number of highly purified and physically characterized mucopolysaccharides and 'native' mucopolysaccharide-proteins on the size and rate of formation of fibril aggregates from collagen solutions in pH 7.6 buffers. They related their observations to the molecular sizes of the mucopolysaccharides used and showed that the agents that had delayed fibril formation were also effective in producing an increase in degree of fibrillar collagen. Lowther and Natarajan (1972) studied the influence of glycoprotein on collagen fibril formation in the presence of chondroitin sulphate proteoglycan and reported that the physical state of the proteoglycans is important in controlling only

the initial stages of collagen fibril formation. Unaggregated proteoglycans inhibit collagen fibril formation 'in vitro' for several hours, but when aggregated chondroitin sulphate proteoglycan containing the glycoproteins was added to tropocollagen solutions, no inhibition of fibril formation was observed. The glycoprotein alone was shown to have no effect on collagen fibril formation. These authors suggested that the interactions of sulphated proteoglycans with tropocollagen may play a role in collagen fibrillogenesis both 'in vitro' and 'in vivo'.

In addition to the abovementioned investigations involving polyanion effects on fibril formation 'in vitro' a number of workers have investigated the effects of unsaturated fatty acids (Grant and Alburn, 1968), pepsin-treated collagen (Bannister and Burns, 1972), non-steroidal anti-inflammatory drugs (Grant et al., 1970) and steroidal anti-inflammatory agents (Cooper, Kanfer and Price, 1970).

B. CORTICOSTEROIDS

The adrenal cortex of man and the higher animals produces a number of steroid hormones which have a wide variety of physiological functions. The three major types of biological responses elicited by the adrenocortical hormones are:-

1) alterations in carbohydrate metabolism;
2) anti-inflammatory activity, and
3) maintenance of proper salt and water balance through effects on the rate of Na^+ excretion from the kidneys.

During the 1950's, a largely empirical search was made for drugs which had a higher anti-inflammatory potency than the natural glucocorticoid hormones, cortisone and hydrocortisone, but which did not alter the electolyte metabolism, a side effect characteristic of these anti-inflammatory drugs. It was shown that a 9α-fluorine atom increases anti-inflammatory activity relative to the natural glucocorticoids but this was accompanied by an increase in sodium

retention. Dehydrogenation of the 1-2 bond also causes an increase in anti-inflammatory activity but without the undesirable increase in sodium retention which accompanies 9α-fluorination. The effects of 1-dehydrogenation and 9α-fluorination are, however, cumulative and whilst the doubly modified steroid has very favourable anti-inflammatory properties, it also causes sodium retention.

6α-Fluorination causes a much smaller increase in anti-inflammatory activity than does 9α-fluorination. The sodium retention properties can, however, be eliminated by the addition of either an α or β 16-methyl group (e.g. Betamethasone and Dexamethasone). The addition of an α-16 hydroxy group has a similar effect (e.g. Triamcinolone).

The relatively low aqueous solubility of adrenocortical and related steroid hormones has often been a hindrance in the study of the interaction of these compounds with proteins in a physiological environment. Hence, the availability of various water-soluble ester forms of several of these steroids facilitates the investigation of their interaction with soluble collagen in aqueous solution.

In the present study, both the hemisuccinate and disodium phosphate esters were utilized in the fibril kinetic investigations whereas only the disodium phosphate received attention in the binding determination experiments.

The water-soluble steroid esters are of interest pharmaceutically because they provide a means of making soluble derivatives out of highly insoluble compounds. Their clinical usefulness is exemplified by the fact that they permit facile parenteral administration where immediate high levels of circulatory steroid are required. In addition, they are extremely useful in aqueous pharmaceutical formulations such as for example, eye-drops.

The compounds used in this study were, hydrocortisone, prednisolone, methyl prednisolone, paramethasone, betamethasone and dexamethasone (Fig. 7).

1. Phosphate esters

The pH profile for all alkyl phosphates is a composite of the individual species profiles, and its shape is determined by their relative concentrations and rates. Four species are possible: the conjugate acid, the free acid, the monoanion, and the dianion. The conjugate acid is formed in highly acidic solution ($pH \ll 1.0$) and thus is only of marginal concern for the steroid-21-phosphates from the pharmaceutical application viewpoint. In the pH range, 1-8, the stability of these compounds can be explained on the basis of the independent reactivities of the free acid and monoanion.

During the present investigations, the influence of two pH regions on binding was investigated; these being pH = 3 and 7. It has been shown by Flynn and Lamb (1970) that at pH = 3, there exists approximately 26.2% of the free acid, 73.8% of the monoanion and negligible amounts of the dianion. At pH = 7, these authors found negligible amounts of the free acid, 9.9% of monoanion and 90.1% dianion. Mechanisms for the hydrolysis of various steroid phosphate esters have also been postulated as a result of their investigations.

Marcus (1960) investigated the degradation of hydrocortisone phosphate in essentially neutral solutions and found that hydrolysis appeared to be the only significant degradative pathway.

2. Hemisuccinate esters

The kinetics of hydrolysis of sodium hydrocortisone hemisuccinate in aqueous solution has been reported by Garrett (1962). It was found that the degradation appeared to involve specific ion catalyzed hydrolysis as the only significant reaction pathway. The major conclusion was that a 21-steroid ester is highly susceptible to alkaline hydrolysis. The stability of hydrocortisone hemisuccinate is given in the following table.

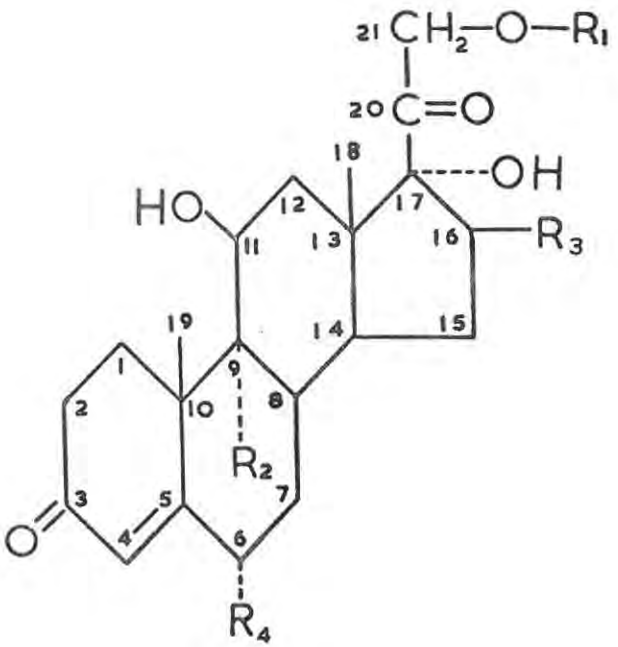
Predicted Half-Life ($t_{\frac{1}{2}}$) of
Hydrocortisone Hemisuccinate at Various pH values at 25°C.

pH	$t_{\frac{1}{2}}$
7	80 days
8	8 days
9	19 hours
10	1.9 hours

Reproduced from Garrett, E.R. (1962)

Mauger et al. (1969) studied the degradation of hydrocortisone hemisuccinate at 70°C and at pH's of 6.9, 7.2 and 7.6. The pH profile indicated that hydrolysis is due to a specific acid catalysis in the pH range of 1 to about 2.5 and specific hydroxyl catalysis from approximately pH = 7.6 to 10.0. In the intermediate range, the compounds may be subjected to an intramolecular attack of the anion on the ester carbonyl or specific hydroxyl ion catalysis of the undissociated hemiester.

Fig. 7



LIST OF CORTICOSTEROIDS

1) Betamethasone Sodium Phosphate [9α-fluoro-11β,17α,21-trihydroxy-16β-methylpregna-1,4-diene-3,20-dione 21-(disodium phosphate)]
(R_1 = PO_3Na_2; R_2 = α-F; R_3 = β-CH_3; R_4 = H)
Double bond between C_1 and C_2.

2) Dexamethasone Sodium Phosphate [9α-fluoro-11β,17α,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione 21-(disodium phosphate)]
(R_1 = PO_3Na_2; R_2 = α-F; R_3 = α-CH_3; R_4 = H)
Double bond between C_1 and C_2.

3) Paramethasone Sodium Phosphate [6α-fluoro-11β,17α,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione 21-(disodium phosphate)]
(R_1 = PO_3Na_2; R_2 = H; R_3 = α-CH_3; R_4 = α-F)
Double bond between C_1 and C_2.

4) Prednisolone Disodium Phosphate[11β,17α,21-trihydroxypregna-1,4-diene-3,20-dione 21-(disodium phosphate)]
(R_1 = PO_3Na_2; R_2 = H; R_3 = H; R_4 = H)
Double bond between C_1 and C_2.

5) Hydrocortisone Sodium Phosphate [11β,17α,21-trihydroxypregna-4-ene-3,20-dione 21-(disodium phosphate)]
(R_1 = PO_3Na_2; R_2 = H; R_3 = H; R_4 = H)

6) Hydrocortisone Sodium Succinate [Sodium salt of 21-(3-carboxy-propionyloxy)-11β,17α-dihydroxypregna-4-ene-3,20-dione]
(R_1 = CO-CH_2-CH_2-COONa; R_2 = H; R_3 = H; R_4 = H)

7) Methylprednisolone Sodium Succinate [Sodium salt of 21-(3-carboxy-propionyloxy)-11β,17α,21-trihydroxy-6α-methylpregna-1,4-diene-3,20 dione]
(R_1 = CO-CH_2-CH_2-COONa; R_2 = H; R_3 = H; R_4 = α-CH_3)
Double bond between C_1 and C_2.

COLOUR CODE FOR PLATES 2 - 6

Upper model = Dreiding type

Colour		Atom
White	=	H
Black	=	C
Red	=	O
Lilac	=	P
Green	=	F

Yellow = double bond.

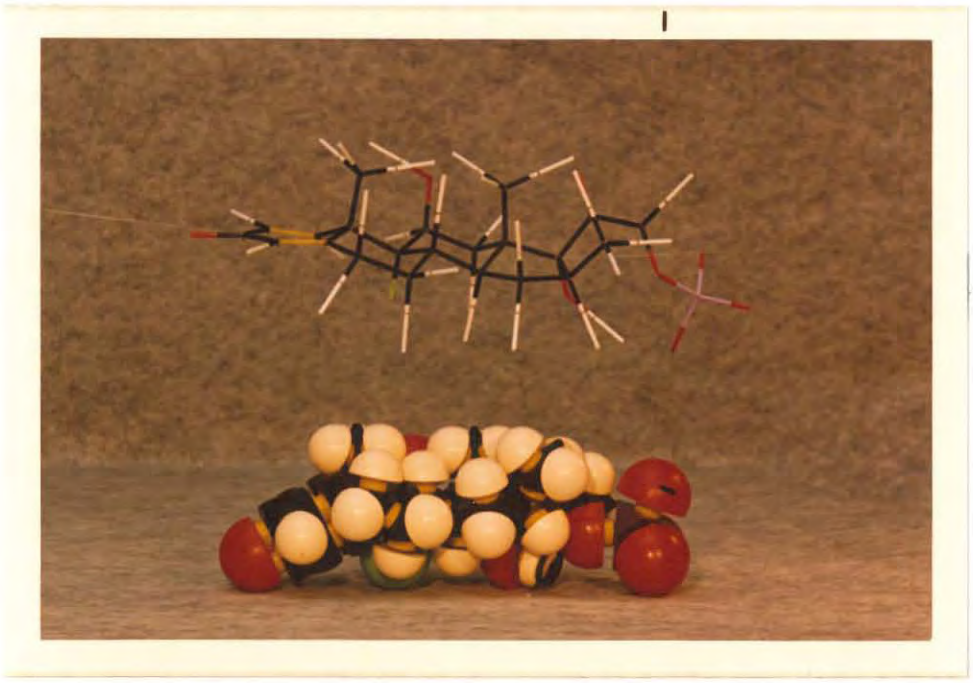
Lower model = Courtauld stereomodel

Colour		Atom
White	=	H
Black	=	C
Red	=	O
Purple	=	P
Red with black dash	=	O^-
Green	=	F

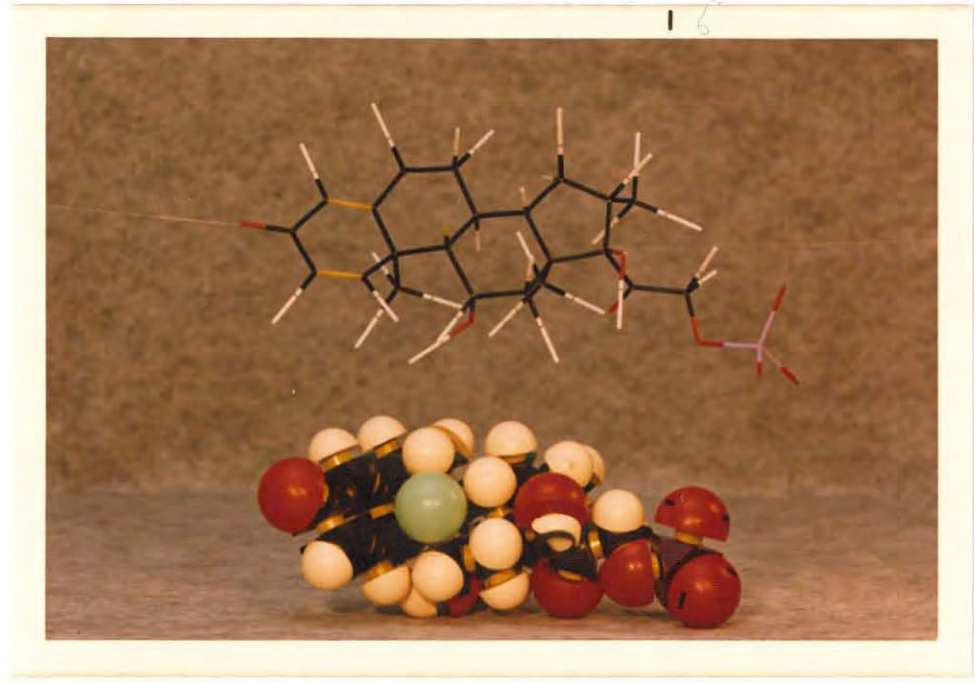
See Fig. 7 for numbering of individual atoms.

PLATE NO. 2

BETAMETHASONE ESTER PHOSPHATE



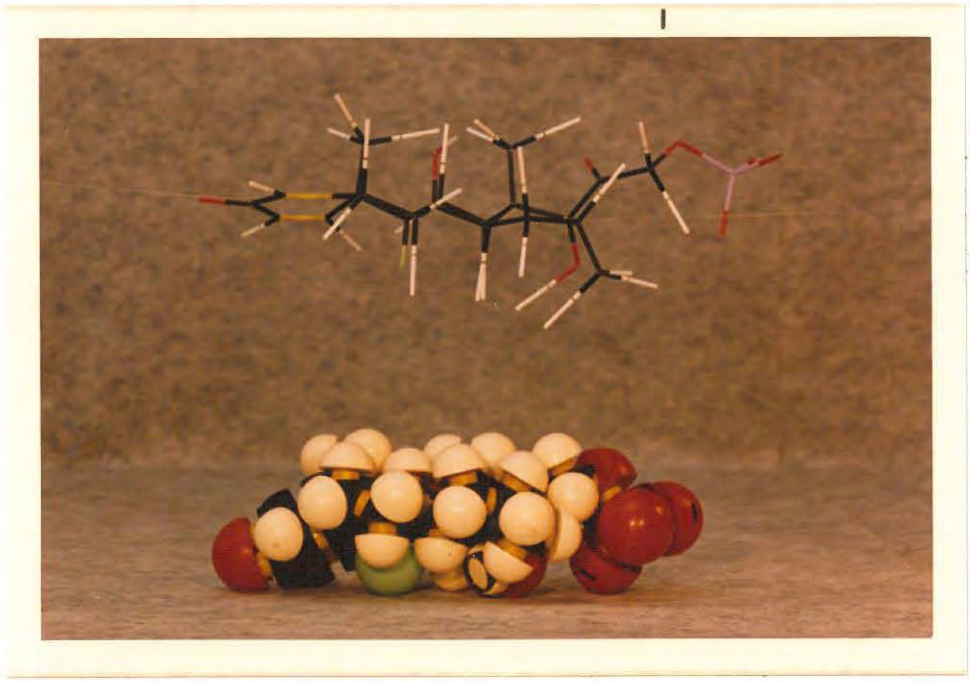
(a) Side view.



(b) Rear view.

PLATE NO. 3

DEXAMETHASONE ESTER PHOSPHATE



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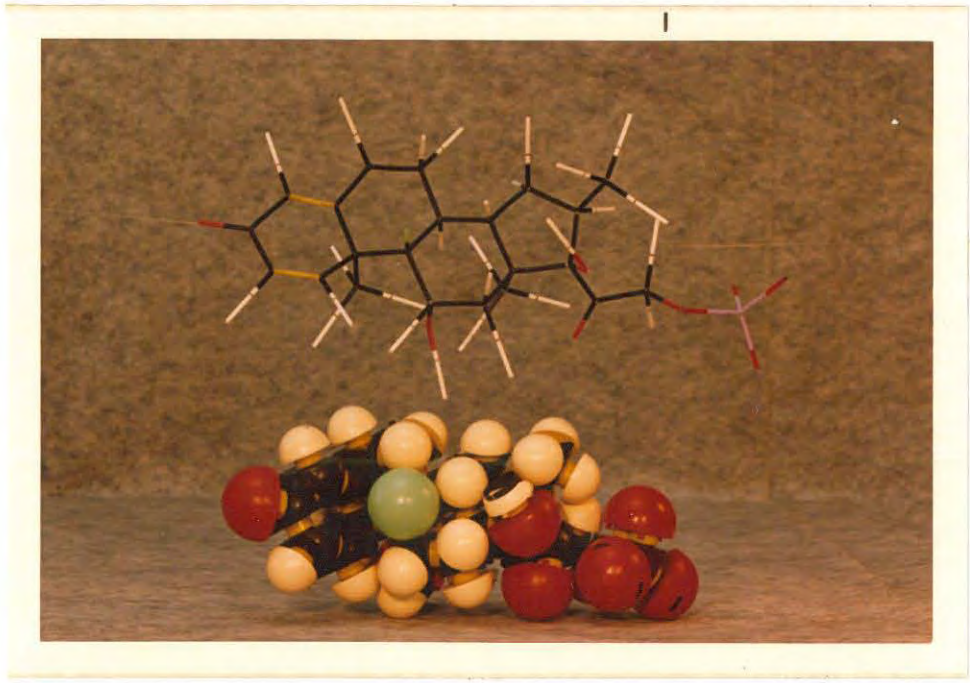
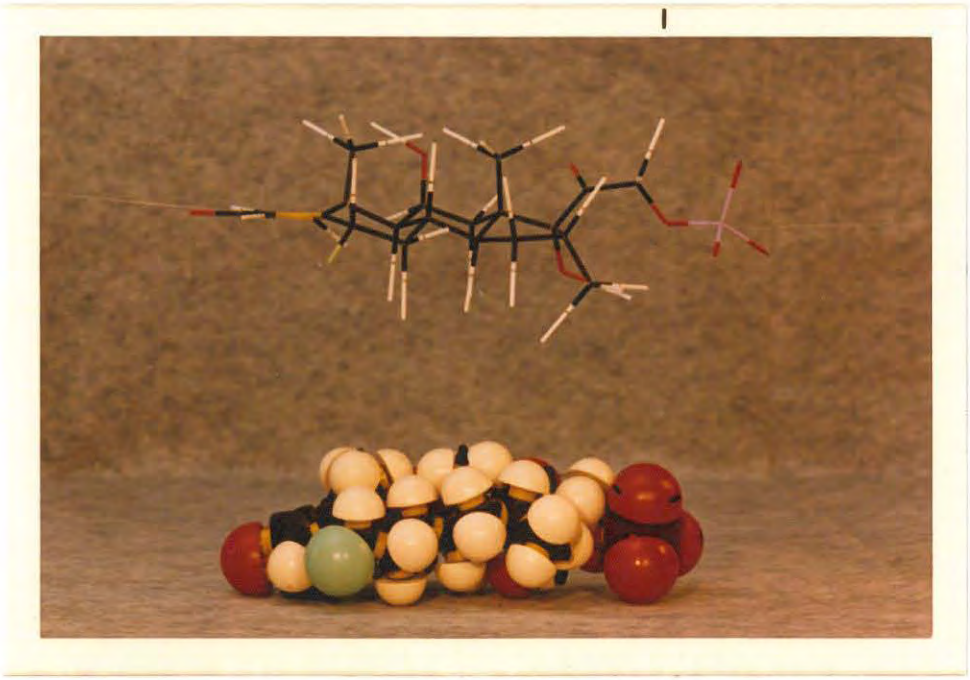


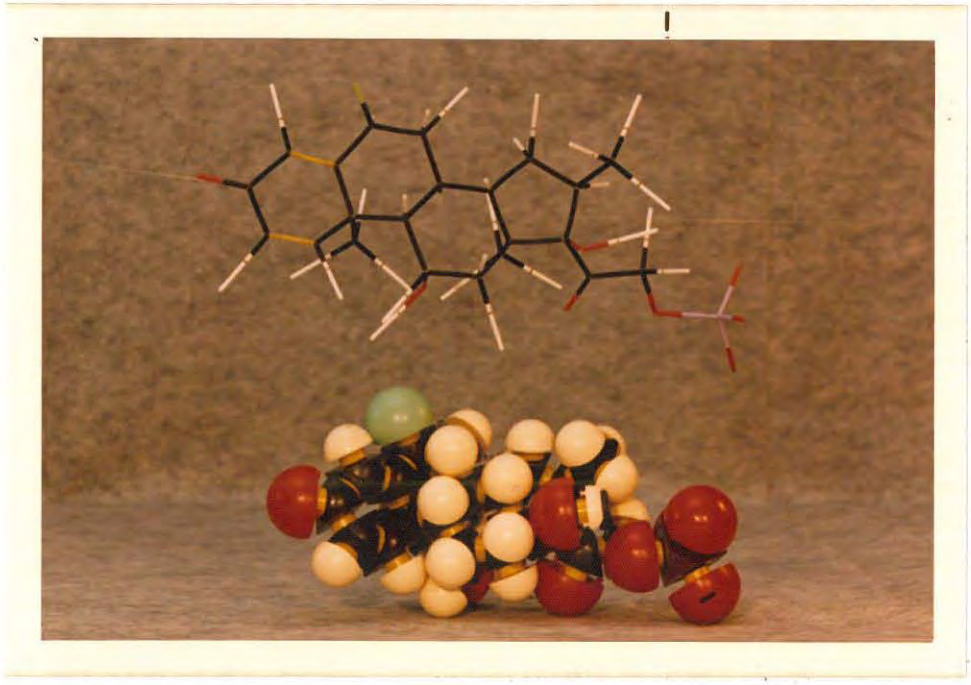
(b) Rear view

PLATE NO. 4

PARAMETHASONE ESTER PHOSPHATE



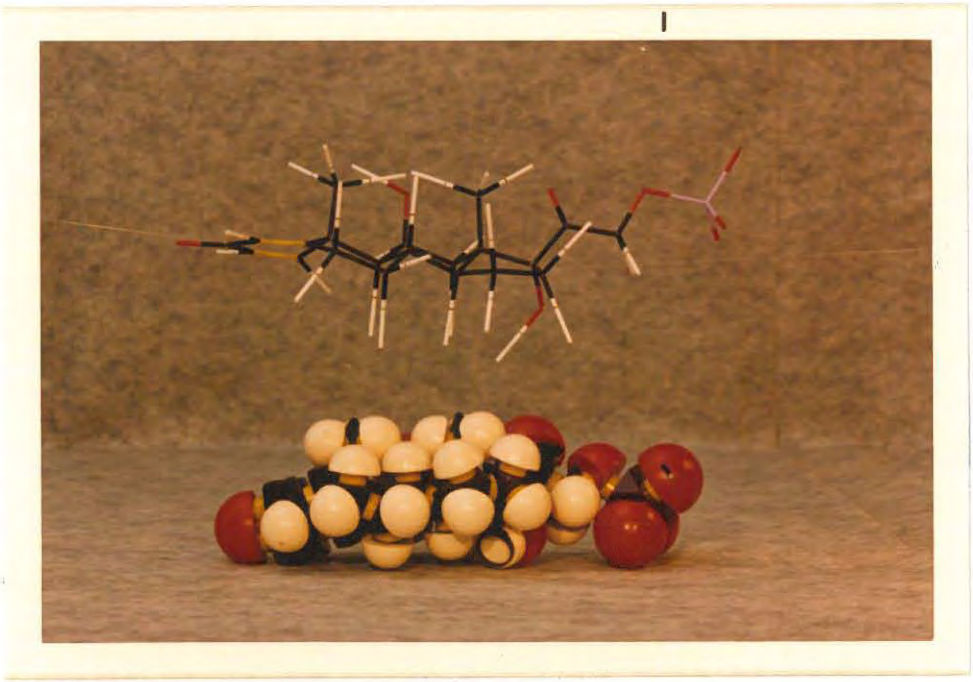
(a) Side view.



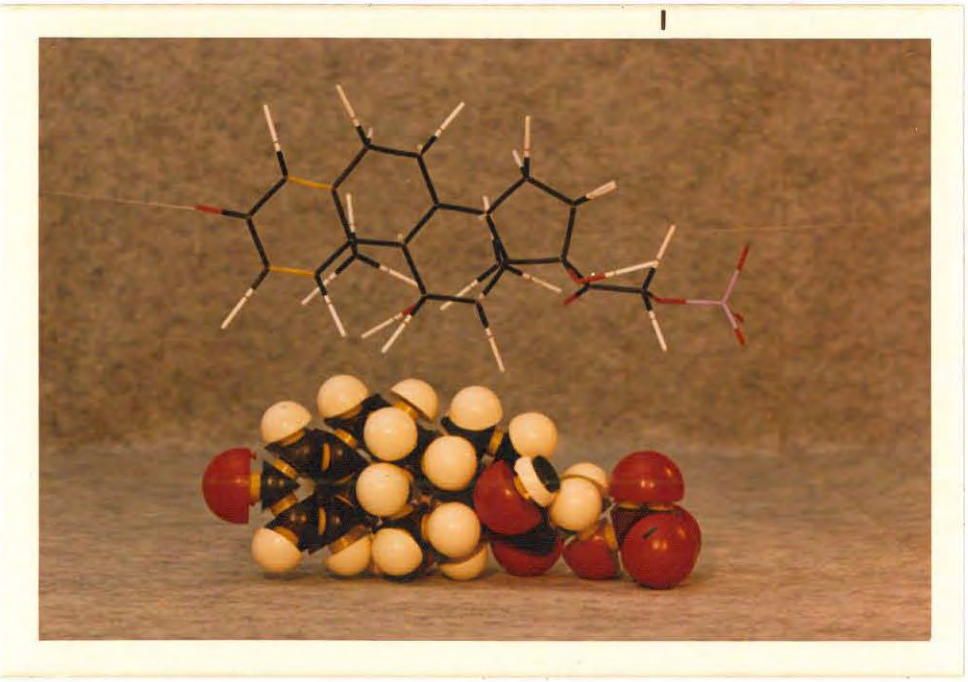
(b) Rear view.

PLATE NO. 5

PREDNISOLONE ESTER PHOSPHATE



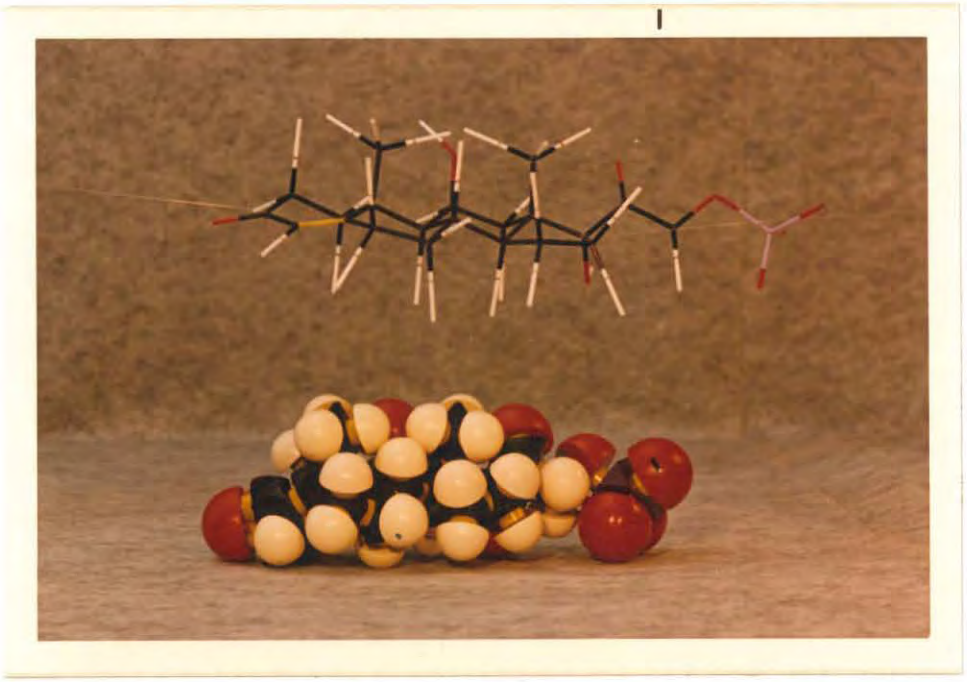
(a) Side view.



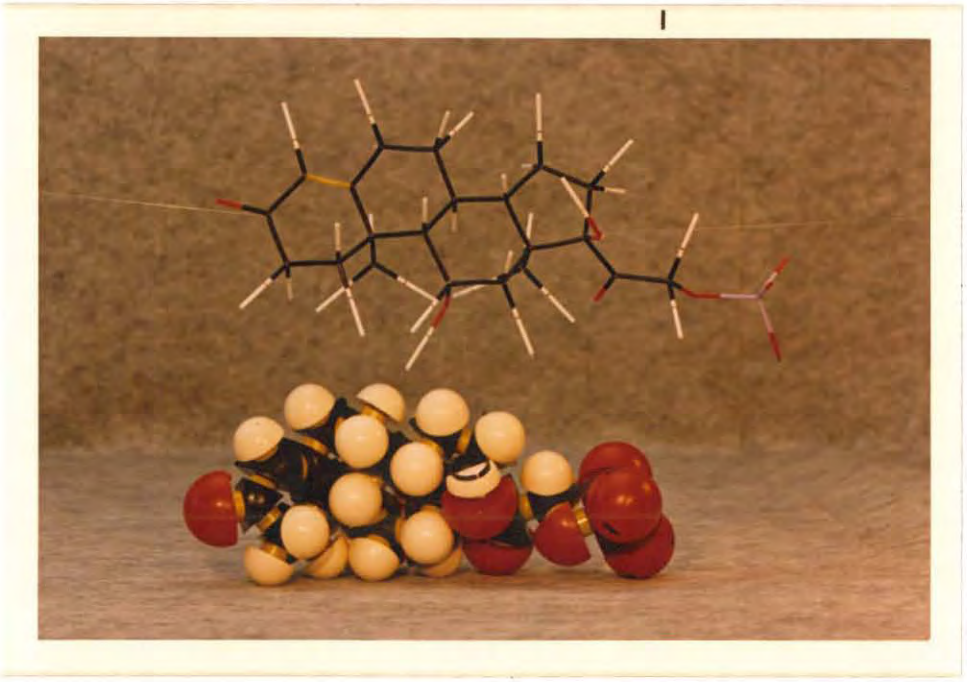
(b) Rear view.

PLATE NO. 6

HYDROCORTISONE ESTER PHOSPHATE



(a) Side view.



(b) Rear view.

CHAPTER II

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A. MATERIALS

1. Proteins

Three preparations of lyophilized, acid-soluble calf-skin collagen (ASC - Preparations P_1, P_4 and P_5) were used. Preparation (P_1) was obtained according to Cooper and Davidson (1965), whereas (P_4) and (P_5) were obtained using the method of Gross (1958) as modified by Piez et al. (1963).

The acid-soluble collagen was purified by phosphate precipitation (Gross, 1958), and the purity checked by amino-acid analysis, chromatography and ultracentrifugation (Cooper and Davidson, 1965; Davidson and Cooper, 1967).

Bovine serum albumin (BSA), fraction V (batch No. B.P.E. 1572, Miles-Seravac, Cape Town) was used in the phenol red calibration experiment.

Standard acid pigskin gelatine (No. 149) which has been fully described by Eastoe (1961), was supplied by the British Gelatine and Glue Research Association. This was used to check the accuracy and precision of the semi-micro-nitrogen analytical procedure as described by Eastoe and Courts (1963).

2. Reagents

All reagents used were either A.R. or Laboratory grade.

3. Phenol red

The phenol red (E. Merck A.G., Darmstadt) was recrystallized according to the method of Orndorff and Sherwood (1923) before use.

4. Corticosteroids

Prednisolone disodium phosphate (MOL. WT. = 484.4) [batch No. EPY(C) 7/7; and batch No. 280 (RB3010) AF/B 30984 J27005 0572] and hydrocortisone disodium phosphate (MOL. WT. = 486.4) [batch No. EPY(C) 7/6; and batch No. 111 (RB3017 AF/B 30984 J27005 0572] were obtained from Glaxo-Allenburys Laboratories Limited, South Africa, betamethasone disodium phosphate (MOL. WT. = 516.4) [control Nos. DOH-M-13-1 and DOH-3-M-7] from The Schering Corporation, South Africa, dexamethasone disodium phosphate (MOL. WT. = 516.4) [product S.P. 2003, lot No. 242, batch No. 233] from M.S.D. (Proprietory) Limited, South Africa,

and paramethasone disodium phosphate (MOL. WT. = 516.4) [lot No. C9-HL-002] from Syntex Pharmaceuticals Limited, Berkshire, England.

Hydrocortisone hemisuccinate sodium (MOL WT. = 484.5) [batch No. E.F.C.B. 152, and lot No. WL 444] was obtained from Glaxo-Allenburys Laboratories Limited, South Africa and The Upjohn Company, Kalamazoo, Michigan, United States of America respectively, whilst 1-dehydro-6α-methyl hydrocortisone (≡ 6α-methylprednisolone) 21-succinate sodium salt (MOL. WT. = 496.54) [lot No. SZ-411] was also obtained from The Upjohn Company.

B. PREPARATIVE AND ANALYTICAL METHODS

1. Preparation of ASC(P_4) and ASC(P_5)

Preparation (P_4) was obtained from the skin of an approximately four-week premature Friesland heifer calf and (P_5) from the skin of a two-week old Jersey bull calf. The skins from the freshly slaughtered calves were washed and immediately cooled in ice. They were defleshed and macerated with ice through an electric mincing machine. The minced skins were extracted independently with about ten volumes of 10% chilled NaCl solution with intermittent stirring for 48 hours at 4°C.

The extract liquors were coarsely filtered through muslin, filtered through non-absorbent cotton-wool and then re-filtered under vacuum through Whatman No. 541 filter paper on a Buchner funnel which was kept cool throughout the procedure. These extractions were each repeated three times on the skin pulp in order to ensure the removal of neutral-salt soluble collagen.

The skin pulp remaining from each of the third NaCl extracts were independently treated with about 10 volumes of 0.5M-acetic acid and stirred intermittently over 48 hours at 4°C. The suspensions were filtered through muslin and then through Whatman No. 541 filter paper. The acid-soluble collagen was then precipitated by the addition of 5% NaCl ($^{W}/_{V}$) and allowed to stand in a cold room at 4°C for 3-5 days. Volume reduction was then carried out by siphoning off the clear solution and the balance plus protein precipitate transferred to tall 2L measuring cylinders for further settling over 2 days.

The clear liquor was again siphoned off and the precipitates were collected by centrifugation at 5°C and for 45 minutes at 6 000g in an M.S.E. High Speed 18 centrifuge.

The skin pulps from (P_4) and (P_5) were extracted a second time with acetic acid as described above, and these precipitates were then combined independently with the precipitates obtained after the first extraction of (P_4) and (P_5), respectively.

2. Purification

The combined precipitates from the acetic acid extractions were redispersed in 0.5M-acetic acid at 4°C keeping the volume of acetic acid to a minimum. Intermittent stirring at 4°C over 2 - 3 days was necessary for complete dissolution. The solutions were then centrifuged at 30 000g for 60 minutes and dialyzed against 0.2M-Na_2HPO_4 with frequent phosphate solution changes until precipitation had occurred in the dialysis bags. The precipitated collagen was removed from the dialysis bags and centrifuged at 6 000g for 15 minutes to consolidate. The consolidated precipitates were then re-dissolved in 0.15M-acetic acid and dialyzed against 0.15M-acetic acid for 24 hours at 4°C to remove phosphate ions before centrifuging at 30 000g for 2 hours at 5°C. Preparations (P_4) and (P_5) were independently lyophilized and stored over silica-gel at 0-4°C.

3. Analytical measurements on total protein

i) Determination of ash

Ash determinations according to the method of Eastoe and Courts (1963) were done on 80 - 100mg lyophilized samples, in triplicate.

ii) Determination of moisture

Moisture determinations according to the method of Eastoe and Courts (1963) were done on 80 - 100mg lyophilized samples, in quadruplicate.

iii) Determination of nitrogen

Using the procedure of Eastoe and Courts (1963), the nitrogen content of standard pigskin gelatine (No. 149-British Gelatine and Glue Research Association)

was determined and checked against the percentage nitrogen reported by Eastoe (1961), after correcting for moisture content. The mean percentage nitrogen obtained from quadruplicate estimations agreed to within 0.12% of the published value. All subsequent nitrogen determinations were carried out using the same procedure.

4. Weighing of protein preparations for fibril kinetics

An approximate weight of material was transferred to a small aluminium-foil weighing boat which was then dried under vacuum over phosphorus pentoxide for at least 12 hours. After attaching a train of U-tubes containing silica-gel, dry air was slowly introduced into the sample desiccator. Weighing was then carried out continuously at half minute intervals and the dry weight of protein calculated by extrapolation to zero time.

The method compared very favourably with that of oven drying at 105°C using an equilibrium moisture content (Davidson, 1967).

5. Preparation of collagen solutions for fibril kinetics

The lyophilized acid-soluble collagen was dissolved in physiological saline with intermittent stirring at 5°C over 24 hours to give a final solution concentration of 1.2 to 1.4mg/ml (pH = 4.2). Clarification of collagen solutions was generally done by centrifugation at 5°C for 1 hour in an M.S.E. High Speed 18 centrifuge at a maximum of 32 000g. However, in the experiments carried out to establish the effects of different clarification procedures on the rate of fibril formation, the solution was clarified using a Spinco model L2-65B ultracentrifuge at 5°C for 1 hour at a maximum of 107 000g in one case, and filtered through a plug of non-absorbent cotton-wool in another.

6. Preparation of collagen solutions for binding experiments

i) Preliminary calibration of concentration measurement by optical rotation

Preparation (P_5) was used exclusively for these experiments. An approximately 2mg/ml solution of (P_5) was prepared by dissolving the sample in 0.05M

phosphate solution containing 0.15M NaCl at 5°C with intermittent stirring over 48 hours. The solution was then clarified by centrifugation at 32 000g for 1 hour at 5°C. Six 5ml aliquots, which included two 'blanks', were analyzed and the mean weight of nitrogen per ml solution calculated. A value of 18.072% nitrogen, being the mean of four replicate determinations on the lyophilized sample by the same general procedure, was used. This value included corrections for moisture and ash content.

Using the value of 18.072% nitrogen found in the dry, ash-free (P_5) samples together with a knowledge of the weight of nitrogen per ml clarified solution, a factor for dry, ash-free protein per ml solution was determined as 2.182mg/ml.

The optical rotation of the solution at six wavelengths was then determined in a water-jacketed tube at 15°C using a Perkin Elmer Model 141 polarimeter. The specific rotation at each wavelength was determined from the mean of 3 readings on each of 3 separate fillings of the polarimeter tube, with each reading being taken 1 hour after filling the tube in order to ensure temperature equilibrium.

The calculations of specific rotation are based on the relation

$$[\alpha]_{\lambda}^{t} = \frac{100}{dc}\,\alpha$$

where α = observed rotation in degrees
d = path length in decimetres
and c = concentration of protein in grams per decilitre.

ii) Summary of optical rotation data

TABLE I

λ(nm)	$[\alpha]_{\lambda}^{15}$
589	-397.48
578	-418.97
546	-477.98
436	-825.60
365	-1330.56
313	-2098.98

The concentration of solution preparations could be determined using any one of the above $[\alpha]^{15}_{\lambda}$ values, all of which gave almost identical results with the exception of $[\alpha]^{15}_{313}$. This was due to the fact that the α^{15}_{313} gave inconsistent readings as this wavelength was at the limit of instrument capability.

The value of $[\alpha]^{15}_{365}$ compared very favourably with that found by Drake et al. (1966) and was therefore used for concentration determinations on subsequent solution preparations.

7. Assay of corticosteroids

The purity of all corticosteroids used in the present study was between 97 - 100%.

Betamethasone and prednisolone disodium phosphate and hydrocortisone hemisuccinate sodium were assayed according to the procedure of the British Pharmacopoeia 1973. Hydrocortisone disodium phosphate was assayed according to the method of the British Pharmaceutical Codex 1973, whereas the λ_{max} and $E^{1\%}_{1cm}$ values for paramethasone disodium phosphate and the percentage purity values for dexamethasone disodium phosphate and 6α-methylprednisolone sodium succinate were obtained from their respective suppliers, since several of these compounds are not commercially available and can only be obtained from specific sources.

All compounds were dried over phosphorus pentoxide in a drying pistol at 90°C for 16 hours at a pressure of 0.1mm mercury, in compliance with B.P. specifications (British Pharmacopoeia, 1973). Stock solutions for the fibril kinetic studies were generally prepared in normal saline or buffer to give final solution concentrations equivalent to 2mg betamethasone disodium phosphate per 1.7ml solution of the respective corticosteroid unless otherwise stated.

i) Spectrophotometric data for corticosteroids

All absorbance measurements were performed on a Beckman Acta M VI U.V. Visible spectrophotometer which was periodically calibrated using a standardized

didymium or holmium oxide filter for wavelength calibration and standardized potassium dichromate solution (Haupt, 1952) for absorbance calibration.

All assays and concentration monitoring were performed at the λ_{max} of each corticosteroid concerned, in the appropriate solvent for purity determinations and at the appropriate pH for concentration monitoring, as summarized in Table II.

ii) Summary of spectrophotometric data

TABLE II

Corticosteroid	MOL. WT.	λ_{max} (nm)	$E^{1\%}_{1cm}$	Solvent
Betamethasone disodium phosphate	516.4	241	297	Distilled water
		241	299	Aq. pH 7.0
		241.5	298	Aq. pH 3.0
Dexamethasone disodium phosphate	516.4	241	294	Aq. pH 7.0
		241	294	Aq. pH 3.0
Paramethasone disodium phosphate	516.4	241	290	Methanol
Prednisolone disodium phosphate	484.4	247	312	Distilled water
		247	312	Aq. pH 7.0
		247.5	312	Aq. pH 3.0
Hydrocortisone disodium phosphate	486.4	248	333	Water
		248	333	Aq. pH 7.0
		248	332	Aq. pH 3.0
Hydrocortisone hemisuccinate sodium	484.5	248	336	Distilled water

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A. INTRODUCTION

1. Fibril formation from collagen solutions - kinetic studies

Many factors such as pH, ionic strength, temperature, the method of preparing the collagen, and the addition of complex molecules influence the formation *in vitro* of fibrils from solutions of soluble collagen (Gross and Kirk, 1958; Bensusan and Hoyt, 1958; Gross, 1958; Bensusan, 1960; Bensusan and Scanu, 1960; Convy and Wynn, 1967; Bowden, Chapman and Wynn, 1968; Wasteson and Obrink, 1968). The precipitation of fibrils occurs in two consecutive steps, a lag period or nucleation step, in which soluble collagen particles aggregate to form nuclei, followed by a growth step represented by a sigmoid precipitation curve in which the nuclei grow into fibrils by accretion of further soluble collagen particles (Bensusan and Hoyt, 1958; Wood and Keech, 1960; Wood, 1960a).

The *in vitro* formation of fibres from solutions of collagen has been shown to be extremely sensitive to the nature of the solution environment and the presence of added chemical compounds, and thus constitutes an interesting system for the study of collagen-small molecule interactions. The effect *in vitro* of several water-soluble anti-inflammatory corticosteroids on fibril formation from solutions of soluble collagen was thus examined. The relatively minor changes in active groups substituted on the basic corticosteroid nucleus in these compounds allows a systematic correlation of the effect of these substituents on fibril formation.

2. Mathematical treatment of fibril kinetics

Mathematical analysis of the rate of fibril formation has been performed by Wood (1960a) and Cassel *et al*. (1962). Wood assumed that reaction of soluble collagen particles with the surface of growing fibrils controlled the rate of growth. He employed the nucleation-growth concept, as applied by Waugh (1957) to the precipitation of insulin fibres, and derived equations which qualitatively accounted for the occurrence of a lag period in precipitation

during which nucleation predominates over growth. These equations also account for the time course of precipitation after the lag period, and the observation that the final distribution of fibril width is determined during the lag period.

Wood and Keech (1960) have shown that a linear relationship exists between the extinction at 400nm (E) of a collagen precipitate at different times during its formation and the corresponding extent of precipitation, p, given by

$$p = (Co - C)/(Co - Cs),$$

where C is the collagen concentration; Co and Cs are the initial and final collagen concentrations respectively. Thus,

$$p = E/E\infty$$

to a close approximation, where E is the extinction and E_∞ the extinction at infinite time. Extinction is therefore a reliable measure of the extent of precipitation and in view of its convenience and precision, the turbidity methods of Wood and Keech (1960) and Bensusan and Hoyt (1958) have been used in this study.

The rate of nucleation is assumed to be

$$\frac{dn}{dt} = k_1 C^x \qquad \text{(EQUATION III-1)}$$

where C = concentration of soluble collagen, n = concentration of nuclei, and x and k are constants, implying that x collagen particles must come together simultaneously to form a nucleus. The rate of growth of each particle is assumed to be proportional to its surface area, A, and to C; thus

$$\frac{dm}{dt} = k_2 AC \qquad \text{(EQUATION III-2)}$$

where m = mass of precipitated particle and k_2 is a rate constant. If the further assumption is made that $A \propto m^{2/3}$, implying that the shape of the particle remains constant,

$$\frac{dm}{dt} = k_3 m^{2/3} C \qquad \text{(EQUATION III-3)}$$

The rate of growth is thus assumed to be controlled by the condensation of the dissolved collagen on to the precipitate and not by its diffusion to the surface of the precipitated particles.

If the growth process is reversible, EQUATION III-3 must be modified to

$$\frac{dm}{dt} = k_3 m^{2/3}(C - Cs) \qquad \text{(EQUATION III-4)}$$

where Cs is the value of C at equilibrium, i.e. the solubility of the collagen.

In the early stages of precipitation, when $C \approx Co$ (the initial collagen concentration) the extent of precipitation, p ($p = (Co - C)/Co$) is given by

$$p = (1/108)k_1 k_3^3 Co^{(x+2)} t^4 \qquad \text{(EQUATION III-5)}$$

For low values of p the predicted course of precipitation is given by EQUATION III-5.

The time course of precipitation after the lag period can be calculated by replacing the distribution of particles by a concentration, n_e, of hypothetical identical particles. The value of n_e and the size of these particles is determined by the conditions that their total surface area and total mass must be the same as those of the actual distribution of particles.

It has thus been shown by Wood (1960a) that the time course of growth, according to the model, is given by

$$\frac{dp}{dt} = K_2 \beta^{2/3} Co^{2/3} p^{2/3}(1 - p) \qquad \text{(EQUATION III-6)}$$

where $K_2 = k_3 n_e^{1/3}$ and $\beta = (Co - Cs)/Co$. On integration this becomes

$$K_2 \beta^{2/3} Co^{2/3} t = \int \frac{dp}{p^{2/3}(1 - p)} = I \qquad \text{(EQUATION III-7)}$$

where

$$I = 1/2 \ln\left\{\frac{R^2 + R + 1}{(R - 1)^2}\right\} + \sqrt{3} \tan^{-1}\left\{\frac{\sqrt{3}}{1 + 2R^{-1}}\right\} \qquad \text{(EQUATION III-8)}$$

and $R = p^{1/3}$.

The integral I may be plotted as a function of p. By the use of this plot to obtain I from p, I may be plotted against time (t) for a set of data at different collagen concentrations. It was, however, found more convenient and accurate to calculate values of I from values of p with the aid of a computer program (FIBRL) written for this purpose and a Hewlett Packard Model 2100A computer.

EQUATION III-7 predicts that the curves are linear over most of their course. The slopes of these curves from a plot of I versus time are proportional to K_2, whereas the intercepts, t_c, mark the ends of the lag periods. At low values of p, relatively small deviations from linearity are due to overlap of the nucleation and growth phases, whereas deviation at values of p approaching unity might be due to the asymptotic nature of the plot I versus p, and hence sensitivity of I to errors in p in this region. The shape of the precipitation curves are, however, well described by EQUATION III-7. This is seen when the appropriate smooth curves of p versus time, computed from a knowledge of the values for K_2 and t_c, are superimposed on the experimentally determined values.

Studies on the kinetics of the heat precipitation of collagen (Cassel et al., 1962) substantiated the concept that a phase transition, which involves no change in molecular conformation, occurs during precipitation.

For a dilute system of particles, growth is governed by processes occurring either at the particle-solution interface or by the diffusion of the solute component to the interface. The growth rate should be controlled by interfacial processes when the particles are smallest whilst the influence of diffusional processes increases as the particle size increases. A detailed analysis of the problems with concurrent interfacial and diffusional processes occurring has been reported (Frisch and Collins, 1953). Cassel et al. (1962), using the approach of Wert and Zener (1950), Turnbull (1956) and Turnbull and Fisher (1949), where the processes are treated independently, showed that the precipitation appeared to be interface-controlled at temperatures above 16°C and diffusion-controlled at 16°C. The latter temperature being the lowest temperature attainable where quantitative studies were feasible. They derived EQUATION III-8 for the interface-controlled process and showed that

$$I = 1/2 \ln\left\{\frac{R^2 + R + 1}{(R - 1)^2}\right\} + \sqrt{3} \tan^{-1}\left\{\frac{-R\sqrt{3}}{2 + R}\right\} \quad \text{(EQUATION III-9)}$$

describes the diffusion-controlled process.

3. Summary of computation utilizing program FIBRL

This computer program (FIBRL) was written to calculate values of p from E and E_∞, I from these values of p, and a linear least squares regression of I versus time (t) according to EQUATION III-7. The same program gave the correlation coefficient for this linear least squares regression (see tables containing data of the rates of precipitation) calculated the value of K_2 from

$$K_2 = \frac{\text{slope from linear least squares regression}}{\beta^{2/3} C_o^{2/3}} \qquad \text{(EQUATION III-10)}$$

and the value of the lag period (t_c) from the intercept at I=0. In addition, the computed values for the parameters, K_2 and t_c, were used in the same program to calculate the smooth curve relationship between p and t. As the collagen was almost completely precipitated in all the experiments, a value of β=1 was used (Wood, 1960a). Several additional computations were made using relatively large variations for the value of β. These did not appreciably affect the final values. As all the present precipitation studies were conducted at either 20°C or 25°C, EQUATION III-8 was used throughout.

B. EXPERIMENTAL

1. Methods of following fibril precipitation

Fibril precipitation was followed by modifications of the turbidity methods of Bensusan and Hoyt (1958) (Method 1) and Wood and Keech (1960) (Method 2). The developemnt of turbidity was monitored at 400 nm using a recording Beckman DB spectrophotometer fitted with a constant temperature cell holder.

i) Modified Bensusan and Hoyt method (Method 1)

Two matched cuvettes (1cm path length) were placed for 30 minutes in the sample and reference beams of the spectrophotometer with the cell-housing being kept at 25°C. The collagen solution was removed from a refrigerator at 4°C and kept in a water bath at 25°C for 30 minutes. The "initiating buffer", consisting of 0.04M-KH_2PO_4-NaOH buffer (pH 6.95, I 0.23), was also placed in a water-bath for 15 minutes at 25°C. At zero time, 1 minute after their removal

from the water-bath, 3.0ml of the collagen solution was added to 3.4ml of the initiating buffer in a glass stoppered test tube which was inverted ten times during 1 minute to mix. The mixture was then placed in a water-bath at 25°C for 4 minutes and an aliquot of 3.2ml then transferred to the sample cuvette kept in the spectrophotometer, this operation being performed in 1 minute. The recorder connected to the spectrophotometer was started exactly 2 minutes after removal of the reaction mixture from the water-bath, the reference cell with its contents of the original collagen solution having previously been allowed to equilibrate at 25°C. The change in extinction with time was recorded. All operations were done in an air-conditioned room at 25°C.

ii) Modified Wood and Keech method (Method 2)

An aliquot of 1.5ml of the collagen solution was placed in a cuvette (1cm path length) in the spectrophotometer and allowed to stand for 30 minutes at 20°C to attain temperature equilibrium. At the same time another matched cuvette was filled with the identical collagen solution and allowed to equilibrate as the 'blank'. At zero time 1.7ml of the initiating buffer kept at 20°C for 30 minutes was added to the cuvette containing 1.5ml of collagen solution which was then inverted three times and replaced in the spectrophotometer. A recording of extinction against time was made. All operations were done in an air-conditioned room at 20°C.

The influence of the corticosteroids on the fibril precipitation was followed either by dissolving these in the initiating buffer, or by dissolving them in physiological saline when no initiating buffer was used. Except where otherwise stated, the corticosteroid was dissolved in initiating buffer or physiological saline to give the equivalent of 2mg betamethasone disodium phosphate per 1.7ml solution of the respective corticosteroid and added to 1.5ml collagen solution containing 1.2 to 1.4mg/ml. At the end of each run the pH of both sample and reference cells were determined on a Beckman Century pH meter.

2. Electron microscopy

Samples of the fibrils obtained from the precipitation experiments were prepared for electron microscopy by drying on formvar-covered copper grids (200 mesh). These were stained by immersion for 1 minute in 0.1% phosphotungstic acid, pH 6.1, and air-dried. The specimens were examined in a Hitachi HU 11B electron microscope.

C. RESULTS AND DISCUSSION

1. Standardization of the procedure for turbidity measurements

The procedure generally adopted to maintain reproducibility was to bracket duplicate runs in the presence of the corticosteroids with blank runs using initiating buffer alone, all on the same or consecutive days. It was thus necessary to have the fibril formation complete within a few hours. A typical example of the reproducibility obtained with strict adherence to procedure is given in Fig. 8A and 8B. All graphs were plotted with the aid of a Calcomp 563 graph plotter in conjunction with an ICL 1901A computer. Fig. 8A depicts duplicate determinations at 25°C using filtered solutions, method 1 and collagen preparation (P_1) while Fig. 8B shows the same data on a normalized basis where the curves represent the best fit of EQUATION III-7 to the experimental data depicted by the symbols. In spite of the deviations at low values of p and at values of p approaching unity when I is plotted versus time (Fig. 8C), the shape of the normalized precipitation curves are well described by EQUATION III-7. Similar observations were seen for all the present precipitation studies. A further demonstration of the reproducibility of the system is illustrated in Fig. 8D. The solid curve was generated from EQUATION III-7 whereas the symbols represent the experimental data plotted on the normalized basis of p versus t. This graph shows the effect of betamethasone disodium phosphate (duplicate determinations) on the rate of collagen fibrillogenesis *in vitro*.

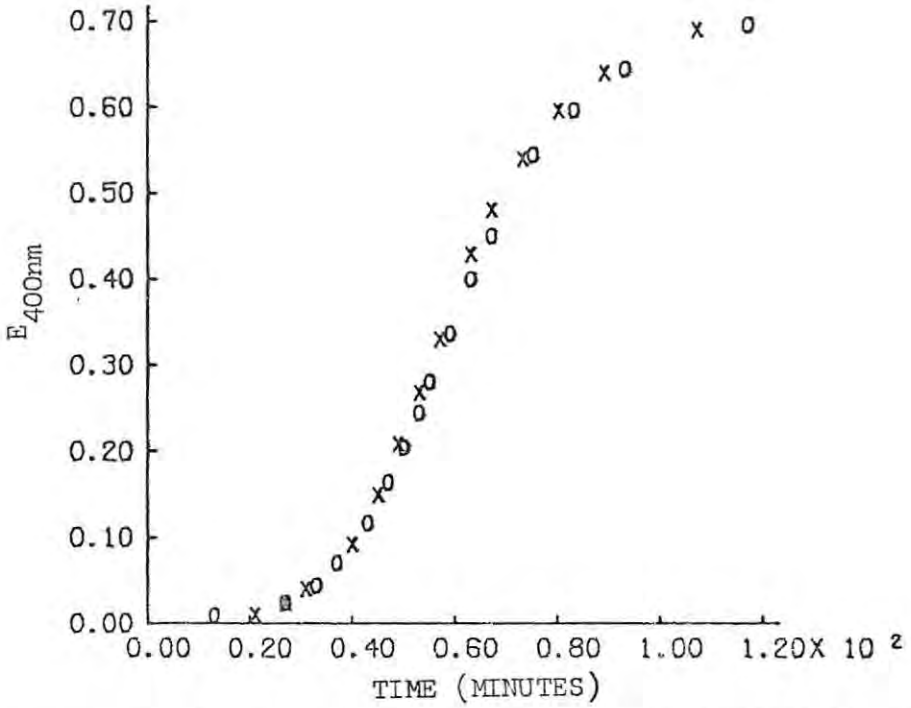

Fig. 8A. Sigmoid curves showing the course of precipitation in two successive, identical experiments. Initial collagen concentration, 0.135%; temperature, 25°C; pH 7; method 1 and filtered solutions of preparation (P_1).

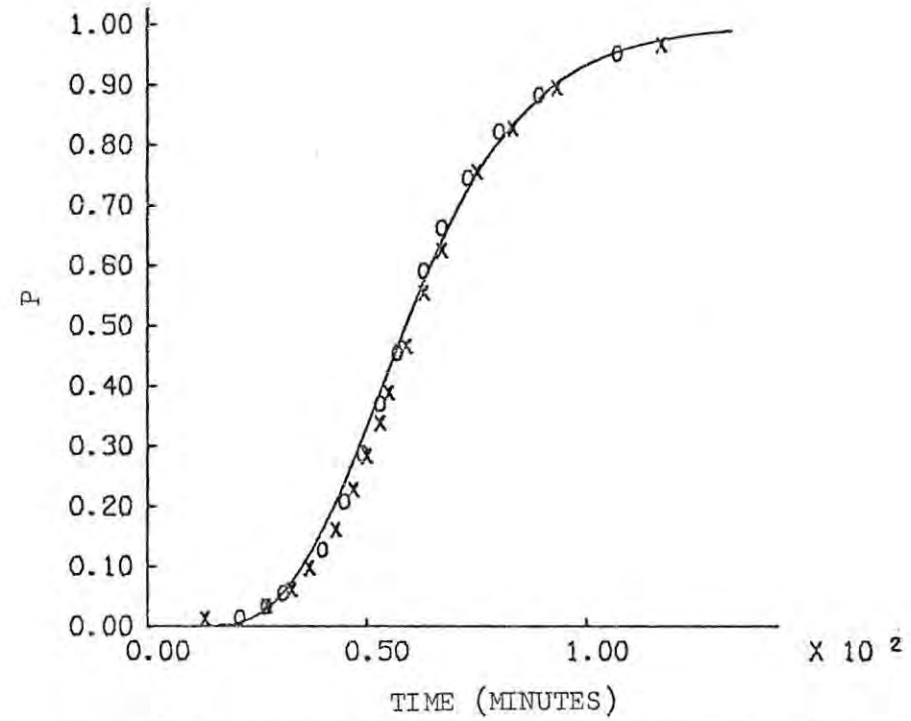

Fig. 8B. Time course of extent of precipitation. The curve represents the best fit of EQUATION III-7 to the experimental data, O, shown in Fig. 8A; X, duplicate determination.

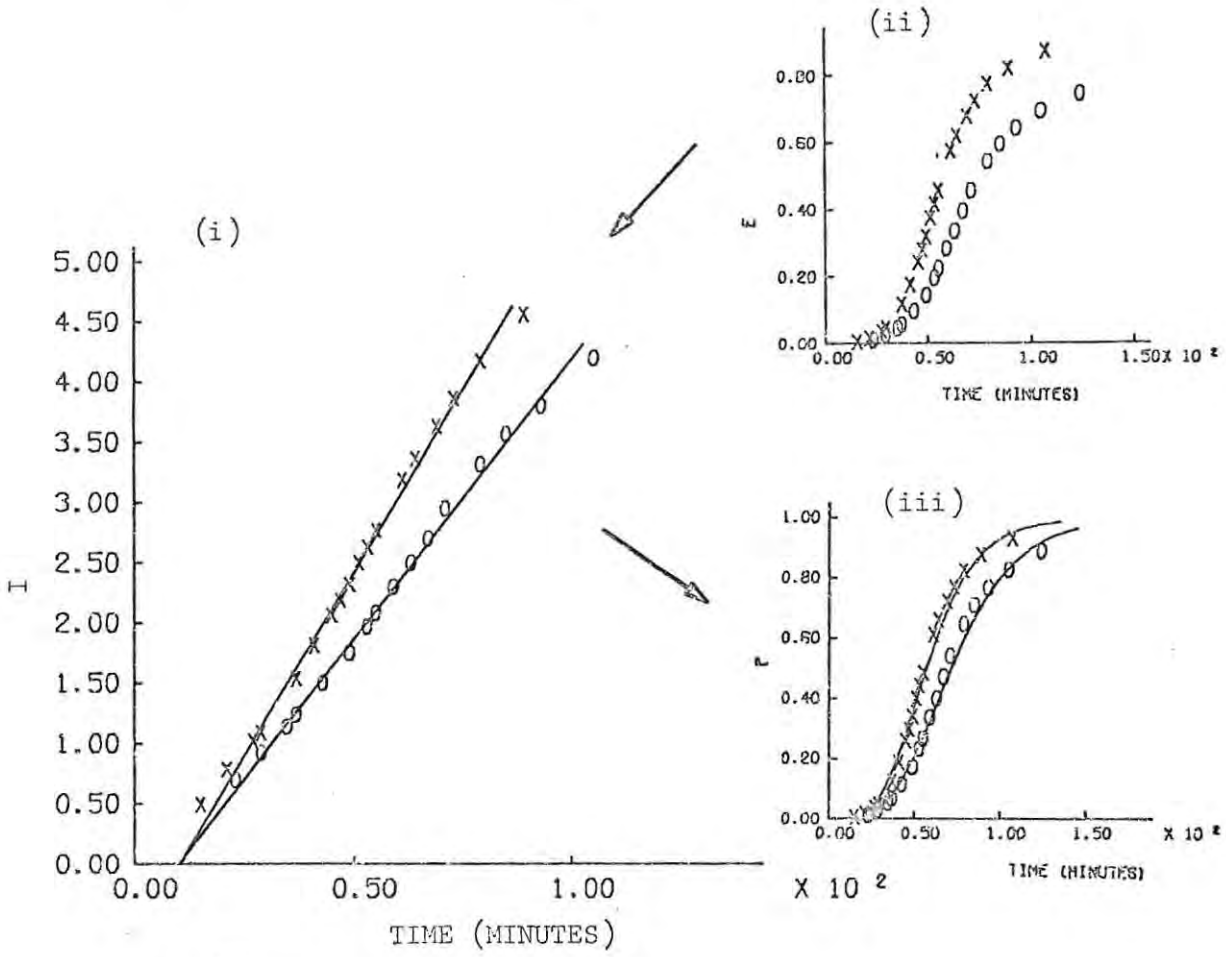

Fig. 8C.

(i) Precipitation at pH 7.04 (O, initiating buffer alone) and pH 6.95 (X, betamethasone disodium phosphate in initiating buffer); collagen concentration, 0.135%; method 1 and solutions of (P_1) clarified by centrifugation at 32 000g; temperature, 25°C. Data are plotted in accordance with EQUATION III-7.

(ii) Time course of change of E; O, initiating buffer alone; X, betamethasone disodium phosphate in initiating buffer.

(iii) Time course of extent of precipitation; O, initiating buffer alone; X, betamethasone disodium phosphate in initiating buffer. The solid curves represent the best fit of EQUATION III-7 to the experimental data shown in (ii).

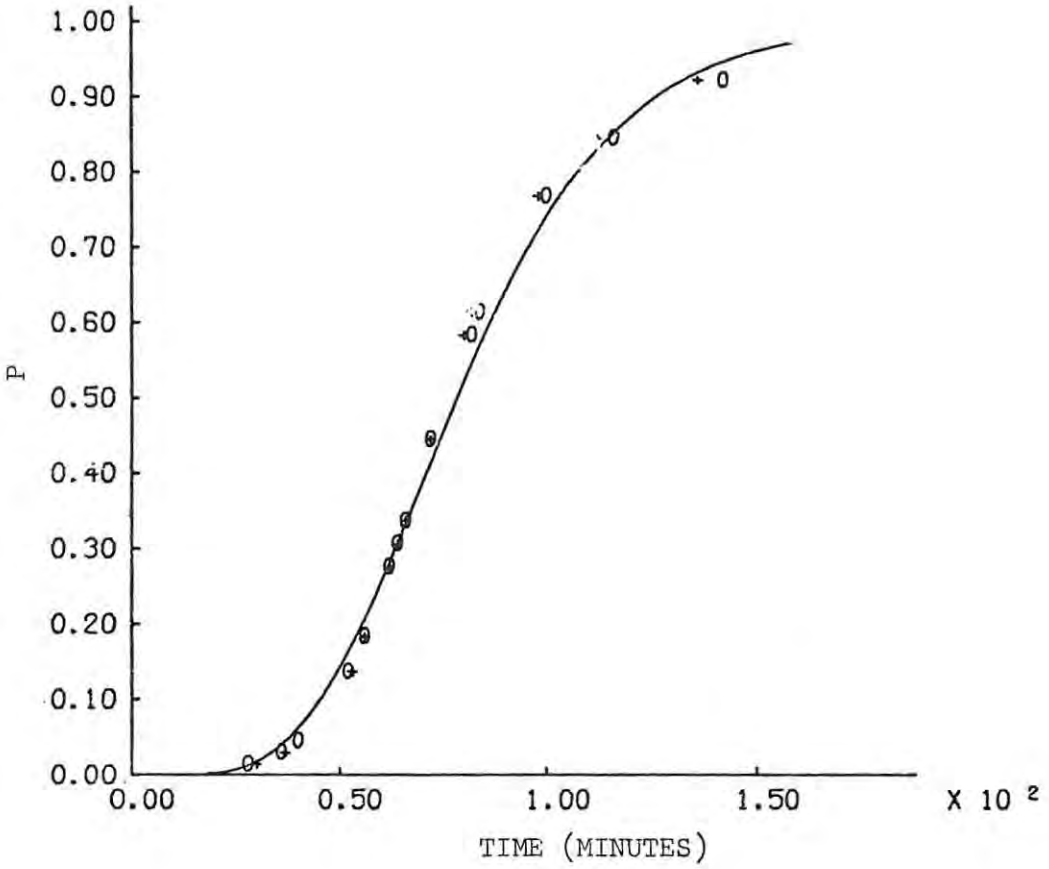

Fig. 8D. Time course of extent of precipitation for two successive, identical experiments involving betamethasone disodium phosphate in initiating buffer, pH 6.95; collagen concentration, 0.140%; solution of collagen (P_4) clarified by centrifugation at 32 000g; method 2; temperature, 20°C. The solid curve represents the average of the best fit of EQUATION III-7 to both sets of experimental data.

All subsequent graphs or data given in the Tables represent the average of duplicate or triplicate determinations. EQUATION III-7 was also used in most cases in order to obtain graphs depicting the precipitation on the basis of p versus t plots. The smooth curves shown in these plots represent the best fit of EQUATION III-7 to the experimental data, indicating that it provides a satisfactory description of the results in the presence or absence of corticosteroids or when the corticosteroids are used in the absence of initiating buffer. The effects of the corticosteroids may therefore be conveniently stated in terms of K_2, $E\infty$ and the lag period t_c. The parameter K_2 is, from EQUATION III-6, proportional to the slope of the graph of p versus t for a given value of p.

2. Factors affecting reproducibility

i) Effect of collagen clarification procedure

Figs. 9A and 9B, corresponding to Run 1 of Table III, shows that clarification of the collagen solution, after ensuring that the collagen had dissolved, had an important bearing on the reaction rate, which corresponds to the findings of Wood and Keech (1960). As shown in Figs. 9A and 9B (Run 1, Table III) the slower precipitation rate involved a longer lag, t_c, which was associated with a lower value of K_2 and a longer $t_{0.5}$. Centrifugation of the solutions presumably removes collagen molecular aggregates which would act as nucleating centres for fibril formation, hence the reaction in the centrifuged solution was slower than in the filtered solution. This point is illustrated again in Figs. 10A and 10B (Run 5, Table V) where the rates of collagen precipitation from solutions centrifuged at 32 000g are compared with solutions centrifuged at 107 000g. This experiment also illustrates the effect of betamethasone disodium phsophate in initiating buffer on the collagen precipitation rate compared to initiating buffer alone. The slower precipitation rates were again associated with a longer t_c, lower K_2 and longer $t_{0.5}$. Centrifugation with increasing centrifugal force reduced the general rate in comparison with filtration, consistent with the increased

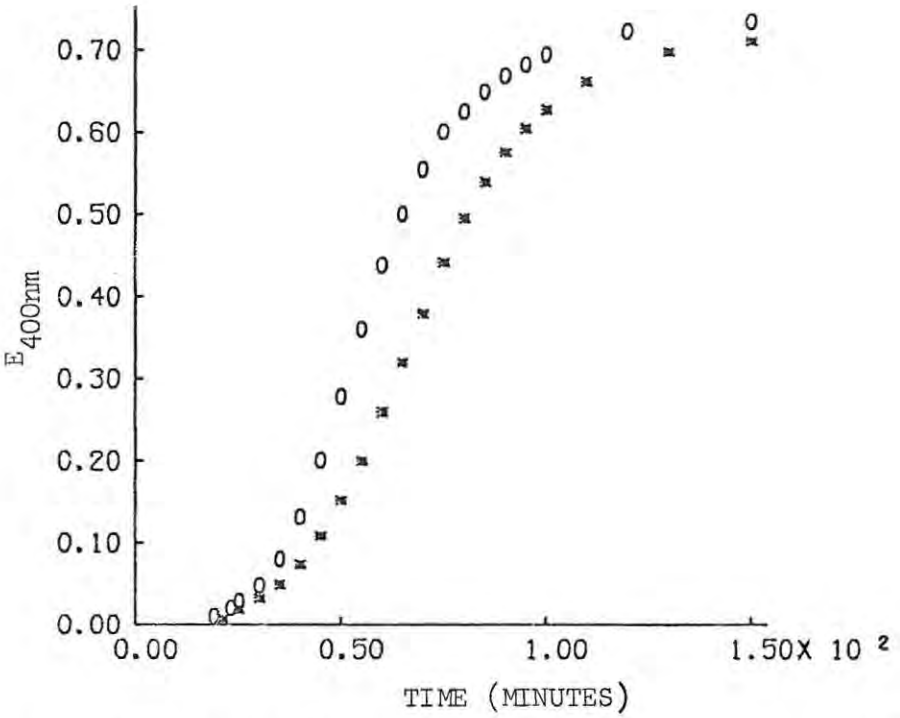

Fig. 9A. Effect of collagen clarification procedure on fibril precipitation rates. Determinations on a filtered solution (O) and a solution centrifuged at 32 000g (*) using preparation (P_1) and method 1 at 25°C. Precipitations by initiating buffer alone, pH 7.04; collagen concentration, 0.135%.

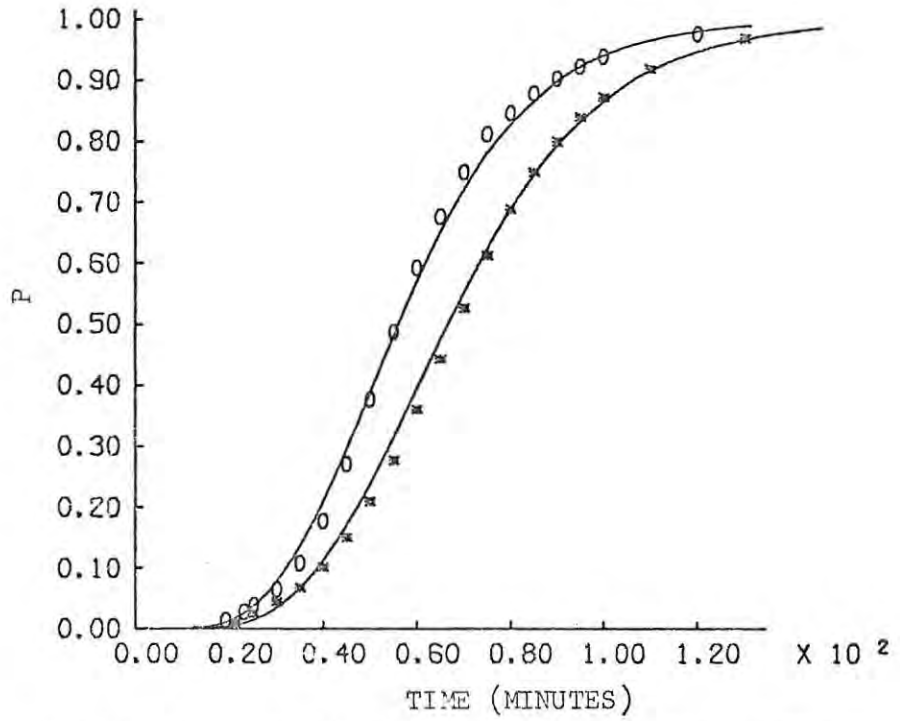

Fig. 9B. Time course of extent of precipitation. The solid curves represent the best fit of EQUATION III-7 to the corresponding experimental data shown in Fig. 9A.

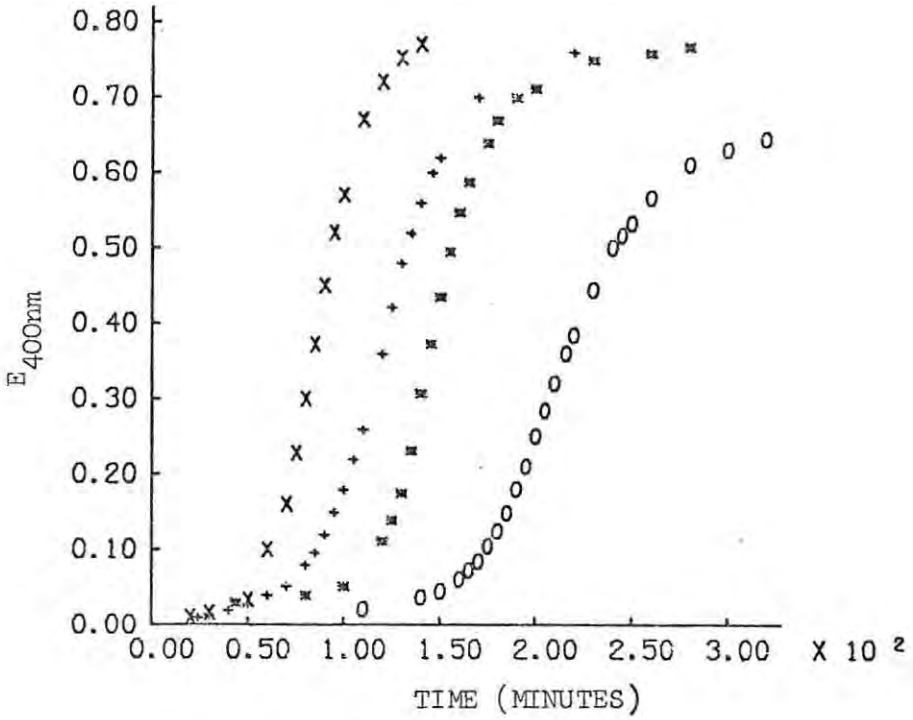

Fig. 10A. Fibril precipitation from solutions of collagen (P_1) in physiological saline using initiating buffer alone (pH 7.04) and initiating buffer containing betamethasone disodium phosphate (pH 6.95) and method 1 at 25°C; +, solution centrifuged at 32 000g and precipitated with initiating buffer alone; X, solution centrifuged at 32 000g and precipitated with initiating buffer containing betamethasone disodium phosphate; O, solution centrifuged at 107 000g and precipitated with initiating buffer alone; *, solution centrifuged at 107 000g and precipitated with initiating buffer containing betamethasone disodium phosphate. Collagen concentration of solutions centrifuged at 32 000g, 0.129%; collagen concentration of solutions centrifuged at 107 000g, 0.128%.

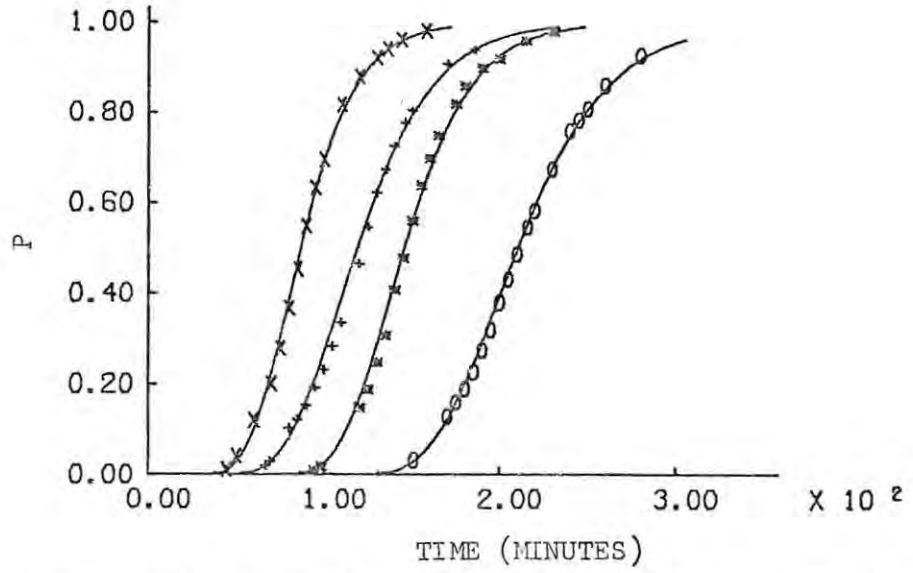

Fig. 10B. Time course of extent of precipitation. The solid curves represent the best fit of EQUATION III-7 to the corresponding experimental data shown in Fig. 10A.

TABLE III

Rate of precipitation of collagen from physiological saline by initiating buffer.

Experimental conditions; pH of reaction mixture 7.04, I 0.23,

reaction temperature 25°C.

Run No.	Pre-treatment of collagen solution	Turbidity method	Collagen pre-paration	E_∞	t_c (min)	K_2	$t_{0.5}$ (min)	Correlation coefficient
1a	filtered	1	P_1	0.740	7.3	0.219	56	0.998
1b	x32 000g	1	P_1	0.720	10.0	0.194	68	0.999
2a	x32 000g	1	P_4	0.660	4.7	0.812	17	0.965
2b	x32 000g	1	P_1	0.730	9.1	0.198	70	0.999

For definition of E_∞, t_c, $t_{0.5}$, K_2 and correlation coefficient, see text.

removal of collagen molecular aggregates which would promote nucleation (Boedtker and Doty, 1956; Mathews et al.,1954). Wood (1960b) concluded from studies on polyanions that those which accelerated precipitation lowered E_∞ whereas in the present case the acceleration of precipitation with the di-sodium phosphate ester salt of betamethasone was accompanied by a significant increase in E_∞ (Table V). This effect does not appear to be due to a pH factor since the pH of the reaction mixtures containing the corticosteroid (pH 6.95) was similar to that for the initiating buffer alone (pH 7.04), while differences in reaction curves and constants are greater than would be expected for the small differences in the pH of the reaction mixtures (Wood and Keech, 1960). This rate difference was particularly evident when the reaction rate was slowed down by centrifuging the collagen solution at 107 000g. Moreover, Wood and Keech (1960) found that increasing the ionic strength above I 0.13 decreased the rate of fibril precipitation at pH 7.1. The addition of ionizable corticosteroid to the initiating buffer at pH 7 should have increased the ionic

strength slightly, but nevertheless the precipitation rate also increased.

ii) Effect of different collagen preparations

When solutions of preparation (P_4) were used, Method 1 gave too rapid a reaction compared with preparation (P_1) (Figs. 11A and 11B, Run 2 of Table III) and therefore the modified Wood and Keech method was developed (Method 2). This observation agrees with those of Gross (1958) and Wood and Keech (1960) who suggested that differences in the rate of precipitation between different preparations of collagen solutions and between samples of the same collagen solution of different ages might also be due to differences in extent of molecular aggregation. These observations emphasize the importance of maintaining constant conditions regarding the age of the collagen solution, type of preparation and clarification procedure as well as adhering strictly to a standard experimental procedure in order to make valid comparisons of the corticosteroid effects. Variations in age of collagen solutions was avoided by using freshly prepared solutions from the same lyophilized collagen preparation in each set of runs.

iii) Effect of temperature

The effect of temperature on the rate of fibril formation is shown in Figs. 12A and 12B (Run 3 of Table IV). In these experiments Method 2 and preparation (P_4) was used. As reported by Wood and Keech (1960) and Cassel et al. (1962), the higher the temperature, the faster the reaction rate. The precipitation process was presumed to be interface-controlled as the experiments were conducted at temperatures above 16°C (Cassel et al.,1962) where reactant diffusion is relatively rapid and hence of lesser importance.

3. Parameters of the precipitation curve

All the precipitation curves had a similar sigmoid shape, consisting of a lag period (t_c), during which no precipitation was recorded, and a sigmoid portion or growth phase described by EQUATION III-7, i.e. the time course of precipitation after the lag period. The lag period (t_c) and K_2 values in each

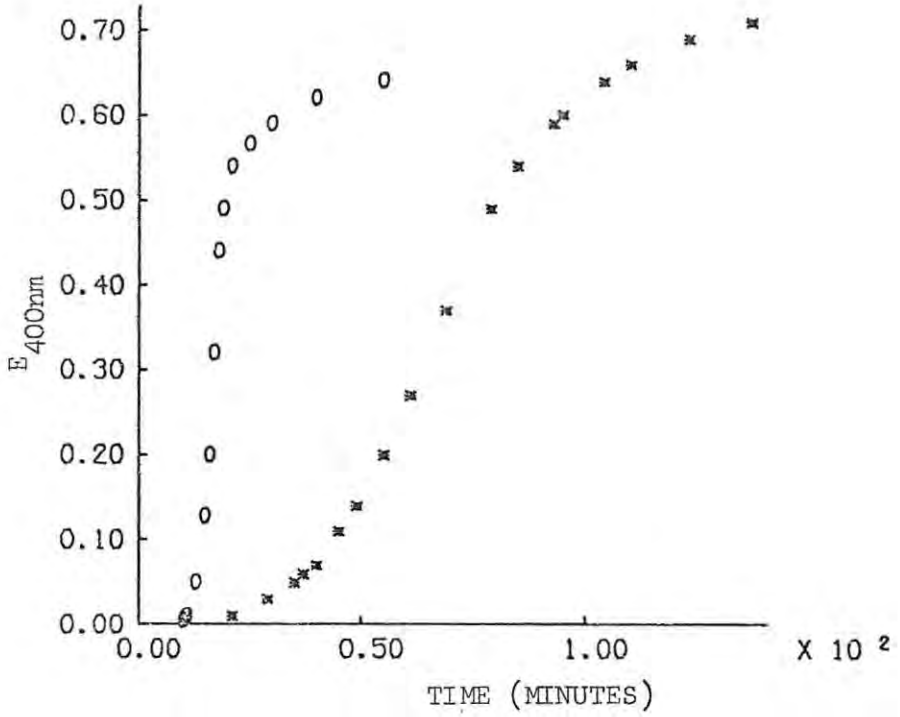

Fig. 11A. Effect of different collagen preparations on fibril precipitation rates. Determinations on solutions of preparation (P_1) (*) and (P_4) (O) centrifuged at 32 000g and using method 1 at 25°C; pH 7.04; concentration of both collagen solutions, 0.12%.

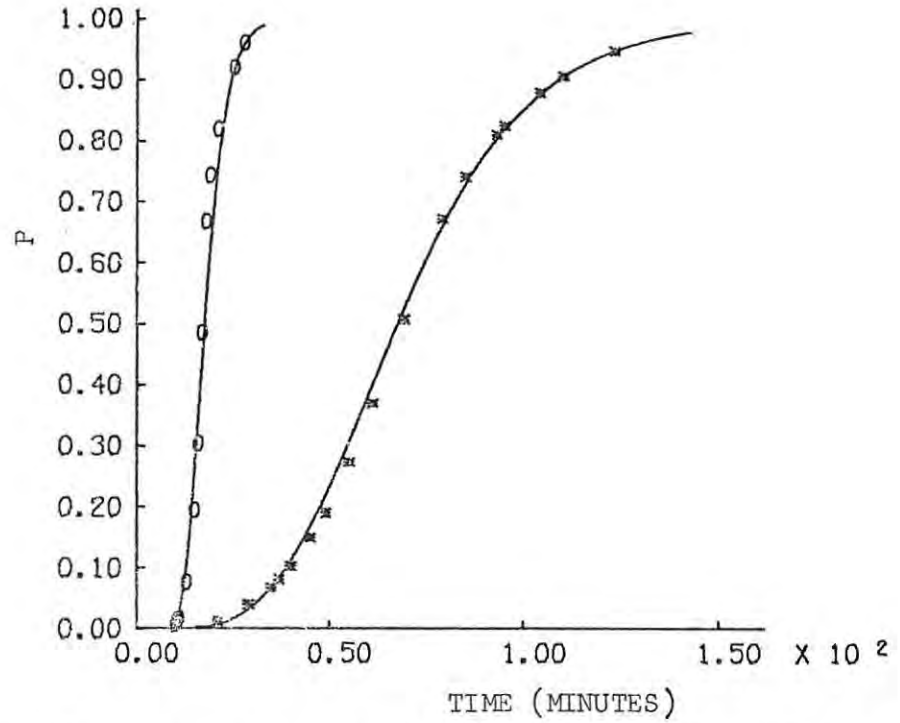

Fig. 11B. Time course of extent of precipitation. The solid curves represent the best fit of EQUATION III-7 to the experimental data shown in Fig. 11A.

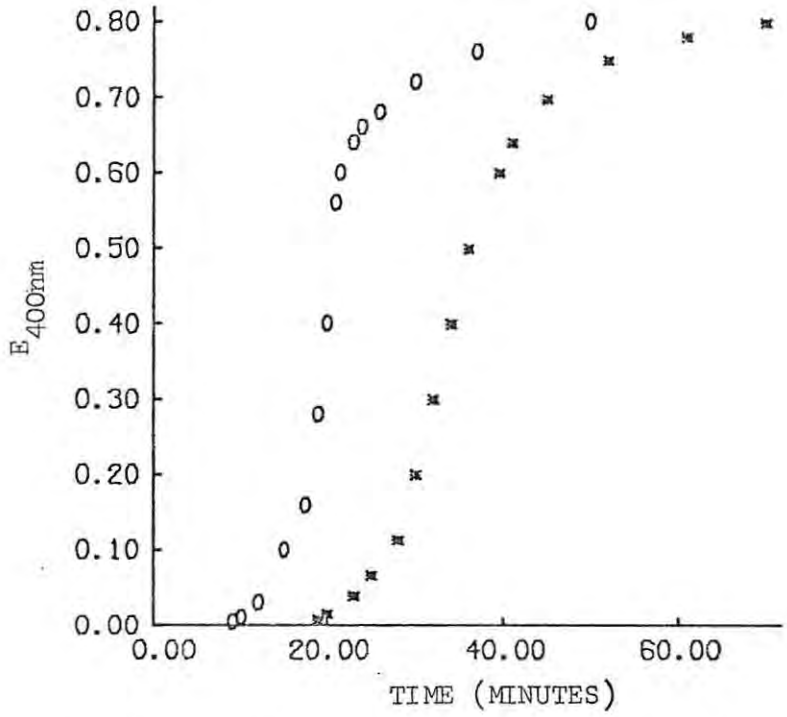

Fig. 12A. Effect of temperature on fibril precipitation rates. Determinations on solution of collagen (P_4) centrifuged at 32 000g and using method 2. O, precipitation at 25°C; *, precipitation at 20°C; collagen concentration, 0.121%; pH 7.04.

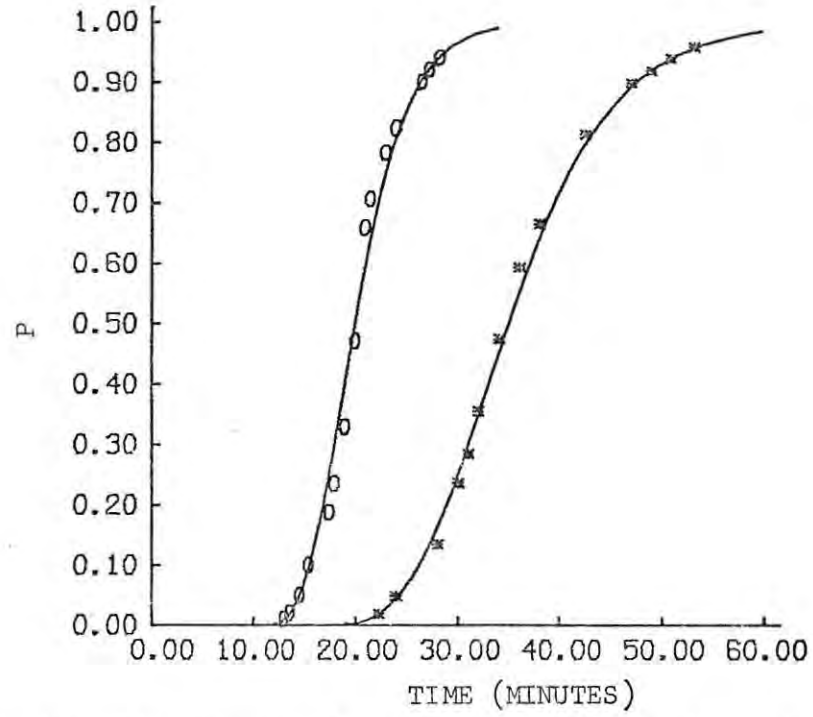

Fig. 12B. Time course of extent of precipitation. The solid curves represent the best fit of EQUATION III-7 to the experimental data shown in Fig. 12A.

TABLE IV

Rate of precipitation of collagen from physiological saline by initiating buffer.

Experimental conditions: pH of reaction mixture 7.04; I 0.23.

Run No.	Pre-treatment of collagen solution	Turbidity method	Collagen pre-paration	E_∞	t_c (min)	K_2	$t_{0.5}$ (min)	Temp. (°C)	Correlation coefficient
3a	x32 000g	2	P_4	0.844	11.0	1.281	20	25	0.971
3b	x32 000g	2	P_4	0.840	17.2	0.647	35	20	0.993

For definition of E_∞, t_c, $t_{0.5}$, K_2 and correlation coefficient, see text.

case, were computed as previously described. The overall precipitation rates could be conveniently compared from the half-precipitation times ($t_{0.5}$) i.e. the time taken for the extinction to rise to one-half of its final value. The extinction at the end of the reaction is given by E_∞. These parameters were subsequently used to compare the various reactions.

4. The action of corticosteroids on fibril formation

i) Corticosteroid effects on fibril formation in the presence of initiating buffer

In the initial study of the effect of the corticosteroids on fibril formation, the anti-inflammatory agents were dissolved in the initiating buffer and the rate of fibril formation compared with that for initiating buffer alone. This study was undertaken to compare the various corticosteroids on the basis of the way in which they modify the rate of precipitation induced by the initiating buffer (i.e. initiating buffer alone serves as the control).

a) The action of betamethasone disodium phosphate

The effect of betamethasone disodium phosphate on fibrillogenesis in vitro is clearly shown in Figs. 10A and 10B (Run 5 of Table V) accelerated formation of fibrils occurring after addition of the corticosteroid to the

TABLE V

Rate of precipitation of collagen from physiological saline by corticosteroids in initiating buffer. Experimental conditions: pH of reaction mixture containing initiating buffer alone, 7.04; pH of reaction mixture containing initiating buffer and corticosteroid, 6.95; I 0.23; Method 1, collagen preparation P_1, reaction temperature 25°C.

Run No.	Initiating solution	Pretreatment of collagen solution	E_∞	t_c (min)	K_2	$t_{0.5}$ (min)	Correlation coefficient
4a	IB	filtered	0.74	9.6	0.229	56	0.997
4b	IB + BDP at half conc.	filtered	0.82	9.0	0.240	52	0.992
5a	IB	x32 000g	0.77	44.8	0.149	124	0.994
5b	IB + BDP	x32 000g	0.82	31.8	0.201	98	0.998
5c	IB	x107 000g	0.66	121.4	0.124	228	0.998
5d	IB + BDP	x107 000g	0.78	79.3	0.167	152	0.991

For definition of E_∞, t_c, $t_{0.5}$, K_2 and correlation coefficient, see text.
Abbreviations:- IB: initiating buffer; BDP: betamethasone disodium phosphate.

initiating buffer at 25°C. The reaction constants reflect an acceleration of fibril formation in the values of t_c, K_2 and $t_{0.5}$, while the final extinction value, E_∞, was greater in the presence of betamethasone disodium phosphate. Similar results were obtained using half the concentration of the corticosteroid although the differences in the reaction curves and constants were not as great (Fig. 13, Run 4 of Table V). Betamethasone disodium phosphate also accelerated fibril formation with collagen preparation (P_4) and the second method of following fibril formation (Figs. 14A and 14B, Run 6 of Table VI) at 20°C. The accelerating effect of the corticosteroid is indicated by a decrease of t_c and an increase of K_2 when compared with the control. In all the above studies, the pH of the reaction mixture was 7.04 (control-initiating buffer only) and 6.95 (betamethasone disodium phosphate dissolved in initiating buffer).

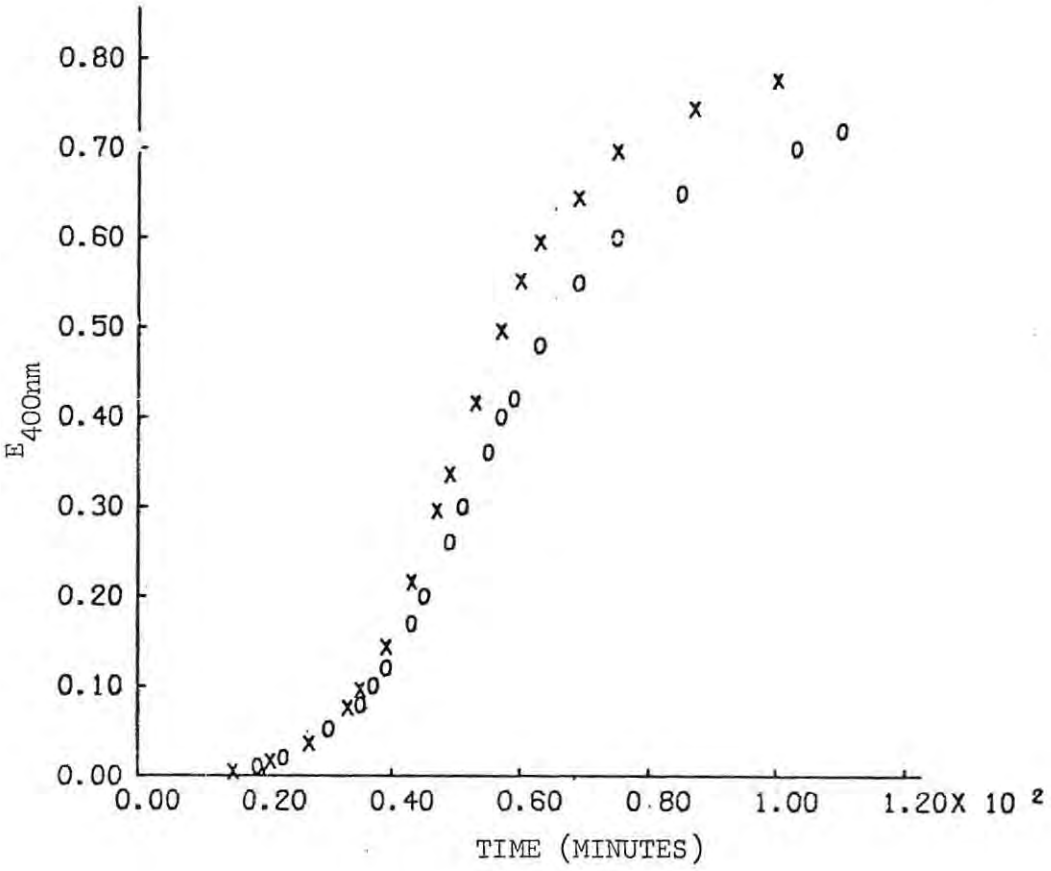

Fig. 13. Fibril precipitation from solutions of collagen (P_1) in physiological saline using method 1 at 25°C; X, 1mg/ml betamethasone disodium phosphate in initiating buffer (pH 6.95); O, initiating buffer alone (pH 7.04).

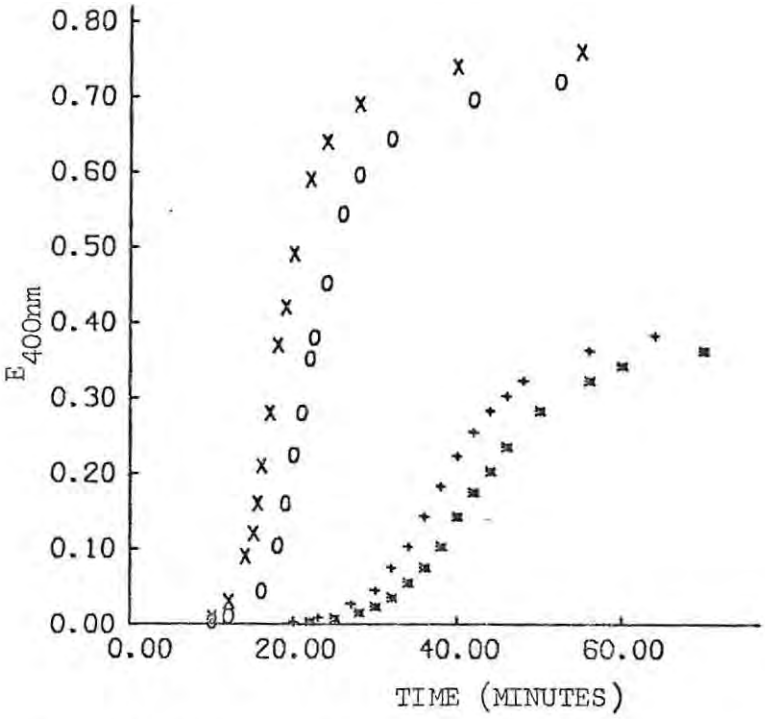

Fig. 14A. Fibril precipitation from solutions of collagen (P_4) in physiological saline using method 2 at 20°C; X, betamethasone disodium phosphate in initiating buffer (pH 6.95); 0, initiating buffer alone (pH 7.04); collagen concentration for (X) and (0), 0.123%; +, betamethasone disodium phosphate in initiating buffer (pH 6.95); *, initiating buffer alone (pH 7.04); collagen concentration for (+) and (*), 0.061%.

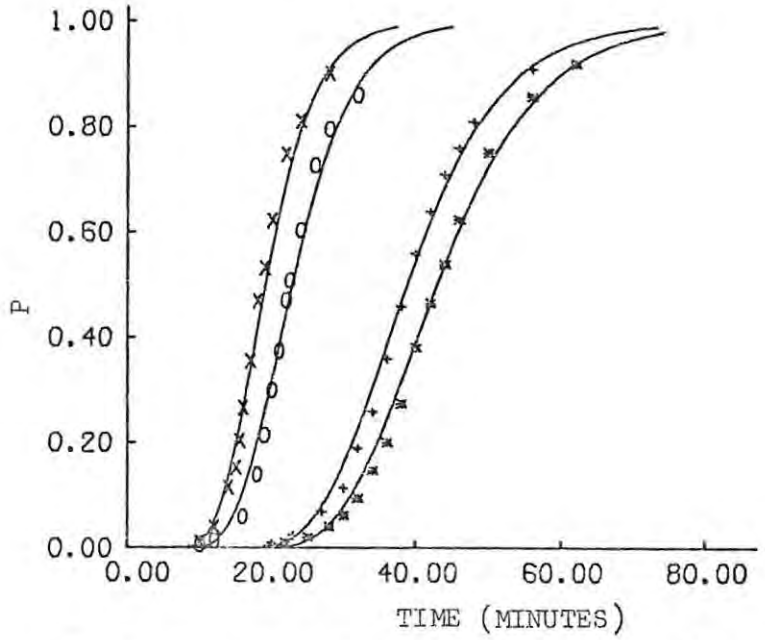

Fig. 14B. Time course of extent of precipitation. The solid curves represent the best fit of EQUATION III-7 to the corresponding experimental data shown in Fig. 14A.

TABLE VI

Rate of precipitation of collagen from physiological saline by corticosteroids in initiating buffer. Experimental conditions: pH of reaction mixture containing initiating buffer alone, 7.04; pH of reaction mixture containing initiating buffer and corticosteroid, 6.95; I 0.23; Method 2; collagen preparation preparation P_4; reaction temperature 20°C; collagen solutions clarified by centrifugation at 32 000g.

Run No.	Initiating solution	E_∞	t_c (min)	K_2	$t_{0.5}$ (min)	Correlation coefficient
6a	IB	0.75	9.6	0.798	22	0.990
6b	IB + BDP	0.79	8.24	0.961	18	0.989
6c*	IB	0.378	18.7	0.747	43	0.994
6d*	IB + BDP	0.40	16.6	0.813	38	0.996
7a	IB	0.73	8.3	0.685	24	0.987
7b	IB + PDP	0.79	5.8	0.714	20	0.990
8a	IB	0.93	15.6	0.173	46	0.990
8b	IB + BDP	1.00	13.5	0.249	50	0.984
8c	IB + HDP	0.96	21.2	0.216	48	0.997
8d	IB + PDP	1.01	11.4	0.219	50	0.980

For definition of E_∞, t_c, $t_{0.5}$, and K_2 and correlation coefficient, see text. Abbreviations:- IB: initiating buffer; BDP: betamethasone disodium phosphate; PDP: prednisolone disodium phosphate; HDP: hydrocortisone disodium phosphate.

* Half the collagen concentration was used in runs 6c and 6d.

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b) The action of prednisolone disodium phosphate

Prednisolone disodium phosphate dissolved in initiating buffer (Fig. 15, Run 7 of Table VI) also accelerated fibril formation. Once again the E_∞ value was seen to be higher in the presence of corticosteroid when compared with the control run. This higher E_∞ was associated with the faster precipitation reflected by the shorter lag, t_c, larger K_2 and shorter $t_{0.5}$, which was contrary to the findings of Wood (1960b) as previously described.

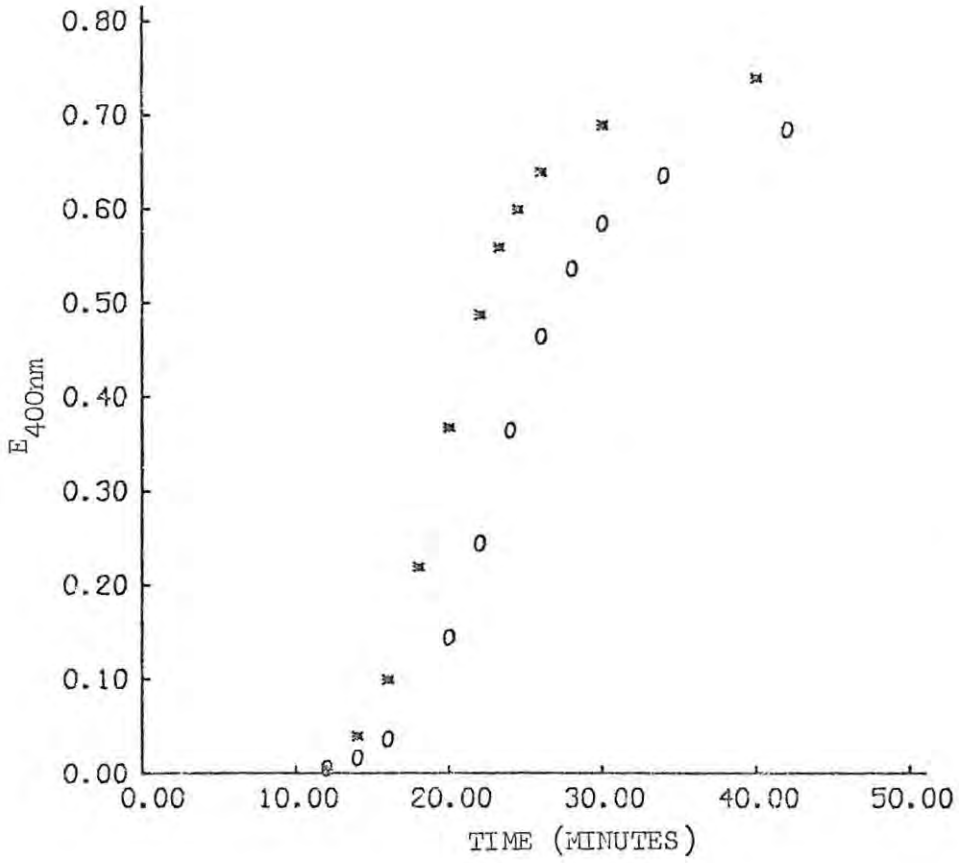


Fig. 15. Fibril precipitation from solutions of collagen (P4) in physiological saline using method 2 at 20°C; *, prednisolone disodium phosphate in initiating buffer (pH 6.95); 0, initiating buffer alone (pH 7.04); collagen concentration, 0.125%.

c) Comparison of effects of betamethasone, prednisolone and hydrocortisone disodium phosphates

Due to the difficulties involved in comparing corticosteroid effects from different experiments (i.e. experiments involving different collagen concentration and ages of solution), duplicate determinations on each of the three above-mentioned corticosteroids were made with the same collagen solution on successive days. These were reproducible and the reaction constants and precipitation curves are illustrated in Table VI, Run 8 and Figs. 16A and 16B respectively which depict the differences. The only structural difference between hydrocortisone and prednisolone is the $C_1 - C_2$ double bond, and between prednisolone and betamethasone, the 9α-fluoro and 16β-methyl substituent of the latter (Fig. 7). The values for the reaction constants appear somewhat anomalous in that the previously established trend of increased rate was always associated with shorter t_c, larger K_2 and shorter $t_{0.5}$. The values of the constants shown in Table VI indicate that although the rate of precipitation in the presence of hydrocortisone disodium phosphate appeared faster than that of the control, the reaction of the former was associated with a longer t_c and larger K_2. Also, the prednisolone disodium phosphate reaction was associated with the shortest lag but had a lower K_2 value than the betamethasone disodium phosphate reaction which appeared to have the fastest precipitation rate. In spite of these anomalies, however, the normalized curves were reasonably well described by the application of EQUATION III-7 to the experimental data.

The order of increasing anti-inflammatory activity of these compounds in vivo is hydrocortisone, prednisolone and betamethasone (Liddle and Fox, 1961; Sarett et al., 1963), which corresponds to the order of increasing fibril precipitation rate found experimentally in vitro. Therefore substitution of these groups has a positive effect on both anti-inflammatory activity and fibril precipitation, but whether this is a significant relationship in vivo requires further investigation.

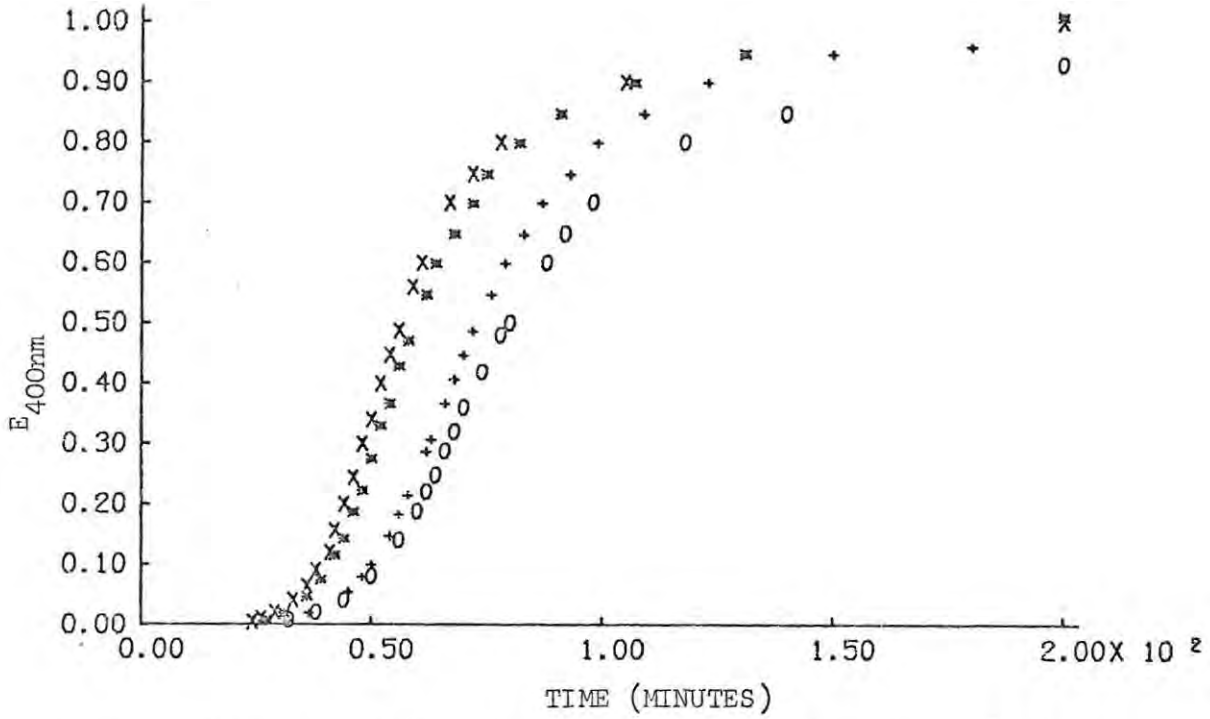


Fig. 16A. Fibril precipitation from solutions of collagen (P_4) in physiological saline using method 2 at 20°C; X, betamethasone disodium phosphate in initiating buffer (pH 6.95); *, prednisolone disodium phosphate in initiating buffer (pH 6.95); +, hydrocortisone disodium phosphate in initiating buffer (pH 6.95); 0, initiating buffer alone (pH 7.04); collagen concentration, 0.125%.

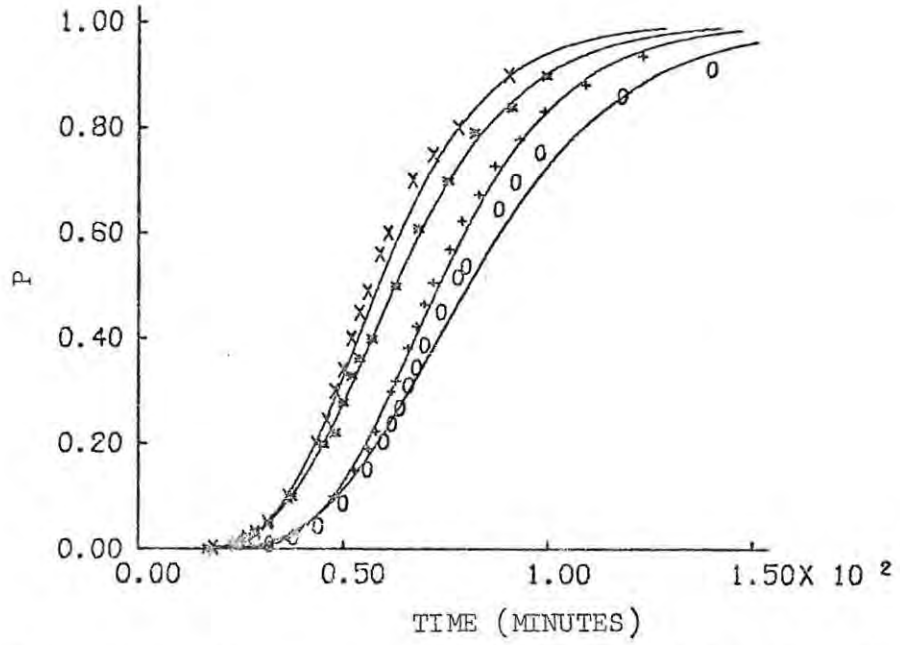


Fig. 16B. Time course of extent of precipitation. The solid curves represent the best fit of EQUATION III-7 to the corresponding experimental data shown in Fig. 16A.

d) Comparison of effects of betamethasone, paramethasone and dexamethasone disodium phosphates

These corticosteroids were compared in order to determine whether the position of the fluorine atom (dexamethasone and paramethasone) or the configuration of the 16-methyl group (betamethasone and dexamethasone) had any effect on the rate of fibril precipitation. Collagen preparation (P_4) and Method 2 at 20°C were used throughout. The results of these experiments are illustrated in Figs. 17A and 17B (Run 9 of Table VII). In spite of the fact that application of EQUATION III-7 was successful, anomalies in the t_c and K_2 values for paramethasone and dexamethasone disodium phosphate are apparent. Figs. 17A and 17B indicate that the order of increasing fibril precipitation rate is dexamethasone, paramethasone and betamethasone disodium phosphates. The pH of the control run was 6.95 as were the reaction mixtures of the individual corticosteroids in initiating buffer. As the normalized control curve was seen to be almost superimposable on the normalized curve resulting when paramethasone disodium phosphate was present, the control run was omitted from Fig. 17B but included in Fig. 17A and Table VII for comparative purposes. The final extinction values (E_∞) in the presence of corticosteroids were again found to be higher than with initiating buffer alone. Although dexamethasone disodium phosphate appeared to produce the slowest rate of precipitation, the lag period for this curve was shorter than the lag phases for both paramethasone disodium phosphate and the control. The value of K_2, however, was lower than the corresponding values for the other corticosteroids and the control.

The corticosteroid containing the 6α-fluoro atom and 16α-methyl group, i.e. paramethasone, accelerated precipitation when compared with the 9α-fluoro, 16α-methyl isomer, dexamethasone, while the 9α-fluoro, 16β-methyl isomer, betamethasone, resulted in a reaction rate greater than with either of the other two corticosteroids. Betamethasone disodium phosphate increased the rate of fibril precipitation in comparison with dexamethasone. With initiating

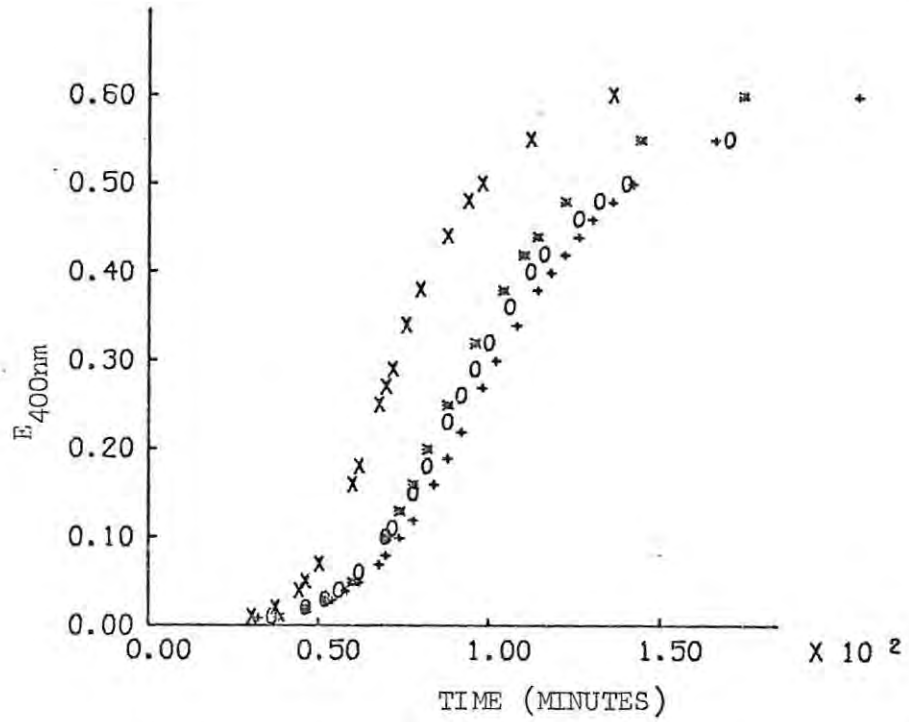


Fig. 17A. Fibril precipitation from solutions of collagen (P_4) in physiological saline using method 2 at 20°C; X, betamethasone disodium phosphate in initiating buffer (pH 6.95); *, paramethasone disodium phosphate in initiating buffer (pH 6.95); O, initiating buffer alone (pH 6.95); +, dexamethasone disodium phosphate (pH 6.95); collagen concentration, 0.140%.

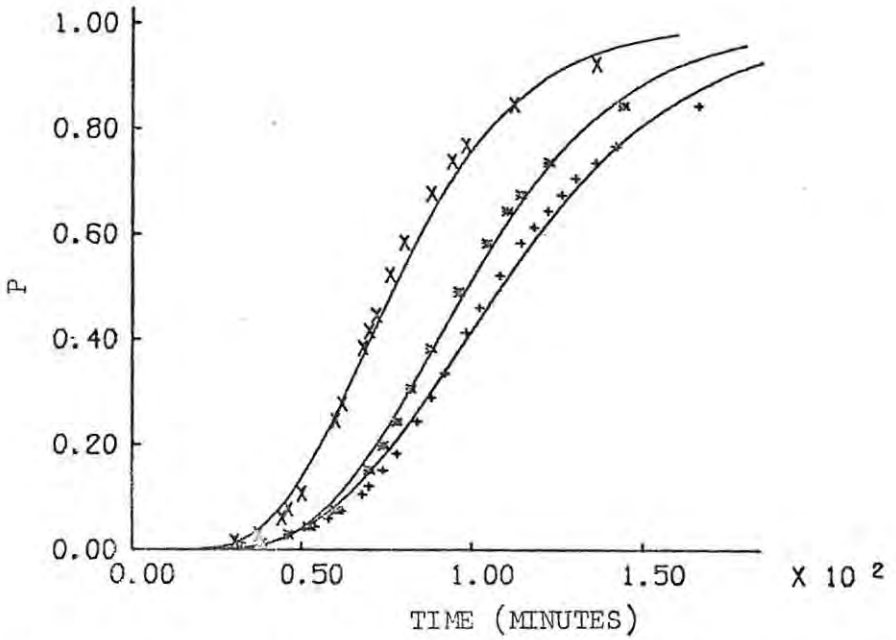


Fig. 17B. Time course of extent of precipitation. The solid curves represent the best fit of EQUATION III-7 to the corresponding experimental data shown in Fig. 17A. (N.B. Control run omitted).

TABLE VII

Rate of precipitation of collagen from physiological saline by corticosteroids in initiating buffer. Experimental conditions: reaction temperature 20°C; Method 2; I 0.23; collagen preparation P_4; collagen solutions clarified by centrifugation at 32 000g.

Run No.	Initiating solution	pH of reaction mixture	E_∞	t_c (min)	K_2	$t_{0.5}$ (min)	Correlation coefficient
9a	IB	6.95	0.62	17.4	0.128	97	0.996
9b	IB + BDP	6.95	0.65	13.6	0.164	75	0.994
9c	IB + Para DP	6.95	0.65	20.7	0.134	96	0.997
9d	IB + DDP	6.95	0.65	15.9	0.113	106	0.995
10a	IB	7.00	0.74	43.3	0.095	151	0.995
10b	IB + HHS	7.00	0.78	31.3	0.122	113	0.993
10c	IB + DHS	7.00	0.735	10.2	0.177	66	0.975

For definition of E_∞, t_c, $t_{0.5}$, K_2 and correlation coefficient, see text. Abbreviations:- IB: initiating buffer; BDP: betamethasone disodium phosphate; Para DP: paramethasone disodium phosphate; DDP: dexamethasone disodium phosphate; HHS: hydrocortisone hemisuccinate sodium; DHS: 1-dehydro-6α-methyl hydrocortisone-21-succinate sodium.

buffer alone, the reaction rate was slower than with betamethasone but slightly faster than with dexamethasone disodium phosphate.

e) Comparison of effects of hydrocortisone hemisuccinate sodium and 1-dehydro-6α-methyl hydrocortisone-21-succinate sodium

The differences between 1-dehydro-6α-methyl hydrocortisone and hydrocortisone are the presence of both a $C_1 - C_2$ double bond and 6α-methyl substituent in the former corticosteroid. The difference in fibril precipitation rate may thus be attributed to the presence or absence of these features.

The effects of the sodium succinate salts of hydrocortisone and 1-dehydro-6α-methyl hydrocortisone dissolved in initiating buffer were

investigated using collagen preparation (P_4) and Method 2 at 20°C. The results are shown in Figs. 18A and 18B, (Run 10 of Table VII) together with the results of the control. The fastest rate of collagen precipitation was elicited by 1-dehydro-6α-methyl hydrocortisone. The rate of precipitation in the control run was slower than either of these two corticosteroids. The rate constants in Table VII summarize these effects. The final extinction value ($E\infty$) for 1-dehydro-6α-methyl hydrocortisone-21-succinate sodium was similar to the corresponding $E\infty$ value of the control whereas hydrocortisone sodium succinate resulted in a significantly larger $E\infty$. Unfortunately, neither 6α-methyl prednisolone disodium phosphate, 6α-methyl hydrocortisone disodium phosphate nor hemisuccinate were available and hence the individual effects of either the $C_1 - C_2$ double bond or the 6α-methyl substituent could not be evaluated.

ii) Corticosteroid effects on fibril formation in the absence of initiating buffer

In order to demonstrate that the corticosteroids can initiate fibril formation on their own, the disodium phosphate derivatives of these anti-inflammatory compounds were dissolved in physiological saline instead of initiating buffer and added to the collagen dissolved in the same solvent. It should be noted that in this study, the corticosteroid effects are compared directly from their influence on precipitation rates per se in the absence of initiating buffer i.e. there is no absolute control run as the run in the absence of corticosteroid which was initiating buffer alone, cannot be directly compared with the effects of the corticosteroids in physiological saline.

The various corticosteroids were dissolved in physiological saline, instead of initiating buffer, and added to the collagen dissolved in the same solvent. The runs in the absence of corticosteroid for each of these reactions involved the use of initiating buffer made up to give a pH in the reaction mixture of 6.2 which is close to the pH of

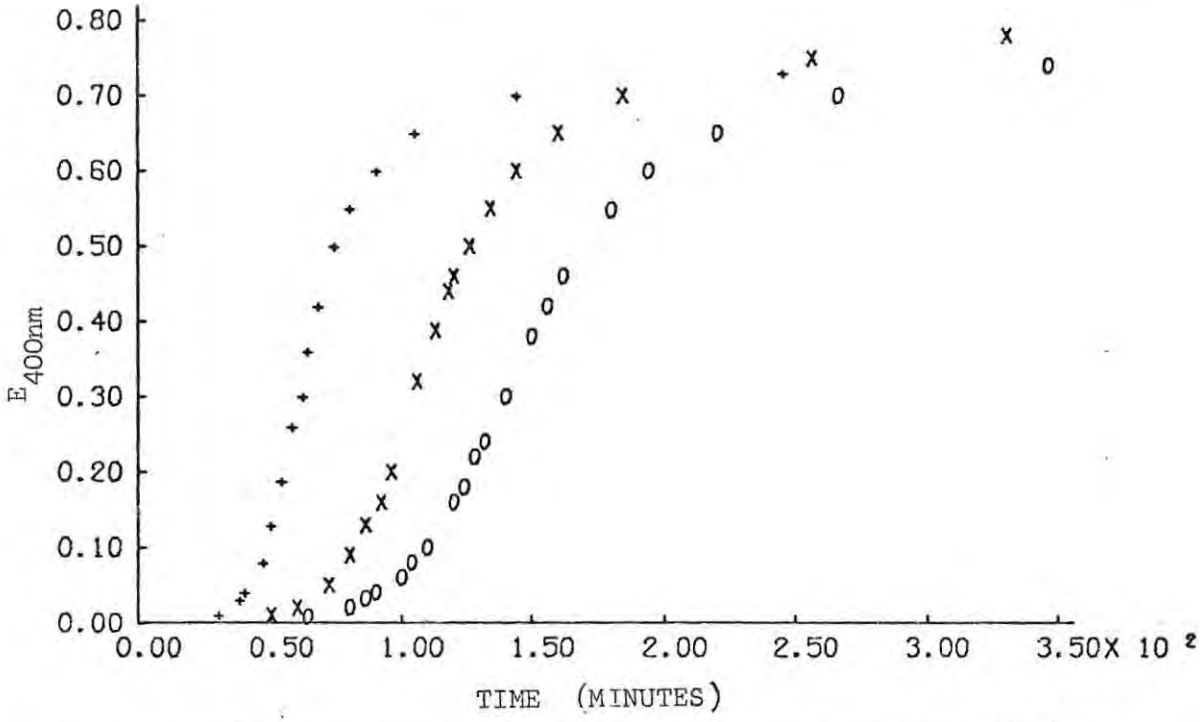


Fig. 18A. Fibril precipitation from solutions of collagen (P_4) in physiological saline using method 2 at 20°C; +, 1-dehydro-6α-methyl hydrocortisone-21-succinate sodium in initiating buffer (pH 7.0); X, hydrocortisone hemisuccinate sodium (pH 7.0); 0, initiating buffer alone (pH 7.00); collagen concentration, 0.141%.

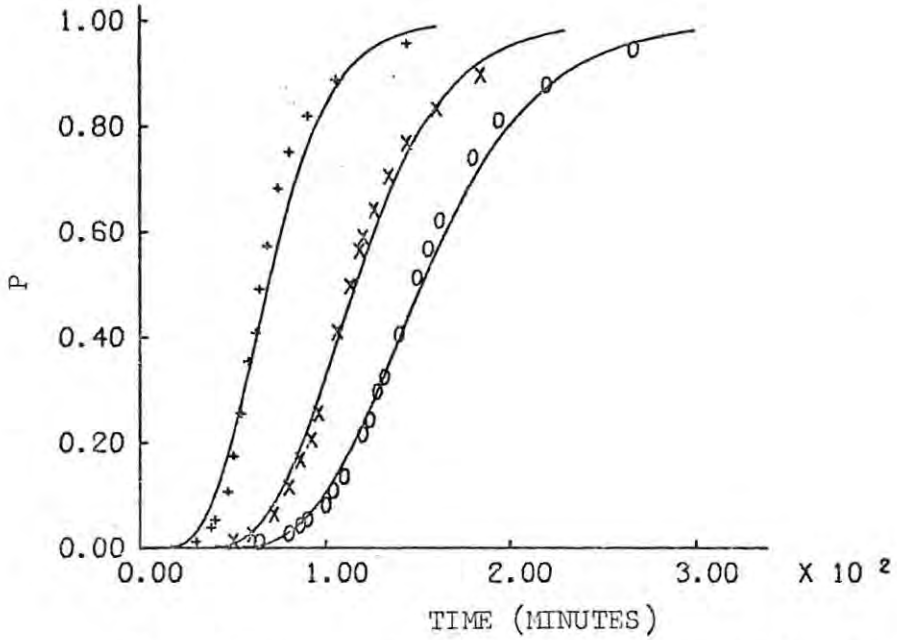


Fig. 18B. Time course of extent of precipitation. The solid curves represent the best fit of EQUATION III-7 to the corresponding experimental data shown in Fig. 18A.

the reaction mixtures containing the corticosteroids (Tables IX and X). In each case the blank solution placed in the reference cell of the spectrophotometer was collagen dissolved in physiological saline, which showed no precipitation of fibrils. Table IX and Figs. 19A and 19B show that in the absence of the initiating buffer the three corticosteroids, betamethasone, prednisolone and hydrocortisone disodium phosphates induced fibril formation at different rates, all of which were slower than that for initiating buffer alone. Furthermore, the E_∞ values in the presence of each of the corticosteroids were significantly higher than the value obtained with the initiating buffer alone. The order of increasing fibril precipitation rate was seen to be identical to that observed when the corticosteroids were dissolved in initiating buffer. The presence of betamethasone disodium phosphate resulted in a faster precipitation rate when compared with the other two corticosteroids. However, this rate of fibril formation was slower than that shown by the control. This is reflected by the values of t_c, K_2 and $t_{0.5}$ in Table IX. There appeared to be very slight differences in these rate constants between prednisolone and hydrocortisone disodium phosphate. The normalized curves illustrated in Fig. 19B indicate that in spite of the absence of initiating buffer, the curves are well described by EQUATION III-7.

a) Effect of corticosteroid concentration

Fibril precipitation was also obtained at a tenth of the concentration of betamethasone disodium phosphate used above, though at a very much slower rate ($t_{0.5}$ 1 250 min.). Preliminary investigations indicated that fibril formation can be obtained at very much lower concentrations of corticosteroids by raising the temperature above the relatively low values used in the current experiments.

During these studies when no initiating buffer was used, the lag and growth phases of the precipitation curves were considerably longer than the

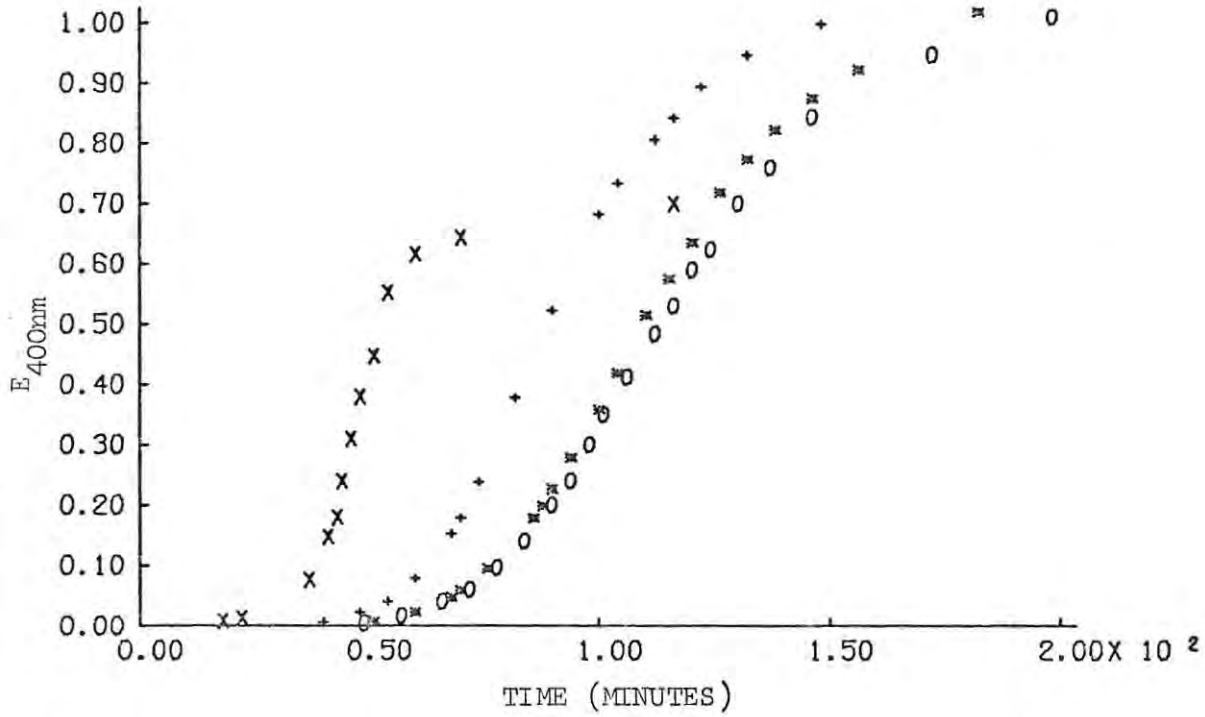


Fig. 19A. Fibril precipitation from solutions of collagen (P_4) in physiological saline using method 2 at 20°C; X, initiating buffer alone (pH 6.30); +, betamethasone disodium phosphate in physiological saline (pH 6.25); *, prednisolone disodium phosphate in physiological saline (pH 6.25); O, hydrocortisone disodium phosphate in physiological saline (pH 6.30); collagen concentration, 0.123%.

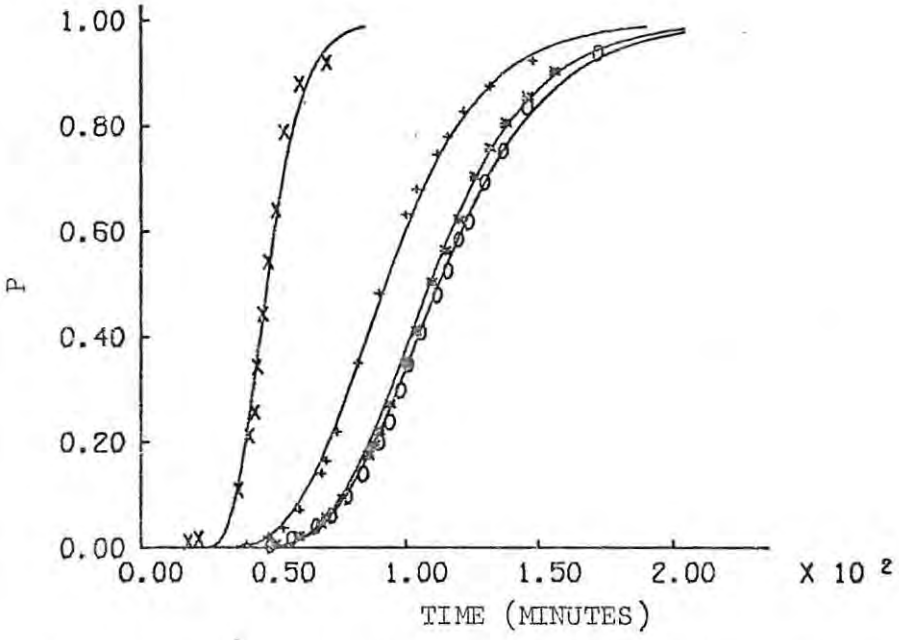


Fig. 19B. Time course of extent of precipitation. The solid curves represent the best fit of EQUATION III-7 to the corresponding experimental data shown in Fig. 19A.

corresponding phases when the corticosteroids were dissolved in initiating buffer. The final extinction value (E_∞), which Wood (1960b) has related directly to fibril width, were much increased in the presence of corticosteroid. Moreover, Wood and Keech (1960) suggested that the coarseness of the precipitates (wide fibrils and high degree of lateral aggregation of fibrils) is associated with high opacity, and vice versa, but emphasized that no simple correlation of these properties with the rate of precipitation existed.

5. Protein factors influencing precipitation

The role of the net collagen charge in the reactions at pH values close to the isoelectric point, which has been reported for acid-soluble collagen at pH 5.8 - 6.0 (Brown and Kelly, 1953) is difficult to define since this value is altered by the binding of various cations and anions (Veis et al., 1958).

Non-covalent bonding such as hydrogen bonds (Gross and Kirk, 1958), electrostatic forces (Wood and Keech, 1960; Gross and Kirk,1958) and hydrophobic bonding (Cassel, 1966; Fessler, 1966; Cassel and Christensen, 1967; Grant and Alburn, 1968) have all been suggested to account for fibril precipitation from solutions of collagen. It appears to be a reasonable assumption that any interaction between collagen and an anionic corticosteroid must involve electrostatic forces leading to a collagen-corticosteroid complex with stability governed by mass action. The precipitation of collagen fibrils, however, depends on collagen-collagen interactions and hence it is difficult to explain how these presumed ionic interactions may account for the increasing rate of precipitation exhibited by the various corticosteroids. On the other hand, however, the observations that the precipitations in the absence of buffer still occurred, suggests that these corticosteroids may exert similar effects to those shown when initiating buffer is present.

6. Steroid structural factors in fibrillogenesis

From studies of the interaction of various reagents with steroids, it has been shown generally that attack by a reagent is at the rear (α-face) of the corticosteroid molecule (Gallagher and Kritchevsky, 1950; Westphal, 1961; 1971). This is presumed to be due to the presence of the C_{18} and C_{19} angular methyl groups which shield the front (β-face) of the corticosteroid molecule (Plates 2-6). This surface is, however, non-polar and thus the possibility of hydrophobic bonding at this surface must also be considered (Schlagel, 1965; 1972).

Furthermore, it is obvious that intermolecular protein bonding forces must be minimal in order for the protein to remain in solution and, hence, precipitation will only occur when intermolecular bonds are formed. These bonds, presumably can result from either protein-ligand or protein-protein interactions. In view of the above discussion, consideration of reactive groups on the corticosteroid molecule may shed some light on the type of bonding involved.

When prednisolone is compared with hydrocortisone, the former caused an increase in precipitation rate (Figs. 16A and 16B; 19A and 19B and Tables VI and IX respectively). This implied that the presence of an additional double bond (Δ^1) in ring A (Fig. 7) of the corticosteroid molecule could account for the observed effects. Unsaturation at this position increases the polarity of the C - 3 ketone as well as increasing the planarity of the molecule across the top of the ring A (Ringold, 1961).

Prednisolone differs from betamethasone in that the latter has both a 9α-fluoro and 16β-methyl group. These resulted in a faster rate of collagen precipitation compared with either prednisolone or hydrocortisone. The presence of the 9α-fluoro causes an increased electronegativity which is imparted to the molecule by the tendency of the fluorine atom to withdraw electrons from its neighbouring elements. The effect of this withdrawal

TABLE VIII

Rate of precipitation of collagen from physiological saline by corticosteroids in initiating buffer. Experimental conditions: reaction temperature 20°C; Method 2; I 0.23; collagen preparation P_4; collagen solutions clarified by centrifugation at 32 000g.

Run No.	Initiating solution	pH of reaction mixture	E_∞	t_c (min)	K_2	$t_{0.5}$ (min)	Correlation coefficient
11a	IB	6.95	0.705	54.9	0.130	136	0.994
11b	IB + DDP	6.95	0.750	52.2	0.134	130	0.996
11c	IB + BDP	6.95	0.752	36.9	0.160	102	0.996

For definition of E_∞, t_c, $t_{0.5}$, K_2 and correlation coefficient, see text.
Abbreviations:- IB: initiating buffer; DDP: dexamethasone disodium phosphate; BDP: betamethasone disodium phosphate.

is to strengthen the acidity of the 11β-hydroxyl and thereby to make the proton of the hydroxyl group more available for stronger hydrogen bonding between the corticosteroid and receptor (Fried and Borman, 1958).

The significance of the 16-methyl configuration in fibril formation is demonstrated in Figs. 17A and 17B; 20A and 20B; 23A and 23B and Run 9 of Table VII, Run 11 of Table VIII and Run 13 of Table X where the rates of precipitation in the presence of either of these epimeric corticosteroids are compared. The results indicate that the precipitation rate in the presence of dexamethasone was slower than in the presence of betamethasone disodium phosphate. This retarding effect may be attributed to the configurational difference between these epimers. There appear to be at least two possibilities of interaction. The first being that the non-polar characteristics of the β-face is increased by the 16β-methyl which would tend to favour hydrophobic interactions whilst the second may be due to a steric hindrance of the 17 - OH in the presence of 16α-methyl which could interfere with a possible hydrogen bonding mechanism

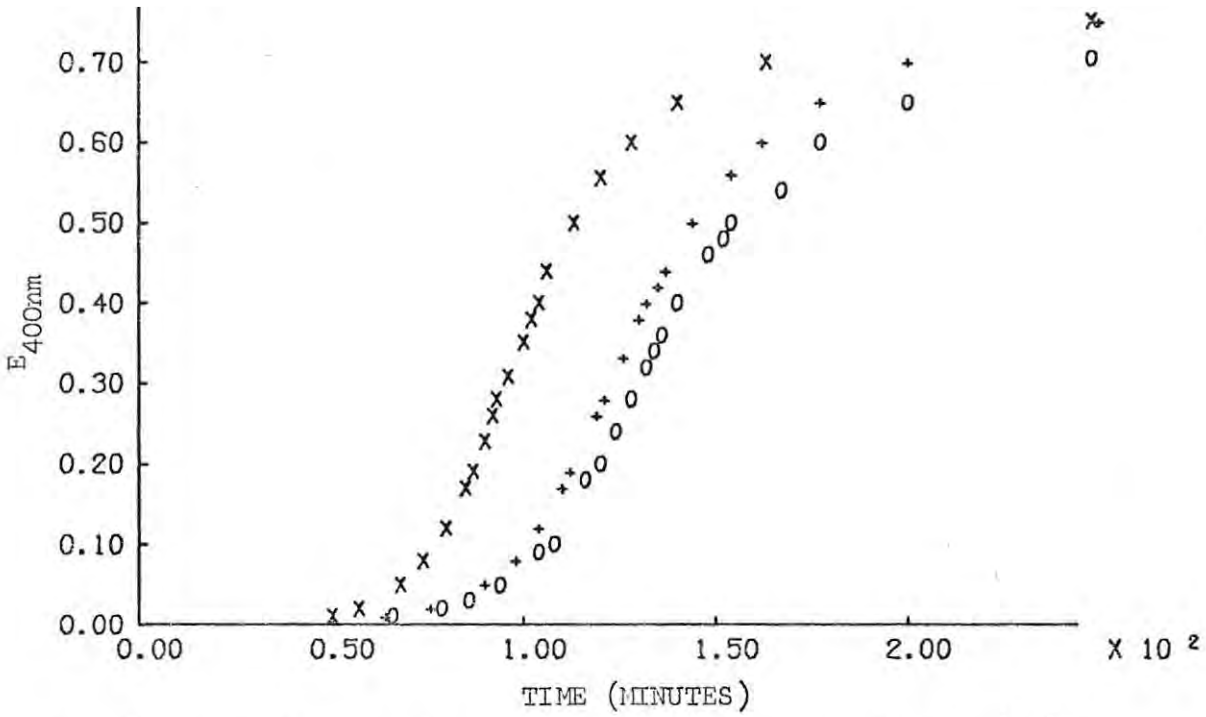


Fig. 20A. Fibril precipitation from solutions of collagen (P_4) in physiological saline using method 2 at 20°C; X, betamethasone disodium phosphate in initiating buffer (pH 6.95); +, dexamethasone disodium phosphate in initiating buffer (pH 6.95); 0, initiating buffer alone (pH 6.95); collagen concentration, 0.138%.

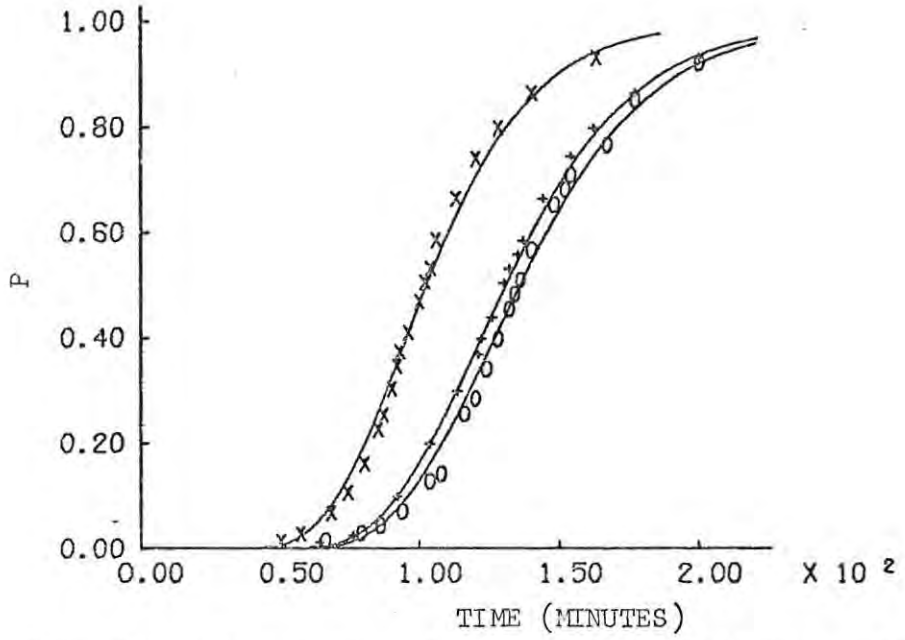


Fig. 20B. Time course of extent of precipitation. The solid curves represent the best fit of EQUATION III-7 to the corresponding experimental data shown in Fig. 20A.

TABLE IX

Rate of precipitation of collagen from physiological saline by corticosteroids in physiological saline. Experimental conditions: collagen preparation P_4; Method 2; collagen solutions clarified by centrifugation at 32 000g; reaction temperature 20°C.

Run No.	Initiating solution	pH of reaction mixture	E_∞	t_c (min)	K_2	$t_{0.5}$ (min)	Correlation coefficient
12a	IB	6.20	0.70	16.1	0.376	48	0.967
12b	BDP	6.25	1.08	29.3	0.181	90	0.998
12c	PDP	6.25	1.02	41.7	0.167	111	0.998
12d	HDP	6.30	1.01	41.2	0.158	114	0.998

For definition of E_∞, t_c, $t_{0.5}$, K_2 and correlation coefficient, see text. Abbreviations:- IB: initiating buffer; BDP: betamethasone disodium phosphate; PDP: prednisolone disodium phosphate; HDP: hydrocortisone disodium phosphate.

via the 17 - OH, possible with the 16β-methyl epimer (Fig. 7 and Plates 2 & 3).

In comparing dexamethasone with paramethasone disodium phosphate, the only structural difference between these isomers is the 9α-fluorine of the former and the 6α-fluorine of the latter, yet these show significant differences in their fibril precipitation rates (Figs. 17A and 17B; 22A and 22B and Run 9 of Table VII and Run 15 of Table X respectively). Table X, Run 15 shows that although the faster rate in the presence of paramethasone disodium phosphate was associated with a shorter t_c, the values of K_2 for both these isomeric corticosteroids were very similar. The position of the electronegative fluorine atom, having a very strong hydrogen bond forming ability (Pauling, 1960) could be responsible for the observed experimental differences. In this instance, a hydrogen bonding mechanism could account for the faster reaction rate observed with paramethasone disodium phosphate. The 6α-fluorine atom in paramethasone has an equatorial configuration

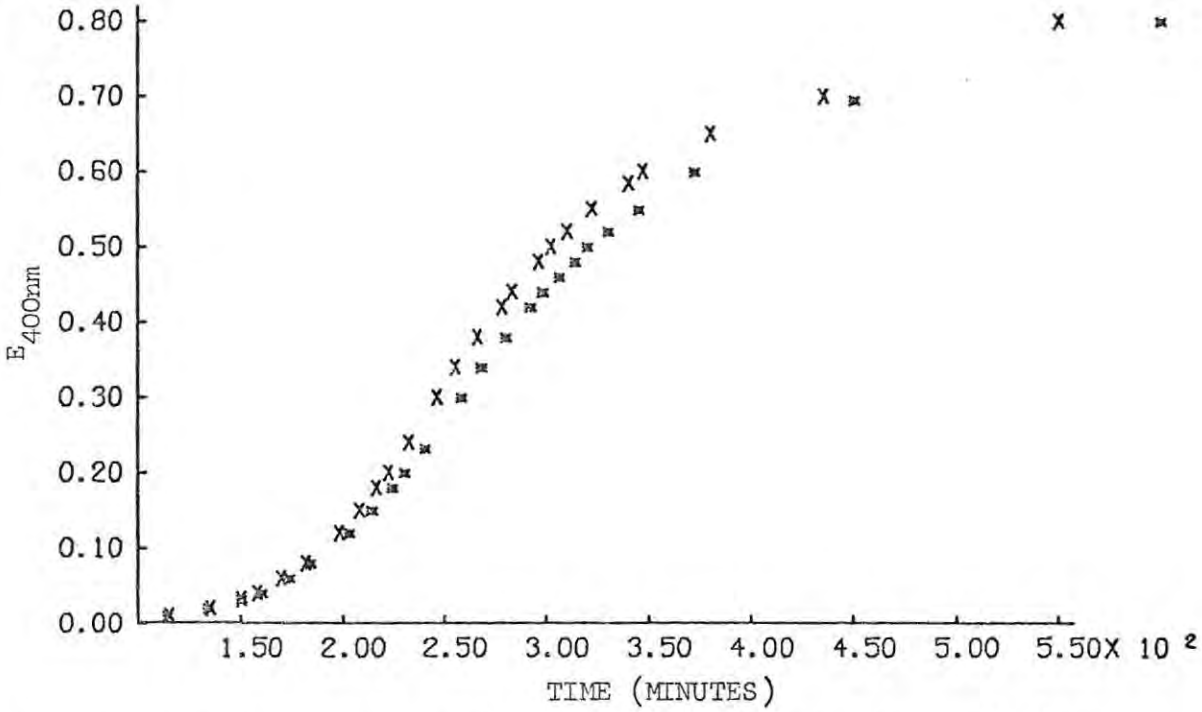


Fig. 21A. Fibril precipitation from solutions of collagen (P_4) in physiological saline using method 2 at 20°C; X, betamethasone disodium phosphate in physiological saline (pH 6.2); *, paramethasone disodium phosphate in physiological saline (pH 6.2); collagen concentration, 0.140%.

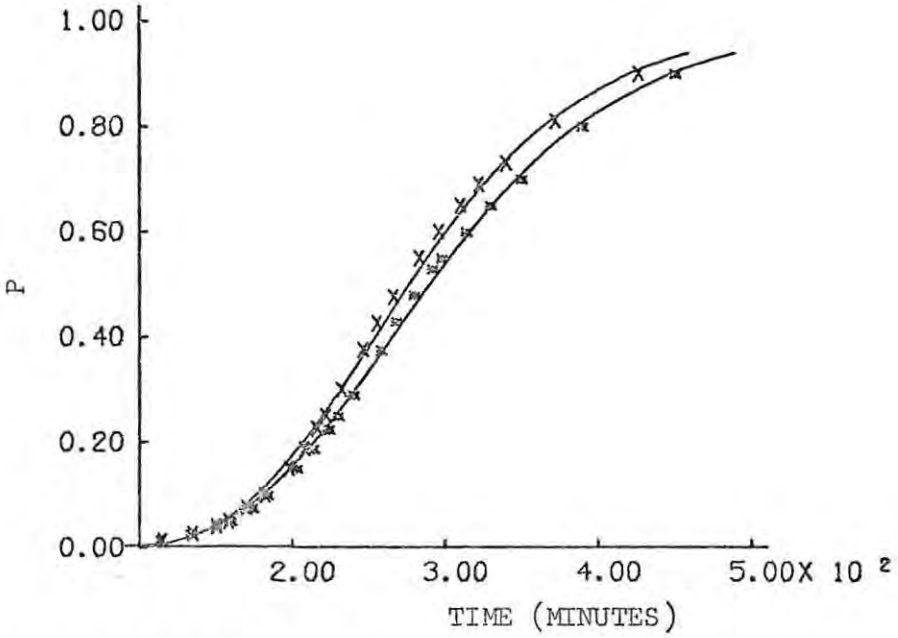


Fig. 21B. Time course of extent of precipitation. The solid curves represent the best fit of EQUATION III-7 to the corresponding experimental data shown in Fig. 21A.

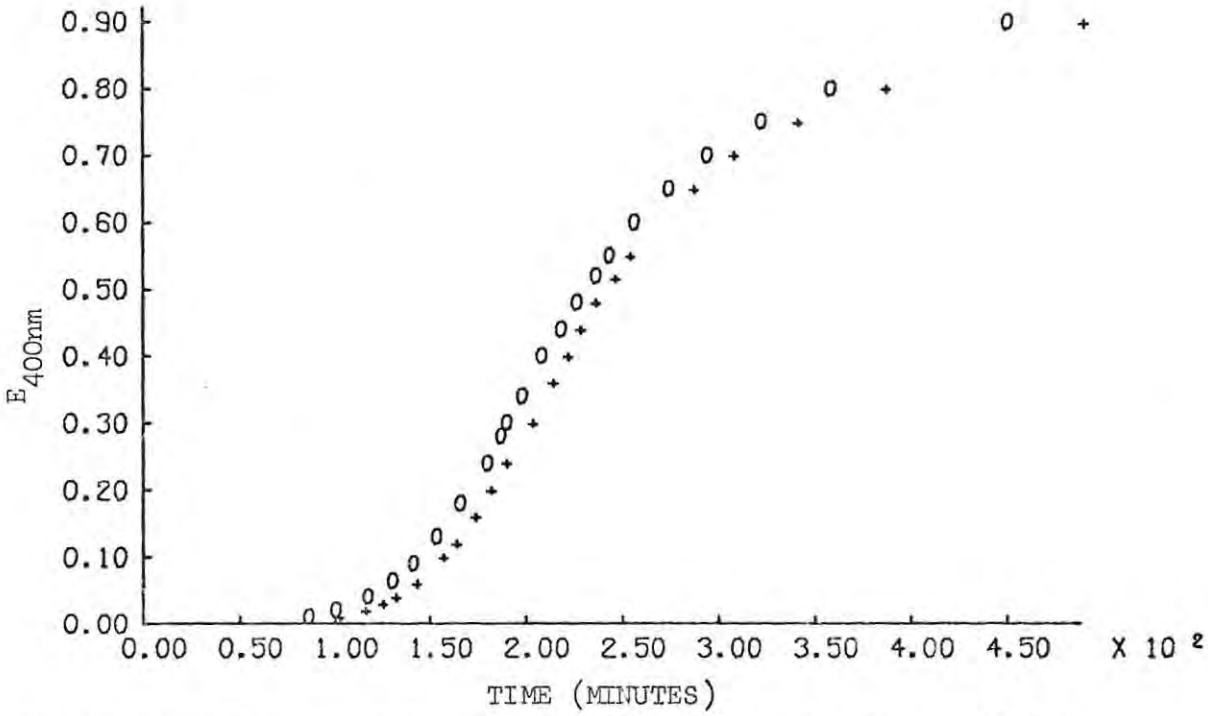


Fig. 22A. Fibril precipitation from solutions of collagen (P_4) in physiological saline using method 2 at 20°C; O, paramethasone disodium phosphate in physiological saline (pH 6.2); +, dexamethasone disodium phosphate in physiological saline (pH 6.2); collagen concentration, 0.141%.

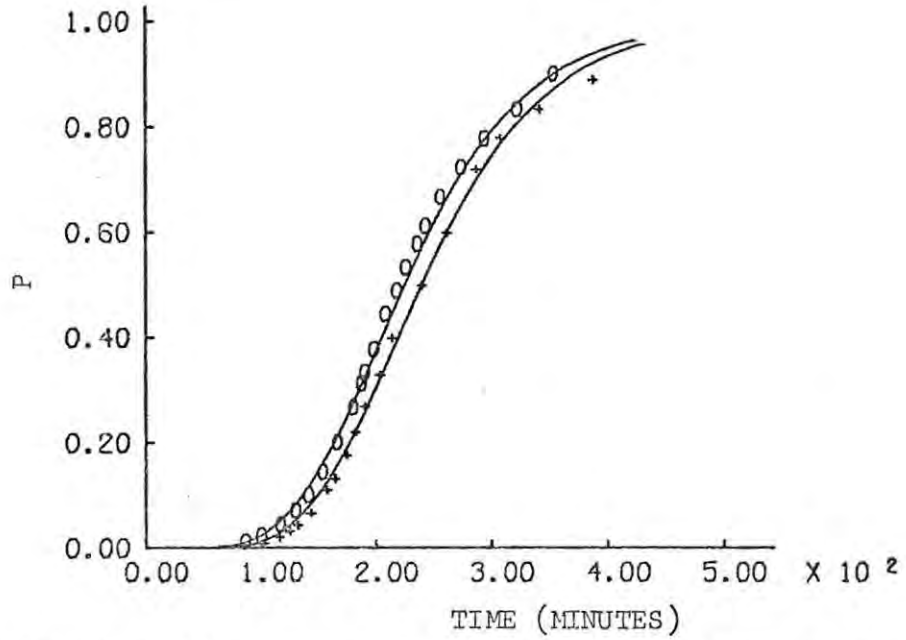


Fig. 22B. Time course of extent of precipitation. The solid curves represent the best fit of EQUATION III-7 to the corresponding experimental data shown in Fig. 22A.

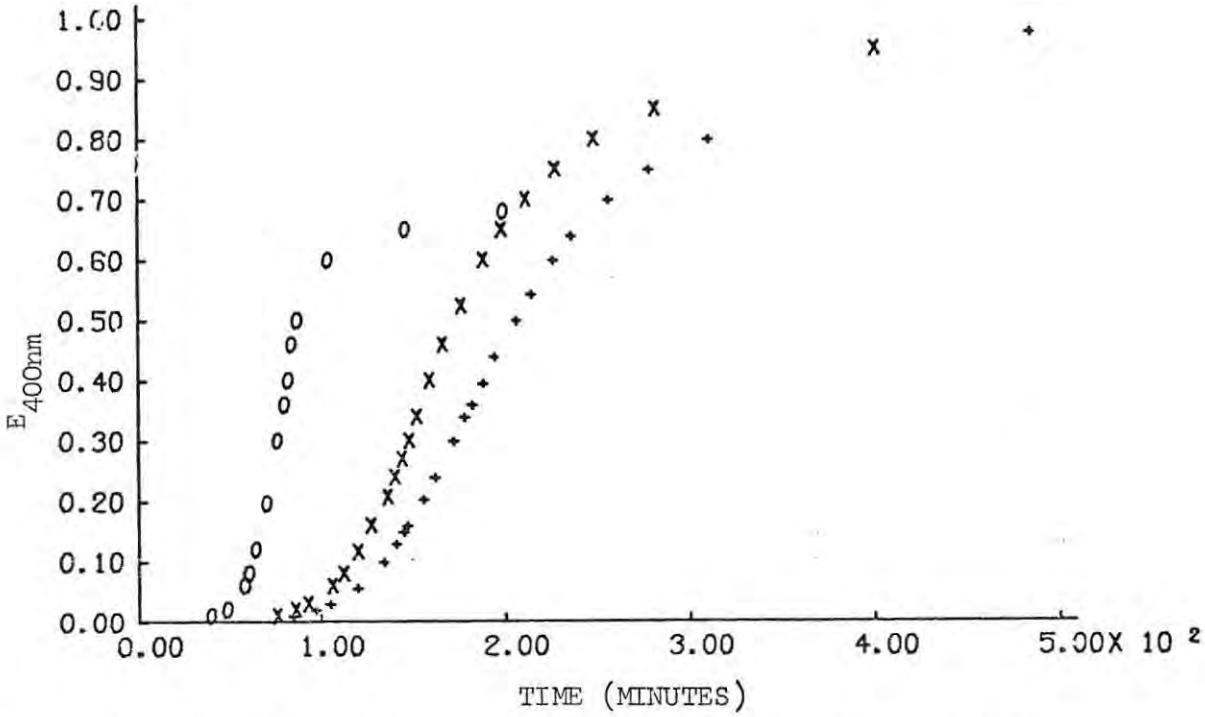


Fig. 23A. Fibril precipitation from solutions of collagen (P_4) in physiological saline using method 2 at 20°C; O, initiating buffer alone (pH 6.2); X, betamethasone disodium phosphate in physiological saline (pH 6.2); +, dexamethasone disodium phosphate in physiological saline (pH 6.2); collagen concentration, 0.132%.

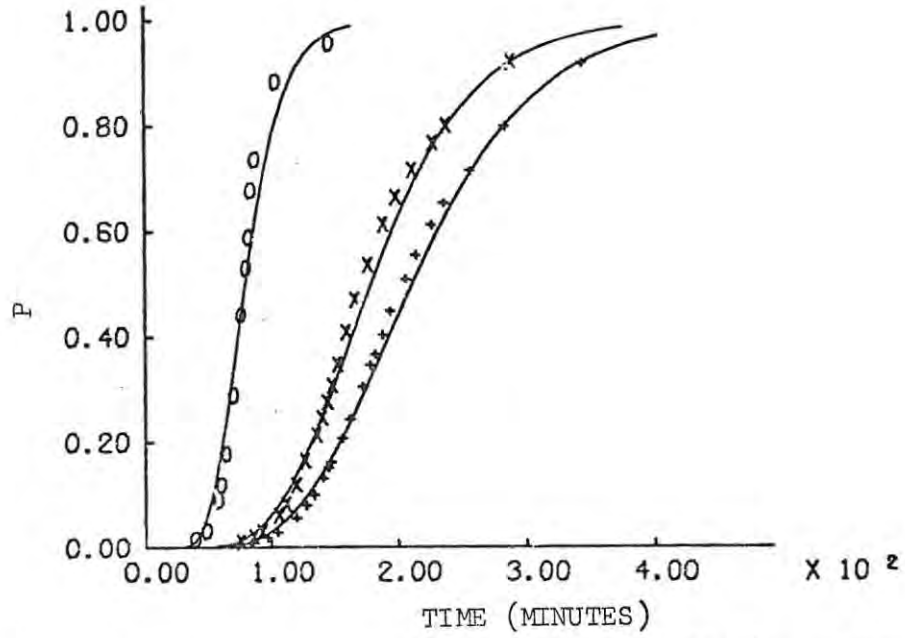


Fig. 23B. Time course of extent of precipitation. The solid curves represent the best fit of EQUATION III-7 to the corresponding experimental data shown in Fig. 23A.

TABLE X

Rate of precipitation of collagen from physiological saline by corticosteroids in physiological saline. Experimental conditions: collagen preparation P_4; Method 2; collagen solutions clarified by centrifugation at 32 000g; reaction temperature 20°C.

Run No.	Initiating solution	pH of reaction mixture	E_∞	t_c (min)	K_2	$t_{0.5}$ (min)	Correlation coefficient
13a	IB	6.2	0.680	25.8	0.201	78	0.972
13b	BDP	6.2	0.978	41.6	0.079	170	0.992
13c	DDP	6.2	0.978	44.0	0.065	203	0.993
14a	BDP	6.2	0.800	72.7	0.051	272	0.998
14b	Para DP	6.2	0 800	67.2	0.047	286	0.998
15a	DDP	6.2	0.898	66.3	0.062	231	0.998
15b	Para DP	6.2	0.900	47.7	0.059	220	0.997

For definition of E_∞, t_c, $t_{0.5}$, K_2 and correlation coefficient, see text. Abbreviations:- IB: initiating buffer; BDP: betamethasone disodium phosphate; DDP: dexamethasone disodium phosphate; Para DP: paramethasone disodium phosphate.

whereas the 9α-fluorine in dexamethasone is axial, indicating that the former fluorine atom may be more accessible to hydrogen bond formation than the latter, which tends to be embedded within the body of the molecule (Plates 3 & 4).

Figs. 21A and 21B (Run 14 of Table X) describe the effects of betamethasone and paramethasone disodium phosphate on fibril precipitation. The rate of fibril formation is faster in the presence of betamethasone disodium phosphate. This faster rate is again associated with a shorter lag phase but the K_2 values are very similar in each case.

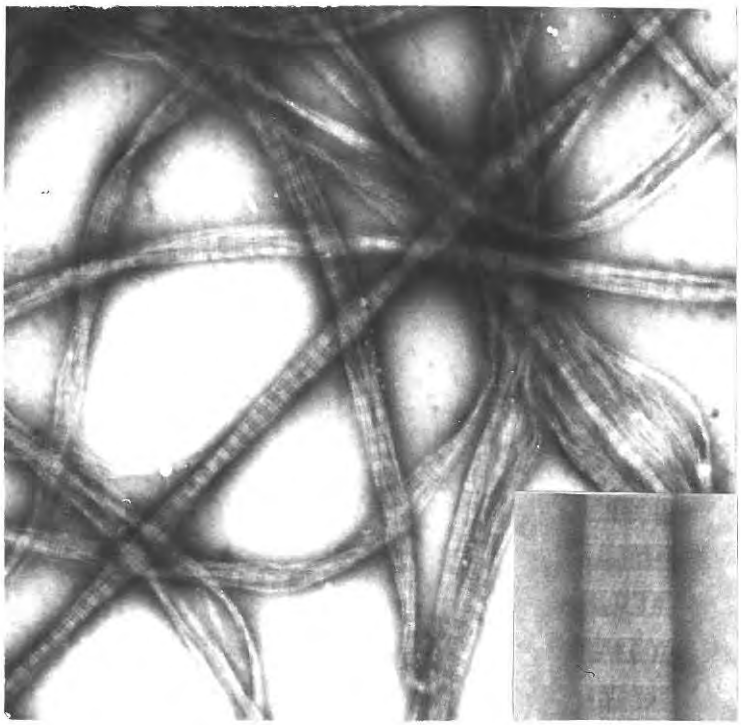
7. Electron microscopy

The fibrils precipitated in the presence of initiating buffer or the corticosteroids resembled native collagen in appearance and in having a repeat

period of about 64nm. The electron micrographs (Plates 7 - 9) depict these native periodicities which occurred independently of the particular corticosteroid used.

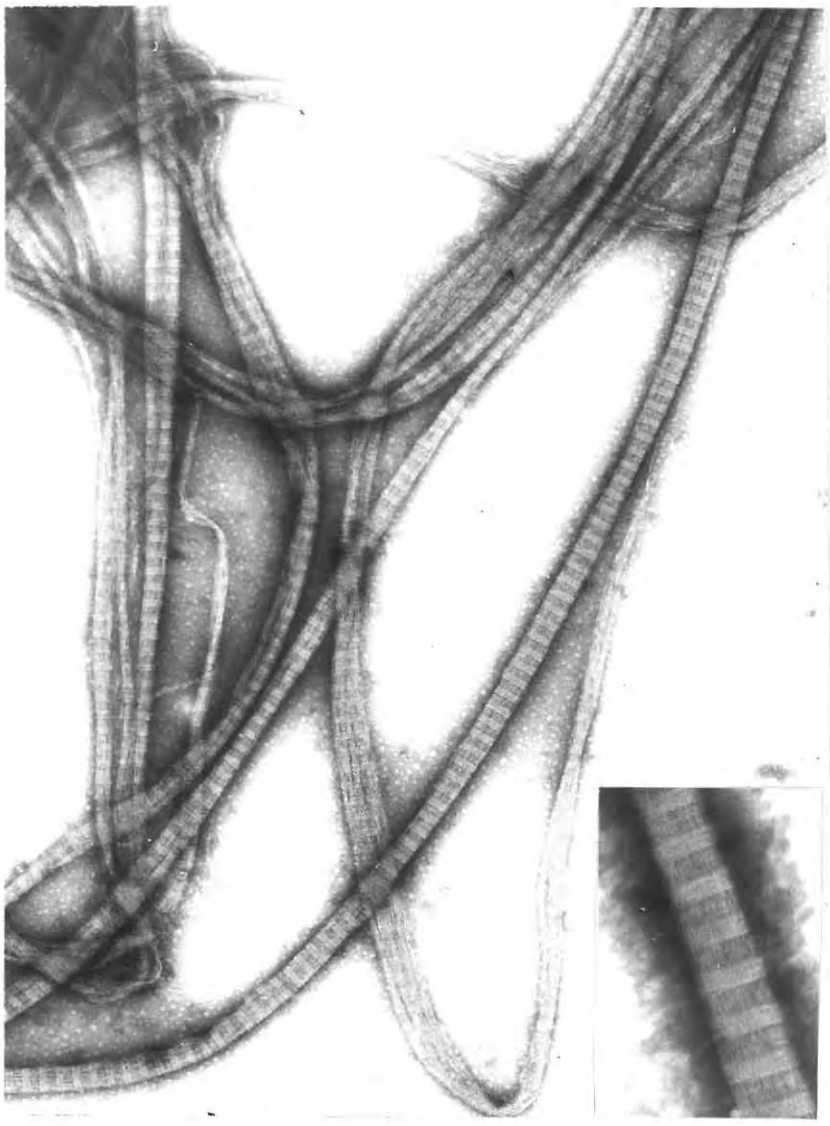
Kuhn et al. (1957; 1959) and Hodge and Schmitt (1958) indicated that amino groups with basic side chains (arginine, lysine) are concentrated in the end regions of the soluble collagen macromolecules and suggested that an anionic (chondroitin sulphate, in that instance) molecule might promote end-to-end aggregation by the interaction of its negatively charged sulphate groups with positively charged groups of two collagen macromolecules. An analogy may thus be drawn with the present system which involves large corticosteroid anions dissolved in initiating buffer. Further proof that the corticosteroids are involved in fibril precipitation is shown in the experiments performed in the absence of buffer. The effect of mucopolysaccharides (Gross and Kirk, 1958; Wood, 1960b; Mathews and Decker, 1968; Wasteson and Obrink, 1968), nucleic acids (Gross, 1956; Randall et al.,1955) and other compounds (Convy and Wynn, 1967; Bowden et al.,1968; Shimizu et al.,1968) on the formation of fibrils from collagen solutions has been studied. Wood (1960b) concluded that polyanions, such as the chondroitin sulphates, which accelerated precipitation lowered E_{∞} relative to the controls and gave thinner fibrils, while those, such as heparin and DNA, which retarded precipitation increased E_{∞} and fibril width, but there were exceptions. While there appeared to be a direct correlation between E_{∞} and fibril width, no simple relationship between rate of precipitation and fibril width existed. It was evident from the polyanions studied that these did not alter the way in which collagen particles aggregated in growth, but that they acted on the nucleation phase in one of two ways. They could accelerate nucleation, thereby increasing the number of nuclei formed and accelerating subsequent precipitation. This would have the effect of decreasing the size of the fibrils. Alternatively, they could alter the shape of the nuclei, thus increasing the

PLATE NO. 7



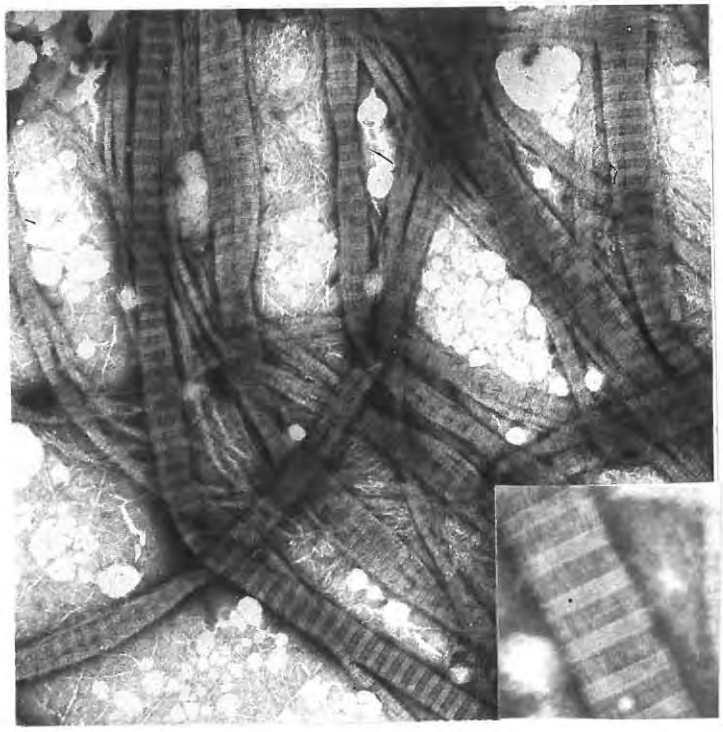
Electron micrograph of native collagen fibrils (± 64nm periodicity) precipitated by initiating buffer alone; pH 7.04 and 20°C. Magnification; x34 500; inset, x97 350.

PLATE NO. 8



Electron micrograph of native collagen fibrils (±64nm periodicity) precipitated by corticosteroid phosphate ester in initiating buffer; pH 6.95 and 20°C. Magnification; x35 100; inset; x95 760.

PLATE NO. 9



Electron micrograph of native collagen fibrils (±64nm periodicity) precipitated by corticosteroid phosphate ester in physiological saline (i.e. in the absence of initiating buffer); pH 6.2 and 20°C. Magnification; x34 720; inset, x97 350.

the axial ratio of the nuclei and their rate of growth, but decreasing the width of the fibrils. These could involve polyanion-collagen interaction through the formation of complexes via electrostatic intermolecular forces as previously mentioned.

It appears reasonable to assume that in view of the fact that E_∞ was always higher in the presence of corticosteroid, particularly so in the absence of initiating buffer, that the presence of these drugs resulted in a variation in fibril width associated with a high degree of lateral aggregation of fibrils, mediated by the presence of corticosteroids in the formation of the rigid gel matrix. These features are, however, not readily apparent from the electron micrographs (Plates 7-9). Morphological differences in the precipitated fibrils (i.e. between control, corticosteroid/initiating buffer and corticosteroid/saline) could not be distinguished during the present studies as a quantitative analysis of fibril diameter and degree of fibril association was not attempted.

Consideration of Figs. 14A and 14B (Run 6 of Table VI) illustrate the effect of protein concentration on the rate of precipitation of collagen. The data indicates that the rate of precipitation in the control runs and the runs in the presence of corticosteroid was proportional to the collagen concentration i.e. half the collagen concentration resulted in half the E_∞ value. Similar results are observed when t_c and $t_{0.5}$ are compared. The excellent agreement obtained from the application of EQUATION III-7 once again confirms the validity of the model. The apparent linear relationship between the parameters t_c, $t_{0.5}$ and E_∞ and collagen concentration suggests that the extent of precipitation at the end of each run remained constant throughout all these studies, over the range of collagen concentrations employed. Furthermore, inspection of the Tables indicate that in almost all the experiments the E_∞ values obtained in the presence of the corticosteroids within a set of runs (i.e. constant collagen concentration) were constant

irrespective of the corticosteroid used. The possibility that differences in E_∞ were due to differences in the extent of precipitation may thus be discounted in the light of these results and hence differences in E_∞ may reasonably be attributed to differences in fibril width.

Since, in spite of the presence of corticosteroids in the reaction mixtures, all the precipitation curves are described by EQUATION III-7, it is concluded that these anti-inflammatory corticosteroids do not alter the way in which the soluble collagen particles aggregate during growth. This conclusion is supported by the observation that all the precipitates consisted of fibrils with 64nm striations (Plates 7-9) characteristic of native collagen.

Although all the major types of non-covalent bonding have been suggested to account for fibril formation from collagen solutions, the relative contributions of these various forces in fibrillogenesis are not readily evaluated. However, it appears probable that electrostatic interactions play a significant role. The electrophoretic studies of Mathews (1965) resulted in evidence for the participation of electrostatic interactions between anionic charges on mucopolysaccharides and cationic charges on the protein. The cationic charged groups of collagen are longitudinally distributed in discrete clusters, as indicated by fibril staining with phosphotungstic acid (Nemetschek et al., 1955; Hodge and Schmidt, 1960). Many more of these charged centres are available for interaction with a large organic anion. Further evidence for significant involvement of electrostatic forces has been provided by Wood and Keech (1960), Gross and Kirk (1958) and Bensusan and Hoyt (1958).

Cassel (1966) and Bianchi et al. (1966) independently postulated that the required entropy gain in transferring collagen molecules from the isotropic phase to the ordered anisotropic phase of native-type fibrils was provided by a disruption of water-structure around the individual rodlike collagen molecules. Supporting the interpretation that disruption of water

structure plays an important role in native-type fibril precipitation, is the fact that the energy of activation for this process decreases with increased addition of various alcohols known to act as 'breakers' of the water structure (Bensusan, 1960). This resulting increase in precipitation rate was correlated with the decrease in the dielectric constant of the medium. It would thus appear that further light may be shed in either experiments involving the determination of the dielectric constant of the medium in the presence of various corticosteroids or the determination of the dielectric constants of the various corticosteroids in the solid state. This may establish whether the degree of charge attenuation between the protein molecules in solution is altered by the presence of any particular corticosteroid.

The role of hydrogen bonding in fibril formation has been postulated by Gross and Kirk (1958) while Bensusan and Hoyt (1958) in their proposed mechanism of collagen formation suggested that conditions which enhance hydrogen bonding favour the formation of collagen fibrils. The interpretation of Gross and Kirk (1958) based on the inhibition of fibril precipitation by urea may, however, be interpreted on the basis that urea is known to weaken hydrophobic bonds (Bruning and Holtzer, 1961; Steinberg and Scheraga, 1962; Cooper, 1970).

Thus, it is seen that in spite of the large amount of data on collagen fibrillogenesis in vitro, additional evidence is required in order to establish the exact mechanisms involved in this complex macromolecular system.

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A. INTRODUCTION

1. Quantitative binding determination

An important property of protein molecules is their ability to interact specifically with small molecules of various chemical structures to form protein-small molecule complexes (Klotz, 1953; Edsall and Wyman, 1958; Tanford, 1961; Steinhardt and Beychok, 1964; Weber, 1965; Steinhardt and Reynolds, 1969; Klotz, 1974). A great deal of quantitative data depicting the association constants and the number of binding sites for a large variety of drugs and proteins has been published (Goldstein, 1949; Meyer and Guttman, 1968a; Steinhardt and Reynolds, 1969). In spite of this information relatively little is known concerning the "active site" on drug molecules or the amino acid residues in the protein associated with the binding process.

These ligand-protein complexes, which essentially involve reversible reactions, are thus considered to be held together by non-covalent bonds. In general, three main types of bonds are considered to be involved in interactions with proteins: (1) hydrogen bonds,(2) ionic bonds,(3) hydrophobic bonds.

Numerous possibilities exist for the formation of hydrogen bonds between steroids and proteins. Steroid hydroxy groups as well as hydroxy, amino and peptide structures of the protein can donate hydrogen atoms, whilst oxo groups, peptide carbonyl and other groupings can function as hydrogen acceptors. Hydrophobic bonds are formed between large areas of the steroid molecule, which is basically a non-polar structure, and aliphatic and aromatic side chains of proteins. Ionic bonds occur when a charged molecule forms a salt bridge with an oppositely charged group on the protein. These bonds are, however, often treated together with hydrogen bonds as interactions between polar groups as it is not always possible to specifiy the electrostatic contribution of the hydrogen bond precisely, and is thus not advantageous to distinguish between hydrogen and ionic bonds (Scheraga, 1963).

In the case of charged steroid derivatives, such as phosphates or succinates,

these may interact with cationic sites in the protein. It must be assumed in these cases, however, that the interaction is not mediated by electrostatic bonding alone; there is evidence that the neutral portion of the steroid molecule participates in the binding process. Both polar and apolar interaction may be involved in the same complex, depending on the structure of the steroid and the protein (Westphal, 1971)

i) Treatment of binding data

The protein-small molecule interactions under consideration, being of a reversible nature, are regulated by the law of mass action, and the mathematical formulations developed for their characterization are thus based on this law (Edsall and Wyman, 1958; Westphal, 1971).

In the simple case the activities of the components of a binding system may be set equal to their concentrations, especially with the idealized case in which all the combining sites (n) on the protein (P) may be considered as equivalent and independent. In the special case when n = 1, the value $\bar{\nu}$ (average number of moles of small molecules, D, bound by one molecule of protein) varies only between 0 and 1. The only reaction taking place is

$$P + D = PD \qquad \text{(EQUATION IV-1)}$$

and the association constant, k, is given by

$$k = \frac{[PD]}{[P][D]} = \frac{1}{K} \qquad \text{(EQUATION IV-2)}$$

EQUATION IV-2 indicates further that the dissociation constant, K, is equal to the reciprocal of k.

Considering the total P (free and complexed) present in solution and defining α as the fraction of binding sites on P which are unoccupied,

$$\alpha = \frac{[P]}{[PD] + [P]} = \frac{K}{[D] + K} = \frac{1}{1 + k[D]} \qquad \text{(EQUATION IV-3)}$$

The average number of moles of D bound, for all the molecules of P and PD present is given by

$$\bar{\nu} = 1 - \alpha = \frac{[PD]}{[PD] + [P]} = \frac{[D]}{[D] + K} = \frac{k[D]}{1 + k[D]} \qquad \text{(EQUATION IV-4)}$$

In this instance, $\bar{\nu}$ may also be regarded as the probability that any molecule of P, chosen at random from the solution, will be found to have a molecule of D attached to it.

If the protein has n binding sites instead of one as previously assumed, and if these binding sites are mutually independent and equivalent, then the probability that D is bound to the available binding sites ranging from i=1 to n, becomes

$$\bar{\nu} = \sum_{i=1}^{n} \bar{\nu}_i = n\bar{\nu}_i \qquad \text{(EQUATION IV-5)}$$

which indicates the total average number of occupied sites per molecule of protein. Then the probability that any site, chosen at random from any molecule present in the solution is occupied by D becomes

$$1 - \alpha = \frac{\bar{\nu}}{n} = \frac{k[Df]}{1 + k[Df]} \qquad \text{(EQUATION IV-6)}$$

or,

$$\bar{\nu} = \frac{nk[Df]}{1 + k[Df]} \qquad \text{(EQUATION IV-7)}$$

or,

$$\frac{\bar{\nu}}{n - \bar{\nu}} = k[Df] \qquad \text{(EQUATION IV-8)}$$

where $[Df]$ is the concentration of unbound or free ligand.

Protein molecules, in general, are however, likely to contain more than a single class of binding sites. The situation where several classes of binding sites occur may be dealt with by assuming m different classes, in which each are binding groups mutually independent and equivalent. The first class having n_1 equivalent and independent groups, each with association constant k_1, the second class n_2 such groups, each with association constant k_2, and so forth.

Starting with the highest binding affinity for the first class of sites, the k values may be written in the following order:

$$k_1 > k_2 > k_3 \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad k_{m-1} > k_m$$

The total number of binding sites in the protein is thus given by,

$$n = n_1 + n_2 + n_3 + \ldots\ldots\ldots\ldots\ldots\ldots \quad n_m = \Sigma n_i \qquad \text{(EQUATION IV-9)}$$

and the average number of sites occupied by D molecules becomes

$$\bar{\nu} = \sum_{i=1}^{m} \frac{n_i k_i [Df]}{1 + k_i [Df]} \qquad \text{(EQUATION IV-10)}$$

EQUATION IV-10 does not account for interactions between sites.

The most common method of treating binding data is by the Scatchard plot (Scatchard, 1949). This is based on a modification of EQUATION IV-8, obtained by multiplying both sides of this equation by $(n - \bar{\nu})/[Df]$ to yield

$$\frac{\bar{\nu}}{[Df]} = k(n - \bar{\nu}) \qquad \text{(EQUATION IV-11)}$$

This equation predicts that a plot of $\bar{\nu}/[Df]$ versus $\bar{\nu}$ should yield a straight line when only one class of binding sites is involved. Furthermore, the ordinate intercept of such a plot will be nk, the abscissa intercept will be n, and the slope will be -k. However, when two classes of sites are involved (i.e. i = 2), the following equations will apply:

$$\frac{\bar{\nu}}{[Df]} = (\Sigma nk + k_1 k_2 [Df] \Sigma n) - \bar{\nu}(\Sigma k + k_1 k_2 [Df]) \qquad \text{(EQUATION IV-12)}$$

or,

$$\frac{\bar{\nu}}{[Df]} = k_1(n_1 - \bar{\nu}) + k_2(n_2 - \bar{\nu}) + k_1 k_2 (n_1 + n_2 - \bar{\nu})[Df] \qquad \text{(EQUATION IV-12.1)}$$

These equations predict a non-linear relationship between $\bar{\nu}/[Df]$ and $\bar{\nu}$. The intercept on the abscissa, when $\bar{\nu}/[Df] \rightarrow 0$, which occurs when $[Df] \rightarrow \infty$ as implied by EQUATION IV-10, gives Σn_i whereas the ordinate intercept, when $\bar{\nu} \rightarrow 0$, which occurs when $[Df] \rightarrow 0$, gives $\Sigma n_i k_i$.

Although graphical extrapolation of these non-linear binding curves have been used by numerous workers to obtain estimates of n's and k's, Klotz (1973; 1974) in his recent publications has re-emphasized the hazards involved using this approach.

The most precise method of establishing binding parameters (when $i > 1$) is to fit the experimentally obtained data using non-linear least-squares computer curve-fitting procedures.

2. Dynamic dialysis

Numerous experimental techniques have been described for the determination and characterization of the binding of small molecules to proteins. Recently, a dynamic dialysis technique was introduced by Meyer and Guttman (1968b). This is based on the determination of the rate of dialysis of a small molecule from a compartment which is impermeable to protein but permeable to the small molecule. The method differs from other non-equilibrium dialysis approaches described by Stein (1965), Agren and Elofsson (1967), Farrell et al. (1971) and Robertson and Madsen (1974) in that the time course of disappearance of small molecule from a protein compartment is followed for extended periods of time.

In the dynamic dialysis system of Meyer and Guttman (1968b; 1970), the relative concentrations of bound and unbound small molecule change continuously and, as a result, data which are obtained from a single kinetic experiment permit characterization of binding behaviour over a wide range of small molecule concentrations. The method is based on the fact that non-diffusable protein-small molecule complexes are reversibly formed in the protein compartment and that the rate of loss of small molecule from the compartment is directly proportional to the concentration of unbound small molecule, provided that care is taken to insure that sink conditions are maintained for the diffusing species, i.e. that back diffusion into the protein compartment is insignificant.

In the absence of protein, the permeation of small molecule through the

dialysis membrane obeys first order diffusion kinetics provided that sink conditions are maintained (Meyer and Guttman, 1968b).

The rate of loss of small molecule $[-d(Dt)/dT]$ from within the protein compartment, is related to the total small molecule concentration (Dt) remaining, by the following equation:

$$\frac{-d(Dt)}{dT} = K(Df) \qquad \text{(EQUATION IV-13)}$$

where K is the first-order rate constant which characterizes the diffusion process and which incorporates the area and thickness of the membrane, and Df is the concentration of unbound or free small molecule within the protein compartment. Hence, a semi-log plot of Dt versus time in the absence of protein should yield a straight line, provided membrane binding is negligible. In the presence of protein, however, the semi-log plot will exhibit marked curvature when measurable binding of the small molecule to the protein occurs. This curvature implies that as the concentration of small molecule in the protein compartment decreases, the fraction of small molecule which is bound increases. The concentration of unbound small molecule (Df) in the protein compartment at any Dt value can then be calculated from EQUATION IV-13 with a knowledge of K and the instantaneous rate $[-d(Dt)/dT]$ at that particular Dt. The value of K may be obtained from the slope of the semi-log plot of Dt versus time in the absence of protein. The instantaneous rate at a value of Dt in the presence of protein can be obtained from a plot of Dt versus time. The calculated values of Dt and Df may then be used to determine the concentration of bound small molecule (Db) from the relationship:

$$Dt = Db + Df \qquad \text{(EQUATION IV-14)}$$

The binding data obtained in this manner may then be used to construct a Scatchard plot from $\bar{\nu}/[Df]$ versus $\bar{\nu}$ which can be mathematically analyzed in terms of the fundamental binding parameters, n and k.

The studies by Meyer and Guttman (1968b; 1970) confirmed that the relative

ease and rapidity of their dynamic dialysis procedure, the minimal amount of sample preparation required, the convenient means of temperature control, and the economical utilization of protein are definite advantages of this approach. In addition, the principal disadvantages of the widely used equilibrium dialysis and ultrafiltration methods are overcome, namely, the time required for the system to attain equilibrium resulting in the risk of degradation products interfering with the results in the former approach and the approximations necessary to assess the changing protein concentration during the course of filtration when the latter method is employed.

Recent reports describing the use of this dynamic dialysis technique include the investigation of the binding of p-substituted acetanilides to bovine serum albumin (Dearden and Tomlinson, 1970), competitive interaction of preservative mixtures with cetomacrogol (Brown and Crooks, 1973), the binding of polymethylene bisquaternary ions to chondroitin (Asghar and Roth, 1971), the binding of sulfonylureas to serum albumin (Crooks and Brown, 1973 and 1974) and the binding of ascorbic acid and fatty acid ascorbyl esters to bovine serum albumin (Tukamoto et al., 1974).

B. EXPERIMENTAL

1. Apparatus

The apparatus for dynamic dialysis was based on that described by Meyer and Guttman (1968b). Two water-jacketed dialysis cells were constructed and connected in series to a Colora Ultra-Thermostat NB-34980 water-bath in conjunction with a Colora-Tauchkühler Cold-Finger refrigeration system. A 100ml jacketed volumetric pipette (Grade A) was also placed in series between the two cells and water at a constant temperature circulated throughout the entire system (Plate 10). The dialysis cells were constructed from 250ml jacketed beakers (internal diameter = 6.5cm, internal height = 9cm). A large rubber stopper was bored out to contain three holes, the centre hole accommodating a ground-glass Quick Fit socket (B19/26) tapered at one end to receive

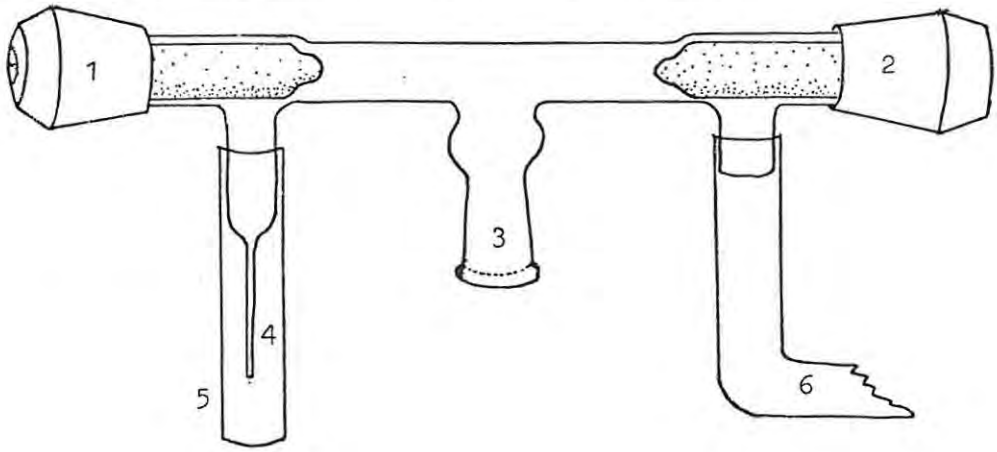
PLATE NO. 10

DYNAMIC DIALYSIS SYSTEM

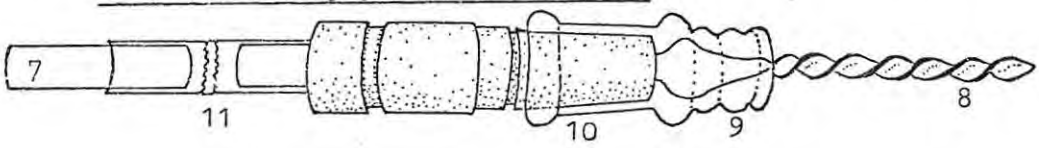
1. Water-jacketed dialysis cell.
2. Rubber stopper.
3. Teflon stirring gland.
4. Dialysis sac.
5. Water-jacketed volumetric pipette (100ml).
6. Flexible shaft (plastic tubing).
7. Filling tube.
8. Magnetic stirring motor (external stirring).
9. Stirring motor (internal stirring).
10. Rotoflo tap system.
11. Cold-finger.
12. Refrigeration unit.

Fig. 24

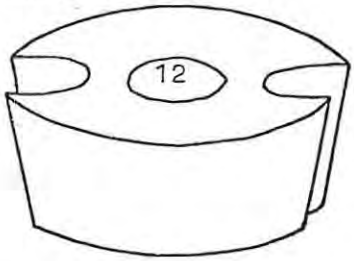
Rotoflo tap system used with 100ml pipette



Teflon stirring gland with stirrer



Rubber stopper for dialysis cell



1. Teflon tap for levelling up to the mark.
2. Teflon tap for filling and emptying.
3. Ground glass socket - fits onto compatible ground glass tube on top of pipette.
4. Fine capillary tube - allows slow air-intake for levelling.
5. Protective plastic tubing for capillary.
6. Plastic tubing for sucking up liquid into pipette.
7. Glass rod - fits into chuck of stirring motor.
8. Twisted glass stirring rod.
9. Dialysis sac attachment site.
10. Ground glass socket.
11. Flexible shaft (plastic tubing).
12. Hole to accommodate ground glass socket with stirring gland.

(Diagrams - courtesy E.M. Haig)

the dialysis sac. The socket was fitted with a Quick Fit teflon stirring gland containing a twisted glass rod. This was used to stir the contents within the dialysis sac and was connected to a variable speed stirring motor, I.K.A. - Type R.M. 18Nr. (Plate 10 and Fig. 24). The shaft connecting the short glass stirrer comprised a length of plastic tubing attached to the top end of the twisted glass rod in order to impart flexibility and thus facilitate the alignment of the dialysis cell with the stirrer motor. The cells were clamped onto I.K.A.-Combimag-RCH magnetic stirrers in order to stir the solution external to the sac. With the cells clamped into position, the central glass socket together with stirring gland, twisted glass rod and attached bag was fitted onto the beaker. The volumetric jacketed pipette was used to re-introduce fresh buffer after sampling. In order to facilitate filling up to the mark and subsequent emptying, two Quick Fit Rotaflo taps were joined together and fitted to the top of the pipette as shown in Fig. 24.

Visking seamless cellulose tubing (Union Carbide, 2.50cm flat width, with average pore size of 24Å) was treated by heating for 1 hour at 90°C in distilled water (Dearden and Tomlinson, 1970; Tukamoto et al.,1974), followed by repeated rinsing with distilled water prior to use in order to ensure the removal of interfering impurities. Excess treated tubing was stored in distilled water in a refrigerator at 0-2°C for further use. The dialysis sac was prepared by tying one end of a portion of the tubing with a double knot. The desired length of dialysis sac was then cut, measuring from the top of the knot (8cm length). The sac was blotted with chromatography paper (Whatman No. 1) to remove excess moisture and was attached with a rubber band to the tapered end of the glass socket in the centre of the rubber stopper. When the stopper was positioned onto the dialysis cell, the join between the sac and the glass holder was well above the level of the liquids.

All pH measurements were performed on a Beckman Century SS pH meter using a micro-combination electrode while the corticosteroids were determined

spectrophotometrically with a Beckman Acta MVI spectrophotometer equipped with a Sipper system and digital printer (Model 3115) unit.

2. Methods

i) Preliminary investigation - Establishment of solution environment

In order to establish the solution environment necessary to prevent collagen precipitation during the course of dialysis, three separate solutions of collagen (P_5) (± 2mg/ml) were prepared to contain 0.05M phosphate together with 0.08M NaCl, 0.15M NaCl and no NaCl respectively. Seven solutions having pH's of 3,4,5,6,7,8 and 9 respectively were then prepared from each of the above solutions by briefly immersing NaOH pellets into the solutions on a magnetic stirrer to yield the desired pH's. The pH was monitored during these titrations using a micro-combination pH electrode. This procedure was adopted in order to minimize dilution during pH adjustment (Russell, 1974). Five millilitre aliquots of each of the resulting 21 solutions were pipetted into small test tubes and treated with betamethasone disodium phosphate to give solutions having a final corticosteroid concentration of 5mg/ml and collagen concentration of ± 1mg/ml. After inverting the test tubes several times in order to mix, the samples were incubated at 20°C in a constant temperature water bath and the solutions were checked periodically for signs of turbidity.

In the series of samples containing no NaCl, only the solution at pH 3 and 4 did not show any signs of precipitation up to 9 hours. The rest of the solutions in this series developed turbidity within 1 hour. In the second series of solutions which contained 0.08M NaCl, only solutions having pH of 5 and 6 developed turbidity within 1 hour whereas the remaining 5 solutions appeared clear after 9 hours. On examination of the third series of solutions which contained 0.15M NaCl, only the solution at pH 5 developed turbidity within 1 hour. The remaining 6 solutions did not

develop turbidity after a period of 9 hours.

An additional series of 4 solutions were prepared to contain 0.05M phosphate and 0.15M NaCl and the pH of each solution adjusted as before to values of 4.5, 5.1, 5.65 and 6.10 respectively. These solutions were treated with corticosteroid and incubated as previously described.

The solutions at pH 4.5 and 5.1 developed turbidity after 4 hours, the solution at pH 5.65 after 9 hours whereas the solution at pH 6.1 remained clear during the same period. As a result of these studies, compatible pH ranges (3.0 and 7.0) and the appropriate NaCl concentration (0.15M) were chosen to ensure solution homogeneity for all future binding studies. Furthermore, these studies indicated that the iso-electric point of the collagen under these conditions was between pH 4.0 and 5.65 to a rough approximation and that the ionic strength of the solution was sufficiently high to ensure that no precipitation would occur at the chosen pH values. This is in agreement with the observations of Wood and Keech (1960); Bensusan and Hoyt (1958), and Gross and Kirk (1958) who found that an increase in ionic strength of collagen solutions has a retarding effect on fibril precipitation at pH 7.0 and 8.0. Wood and Keech (1960) also found that when the ionic strength was increased at pH 6.0 there appeared to be an increase in the rate of precipitation due to a salting-out effect. In the present work, however, the solutions at pH 3.0 remained clear implying that the ionic strength was still below the value necessary to cause salting-out.

The dissolution rate and solubility characteristics of collagen required that a relatively low concentration of protein solution be used. Hence all binding studies were conducted to give a final collagen concentration $\not>$ 1mg/ml. In addition, any increase in collagen concentration is accompanied by an increase in viscosity and the possibility of aggregation with subsequent precipitation is also enhanced (Wood and Keech, 1960).

The use of 0.05M phosphate buffer together with the relatively high neutral salt and low protein concentration reduces the possibility of interference from the Donnan effect (Donnan, 1924) during the dialyses studies (Rosenberg and Klotz, 1960).

As a result of the use of NaCl, necessary to keep the protein in solution, its presence should be borne in mind with regard to interaction with collagen. Similarly, it is pertinent to include the presence of buffer ions as probable interacting species. During studies of the interaction between various steroids and α_1-Acid Glyco-protein (AAG), Westphal et al. (1966) showed that the apparent association constant rises in proportion to the salt concentration. Klotz and Urquhart (1949) described the effects of various anions including sodium chloride and phosphate ions on the binding of methyl orange and azosulphathiazole to albumin. The osmotic experiments of Scatchard et al. (1946) produced evidence of the binding of chloride by bovine albumin. Recently Kragh-Hansen and Moller (1973a) have described the effect of pH and inorganic ions on the combination of phenol red with proteins. A consecutive report by these same authors (Kragh-Hansen and Moller, 1973b) described the role of electrostatic forces for binding of phenol red by human serum albumin. Weinstock et al. (1967) studied the ion binding characteristics of reconstituted collagen. They found that phosphate and chloride ions were indeed bound by collagen. Physico-chemical studies on other proteins have shown that distinct effects on the dissociation of side-chain groups can be caused by local environment (Alberty, 1953). Collagen exhibits marked changes in its isoelectric and isoionic points in the presence of different salt solutions (Bensusan and Hoyt, 1958; Veis et al., 1958, and Davison and Drake, 1966). This also indicates that variable binding of ions by collagen may occur. A comprehensive account of the effects of neutral salts on the structure and stability of macromolecules in solutions has been reported by von Hippel and Schleich (1969).

In view of the fact that all the present experiments were conducted under identical conditions of buffer ions and NaCl concentration, the contribution of these various ions remains constant and hence does not affect any foregoing comparisons of the binding behaviour of the various corticosteroids or the pH effects.

ii) Evaluation of experimental variables

Meyer and Guttman (1970), have described the influence of experimental variables such as pH, temperature, buffer concentration, stirring rate, viscosity, size of dialysis sac, volumes of liquid within the system and membrane binding of the small molecule on the rate of dialysis. Generally, the same standardized experimental conditions were applied to the present studies. It was felt, however, that in view of the fact that a totally different protein was being used in the present studies as well as different small molecules, the influence of internal stirring rates, the possibility of dilution of the dialysis sac contents due to protein osmotic effect, and the binding of corticosteroids by the dialysis membrane should be investigated.

All binding experiments were conducted at constant pH, temperature, liquid volumes both inside and external to the dialysis sac, sac size, stirring rates, NaCl and phosphate concentrations.

Two hundred millilitres of phosphate buffer (0.05M) containing NaCl (0.15M) at either pH 3.00 or 7.00 (pH adjusted with solid NaOH to the required value) was pipetted into each of two jacketed dialysis cells. All dynamic dialyses were performed in duplicate. Periodically, during the course of dialysis, 100ml of the external solution was removed through one hole of the stopper. The sample was immediately replaced with 100ml of fresh buffer from the jacketed volumetric pipette which was circulated with water at the same temperature as that of the dialysis cells. From preliminary experiments it was observed that sink conditions were approximated if the concentration of unbound small molecule in the external solution was not

allowed to exceed 5% of that in the internal solution. This observation was similar to that of Brown and Crooks (1973) whereas Dearden and Tomlinson (1970) used a sampling frequency which did not allow the concentration of drug in the external solution to rise above 1% of that in the dialysis sac. Meyer and Guttman (1970), on the other hand found for their systems that sink conditions were approximated provided the concentration of unbound small molecule in the external solution was not allowed to exceed one-tenth of that in the dialysis sac. During the present studies a sampling frequency of 100ml/15 minutes with replacement for the first two hours and every half hour thereafter, until completion of the experiment, was required.

The concentration of small molecule remaining in the dialysis sac, at the end of each sampling interval, was calculated from a knowledge of the concentration and volume of the sample removed from the external solution, the total amount of drug which had been previously removed by sampling, and the initial amount and concentration of drug present inside the dialysis sac. These calculations were facilitated by the use of a Hewlett-Packard Model 9100B desk-top programmable calculator employing a program written specifically for this purpose.

The initial concentration of small molecule pipetted into the dialysis sac was determined by assay of the stock solution used to fill the sac and then checked by assaying the amount of small molecule remaining inside the sac at the termination of a run together with a knowledge of the amount of small molecule which had dialyzed during the course of a run. In general, both procedures agreed to within one to two percent.

a) Establishment of internal stirring rate required

Meyer and Guttman (1970) showed that it was necessary to stir both the contents within the dialysis sac as well as the solution external to the sac for their particular systems. In order to confirm that reproducibility was not affected by poor choice of internal stirring rates for the corticosteroid

systems, two studies were undertaken. The external solution was stirred at a constant rate with the aid of a magnetic stirrer and magnetic teflon coated stir bar whilst the stirring rate of the internal solution was maintained at either 100 r.p.m. or 500 r.p.m.

A dialysis sac (8cm length) was prepared as previously described. Seven millilitres of a solution of prednisolone disodium phosphate (4.015mg/ml) in 0.05M phosphate containing 0.15M NaCl at pH 7.00 was pipetted into the sac. Two hundred millilitres of the same buffer solution was used as the external solution and the temperature of the system maintained at $20^{\circ} \pm 0.1^{\circ}C$. At the termination of each run the dialysis sac was thoroughly rinsed with distilled water and re-used for the following run. In the first investigation the contents of the dialysis sac was stirred at 100 r.p.m. whilst in the second run, an internal stirring rate of 500 r.p.m. was used. Fig.25 indicates that there was no apparent difference between these two runs. Furthermore, this study indicates the excellent run to run reproducibility when the same sac is used.

As a result of these observations, subsequent dynamic dialysis studies were conducted using a relatively constant stirring rate for the external solution whilst the contents of the dialysis sac were stirred at approximately 150 r.p.m.

b) Osmotic effect of collagen solutions

An investigation was undertaken in order to ascertain whether the internal contents of the dialysis sac were diluted during the dialysis.

A stock solution of collagen (P_5) (1.074mg/ml) was prepared in 0.05M phosphate buffer containing 0.15M NaCl. The concentration of this solution was obtained from the optical rotation at $15^{\circ}C$ using a wavelength of 365nm. The solution was then adjusted to pH 7.00 as previously described and the optical rotation again checked as above. The values obtained agreed within 2%.

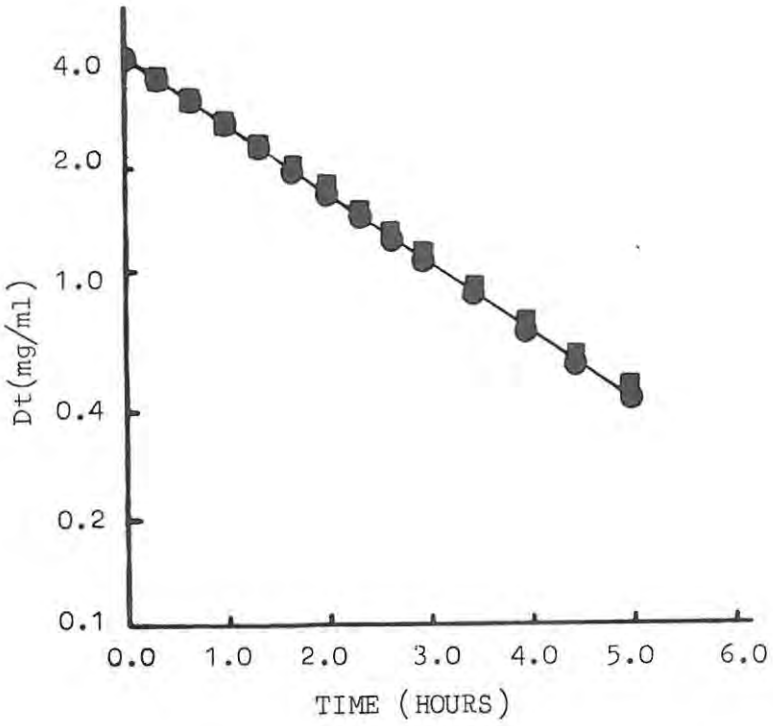


Fig. 25. The influence of internal stirring speed on the rate of dialysis of prednisolone disodium phosphate in 0.05M phosphate buffer containing 0.15M NaCl at pH 7.0 and 20°C. ● , internal stirring at 500 r.p.m.; ■ , internal stirring at 100 r.p.m. The same sac was used for each run.

Two dialysis sacs were prepared as before and into each was pipetted 7.0ml of collagen solution. The first sac was suspended as previously described in an empty dialysis cell. The second sac was suspended in an identical dialysis cell containing 200ml of 0.05M phosphate buffer plus 0.15M NaCl and both the internal and external solutions were stirred as previously described. The temperature of the cells was maintained at 20°C whilst the remainder of the stock collagen solution was placed in the circulating water-bath. At the end of 6 hours, samples were withdrawn from each of the two sacs as well as from the stock solution and the optical rotation of each solution measured as before. The results of this study indicated that no change in collagen concentration had occurred.

c) Binding of corticosteroids by dialysis membranes

Curvature in the semi-log plot of Dt versus time in the absence of protein is indicative of membrane binding (Meyer and Guttman, 1970). During the present investigations curvature of the semi-log plot of Dt versus time for all the small molecules studied was never observed during the control runs.

iii) Application of dynamic dialysis to corticosteroid-collagen systems

All runs were performed in duplicate using the specially constructed apparatus and methods as previously described. Each binding study was preceded by control runs in which the internal solutions contained only the small molecule and the appropriate buffer. This was effected by pipetting exactly 7.0ml of the control solution into an 8cm dialysis sac. The control solution was prepared to contain the particular small molecule in 0.05M phosphate buffer plus 0.15M NaCl at the appropriate pH. The system was maintained at 20° ±0.1°C and each control run was monitored for 2 to 3 hours. The samples were analyzed spectrophotometrically at the appropriate λ_{max} and each concentration was the average of three readings facilitated by the use of the Sipper System connected to the Beckman Acta MVI Spectrophotometer. The results of the control runs were used to calculate

the apparent first-order rate constant for each sac. The sac was then rinsed thoroughly with distilled water, blotted to remove excess moisture and re-filled with exactly 7.0ml of small molecule-protein solution prepared in 0.05M phosphate buffer containing 0.15M NaCl at the same pH as the control. The sac was then suspended into the dialysis cell containing 200ml of fresh buffer and the dialysis of the small molecule from the protein compartment monitored spectrophotometrically for six hours. At the termination of each run, the external and internal solutions were checked for pH changes. No changes were found.

iv) Data treatment

The slopes for all the control runs were obtained using a linear regression program written for the Hewlett-Packard Model 9100B desk-top programmable calculator. The data from plots of Dt versus time from the semi-log plot in the presence of protein was fitted to an empirical tri-exponential equation,

$$Dt = Ae^{-at} + Be^{-bt} + Ce^{-ct} \qquad \text{(EQUATION IV-15)}$$

with the aid of an ICL 1901A computer (Guttman, 1970; Meyer, 1972). This involved the use of a non-linear regression program based on an algorithm for least squares determinations of non-linear parameters (Marquardt, 1963). A listing of this program was gratefully supplied by Dr. M.C. Meyer (1972). The program received was written in Fortran II for use with an IBM 1620 computer. Hence, this program was modified to ICL Fortran IV in order to make it compatible with the ICL 1901A computer facility. The same program was then used to obtain the values of the binding parameters, n and k. This involved modifying the particular subroutine in which the function being fitted is defined e.g. for estimates of n's and k's, EQUATION IV-10 was inserted in place of EQUATION IV-15. As all the plots of $\bar{\nu}/[Df]$ versus $\bar{\nu}$ exhibited curvature, the equation for 2 classes of sites was employed throughout. An additional Fortran IV program was written for the ICL 1901A computer

in order to firstly compute the instantaneous rates at various values of Dt from the differential of the tri-exponential equation,

$$\frac{-d(Dt)}{dt} = Aae^{-at} + Bbe^{-bt} + Cce^{-ct} \qquad \text{(EQUATION IV-16)}$$

and then using these values together with a knowledge of -K, protein concentration and the corresponding molecular weights of the small molecules and protein, values for $\bar{\nu}/[Df]$ and $\bar{\nu}$ were obtained. The graphs of $\bar{\nu}/[Df]$ versus $\bar{\nu}$ were then plotted with the aid of a Calcomp 563 graph plotter connected to the ICL 1901A computer. The binding parameters obtained by the computer fitting of the data were used to generate theoretical curves which were then superimposed as solid lines on the Scatchard plots of the experimental data.

The use of the aforementioned non-linear regression computer program to fit the escape data to the tri-exponential function (EQUATION IV-15) and subsequently for estimation of the binding parameters (EQUATION IV-10) was associated with a number of difficulties due to the inherent mathematical instability of the curve fitting approach (Terry, 1972). Further difficulties, associated with the solution of tri-exponential equations have been described by Lanczos (1967). It was found that the initial parameter estimates had a significant influence on the final results, hence a good choice of these initial estimates was extremely important in obtaining an optimum fit to the appropriate curves. Details of the strategy adopted when using this program are described in Chapter V.

C. RESULTS AND DISCUSSION

1. Calibration of the dynamic dialysis technique

In order to establish the functionality of the apparatus and reproducibility of the dynamic dialysis technique, an investigation of the

binding of phenol red to BSA was undertaken. The conditions for this system were similar to those reported by Meyer and Guttman (1970).

Control solutions of phenol red were prepared (re-crystallized as previously described) in pH 7.3, 0.04M phosphate buffer. The concentration of BSA used in the binding runs was 0.55% determined spectrophotometrically at λ_{280nm} using a value of $E^{1\%}_{1cm} = 6.6$ (Clark et al., 1962; Chignell, 1969; Reynolds et al., 1970). Seven millilitres of the control solution was placed into each of two 8cm dialysis sacs and the dialysis followed for 3 hours. The sacs were then thoroughly rinsed with distilled water, blotted to remove excess moisture and re-filled with exactly 7.0ml of phenol red-BSA solution prepared in the same buffer and at the same pH as the control. The sacs were then re-suspended in their respective dialysis cells which contained 200ml of fresh buffer and the dialysis followed for 7.5 hours. The temperature of this system was maintained at $25^{o} \pm 0.1^{o}C$.

Values for $\bar{\nu}/[Df]$ and $\bar{\nu}$ were calculated as previously described, assuming a molecular weight of 69 000 for BSA (Dearden and Tomlinson, 1970; Kostenbauder et al., 1971) and are illustrated by the Scatchard plot shown in Fig.26. These values were fitted to EQUATION IV-10 assuming that two classes of binding sites are involved as implied by the resulting curvature of the Scatchard plot. The n and k values thus obtained were utilized to generate theoretical values for $\bar{\nu}/[Df]$ and $\bar{\nu}$ which were superimposed in the form of a solid curve, onto the experimental data as shown in Fig. 26. The computed values for n and k were found to be in reasonable agreement with those values obtained by Meyer and Guttman (1968b) and Rodkey (1961) for the binding of phenol red to BSA. Table XI lists the various constants for this system together with those presently determined.

The results of these studies confirm the validity of the dynamic dialysis technique for the determination of small molecule-protein interactions under the present prevailing conditions.

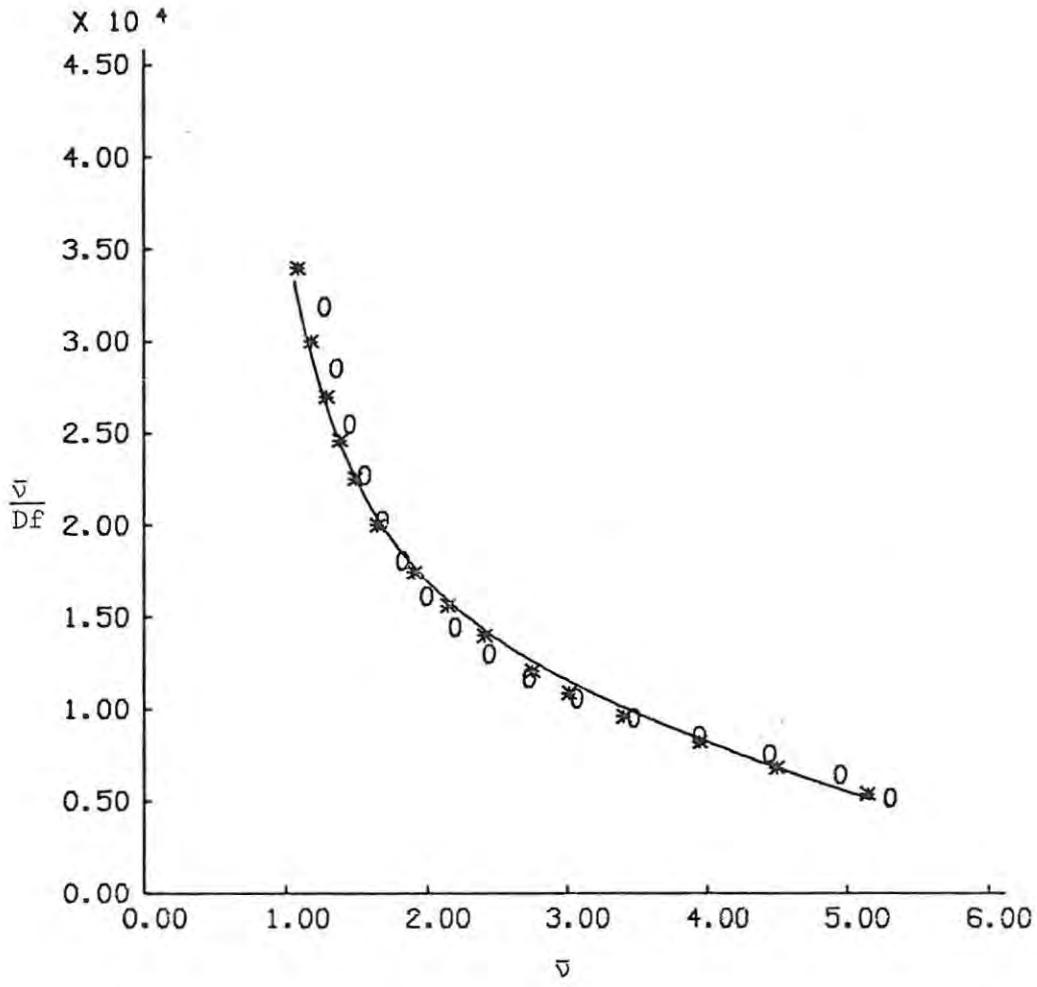


Fig. 26. Scatchard plot of the binding of phenol red to BSA at pH 7.3 at 25°C. The solid curve was computed from the binding parameters, $n_1 = 1$; $k_1 = 1.5 \times 10^5$; $n_2 = 6$; $k_2 = 1.96 \times 10^3$. * and O represent the experimental data obtained from duplicate determinations.

TABLE XI

Summary of binding parameters for phenol red-BSA systems at 25°C (pH 7.3)

n_1	n_2	k_1(litre/mol)	k_2(litre/mol)	Source
1	6	1.74×10^5	1.97×10^3	Meyer and Guttman (1968b)
1	6	1.1×10^5	1.2×10^3	Rodkey (1961)
$0.8 \approx 1$	$6.4 \approx 6$	1.5×10^5	1.96×10^3	Present

2. Binding of corticosteroids to collagen

Duplicate determinations using two separate dialysis cells were carried out as previously described. The binding behaviour of each corticosteroid was studied at two pH values, these being 3.0 and 7.0 respectively. Although every care was taken to ensure that the two sacs were of the same dimensions, small differences in membrane surface area inevitably occurred due to the tying off of one end and the position of attachment of the dialysis sacs to the central glass holders. In spite of this possibility, the variation in the rate constant (K) between duplicate controls was usually small (<5%) in most instances. In addition, the sacs were never removed from their respective holders after each control run and all rinsing was effected with the sacs mounted in their original positions. New sacs were used, however, for each pH study and when a different corticosteroid was employed.

As a result of the close agreement between the K values of duplicate runs and in order to reduce the number of lengthy curve-fitting computations, it was found convenient to normalize a run in the presence of collagen in terms of its duplicate by multiplying the Dt values from sac 2 (or sac 1) by the ratio $K_{sac\ 2}/K_{sac\ 1}$ (or $K_{sac\ 1}/K_{sac\ 2}$). These normalized Dt values were then added to those obtained from the untreated duplicate and the average Dt values plotted versus time on semi-log paper. This data treatment was facilitated by the fact that the sampling frequencies were kept identical during the experiments. The normalized and averaged values obtained in this way were seen to be almost

identical to the values of either individual run and hence only the average data values are shown in all subsequent plots.

Each normalized and averaged "escape curve" was then fitted to the empirical tri-exponential function (EQUATION IV-15) and values for the Scatchard plot computed as previously described assuming a molecular weight of 300 000 for collagen (Boedtker and Doty, 1955 and 1956; Hannig and Engel, 1961; Lewis and Piez, 1964). Finally, the n and k values were computed from which the theoretical data was generated and superimposed on the Scatchard plot as a solid curve.

i) Binding of betamethasone disodium phosphate

A stock control solution of corticosteroid was prepared in 0.05M phosphate containing 0.15M NaCl at pH 3.00, and 7.0ml of this solution was pipetted into each of two dialysis sacs. The dialyses were followed for 3 hours. The concentration of collagen used in the binding runs was 0.88mg/ml and the temperature of the system was maintained at $20^{\circ} \pm 0.1^{\circ}C$.

In order to illustrate the reproducibility of the system, the Scatchard plot shown in Fig.27 depicts the data obtained from a computer fit of the normalized and averaged "escape curve". The solid curve was generated from average values of the n's and k's obtained from individual runs processed independently as previously described. This plot depicts the binding behaviour of betamethasone disodium phosphate to collagen under the aforementioned conditions. Averages of the binding constants for this system together with the binding parameters obtained from each of the replicate "escape curves" are shown in Table XII. The free energy of binding (ΔG) for the first class of sites was obtained from the relationship,

$$\Delta G = -RT\ln k_1 \qquad \text{(EQUATION IV-17)}$$

where R = Universal gas constant, T = absolute temperature and k_1 is the association constant for the first class of binding sites.

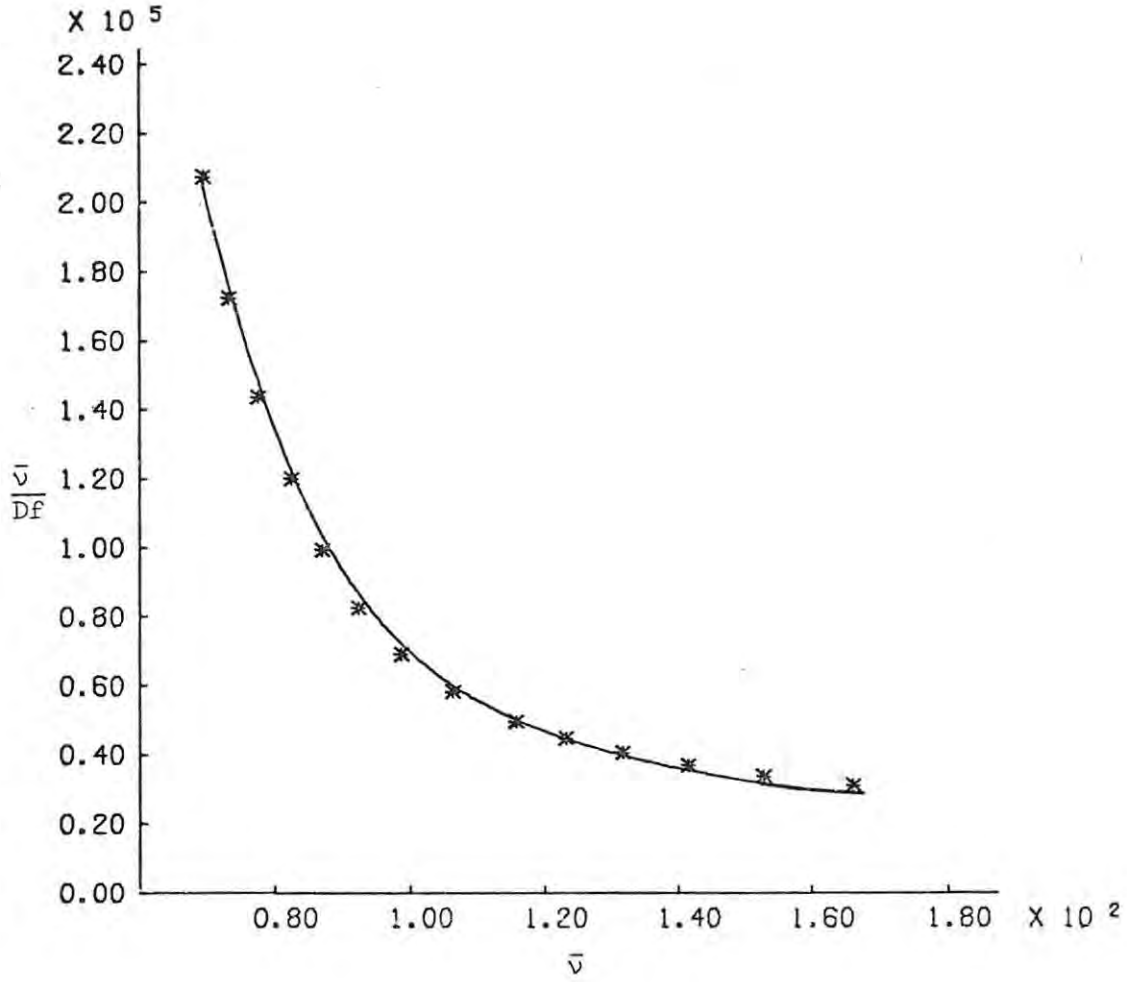


Fig. 27. Scatchard plot of the binding of betamethasone disodium phosphate to collagen at pH 3.0 and 20°C. The solid curve was computed from the average values of the n's and k's from two duplicate runs (Table XII). *, normalized and averaged experimental data.

TABLE XII

Summary of binding constants for betamethasone disodium phosphate - collagen interaction (pH 3.00)

Sac No.	Cortico-steroid	n_1	k_1 (litre/mol)	n_2	k_2 (litre/mol)	ΔG (kJ/mol)
1	BDP	79.39	1.16×10^4	3.77×10^2	5.08×10^1	-22.82
2	BDP	77.71	1.23×10^4	4.04×10^2	4.59×10^1	-22.97
Average	BDP	78.55	1.19×10^4	3.90×10^2	4.84×10^1	-22.90

Abbreviations:- BDP: betamethasone disodium phosphate

The value for ΔG was found to be -22.9kJ/mol, the negative sign implying spontaneous interaction (Glasstone and Lewis, 1964).

The binding of betamethasone disodium phosphate to collagen at pH 7.00 was also investigated. All experimental conditions were identical to those previously described. The "escape curve" for betamethasone disodium phosphate under these conditions in the presence of collagen and in its absence is shown in Fig. 28A. From a non-linear least squares analysis of the experimental $\bar{\nu}/[Df]$ versus $\bar{\nu}$ values, it was determined that the interaction, could be appropriately characterized by the binding parameters $n_1 = 48.47$, $n_2 = 31.9$, $k_1 = 1.96 \times 10^4$ litre/mol, and $k_2 = 4.19 \times 10^2$ litre/mol. ΔG for the first class of binding sites was found to be -24.1kJ/mol. The Scatchard plot for this system is shown in Fig. 28B. The solid curve was generated from the appropriate binding parameters.

Meyer and Guttman (1970) in their investigation of the binding of caffeine to BSA pointed out that the small difference in kinetic behaviour in the absence and presence of protein was a reflection of the weak nature of the interaction. Their interpretation may not, however, apply when a protein other than BSA is studied. Most of the "escape curves" obtained during the present corticosteroid-

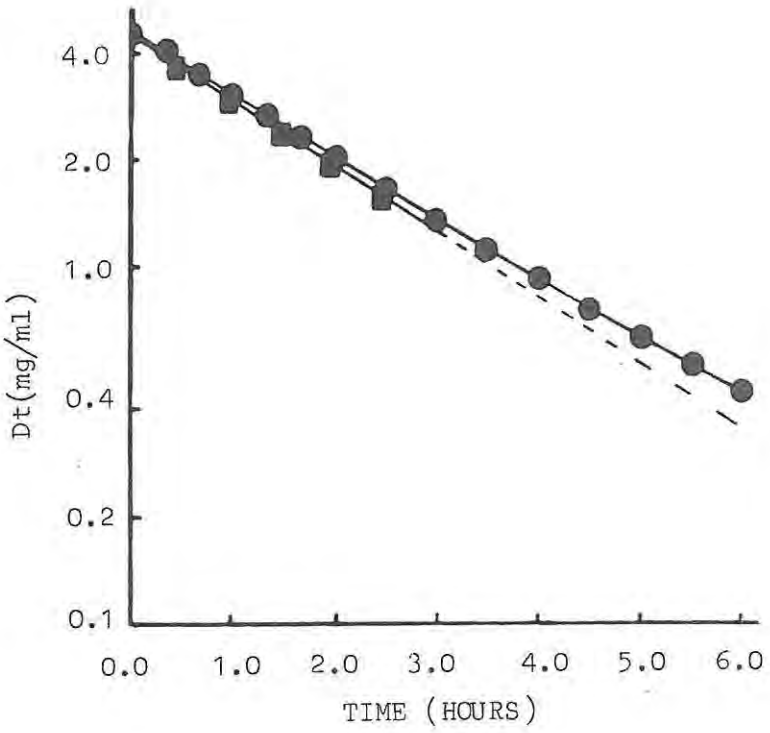


Fig. 28A. The loss of betamethasone disodium phosphate from inside a dialysis sac at pH 7.0 and 20°C. ■ , control; ●, in the presence of 0.084% collagen.

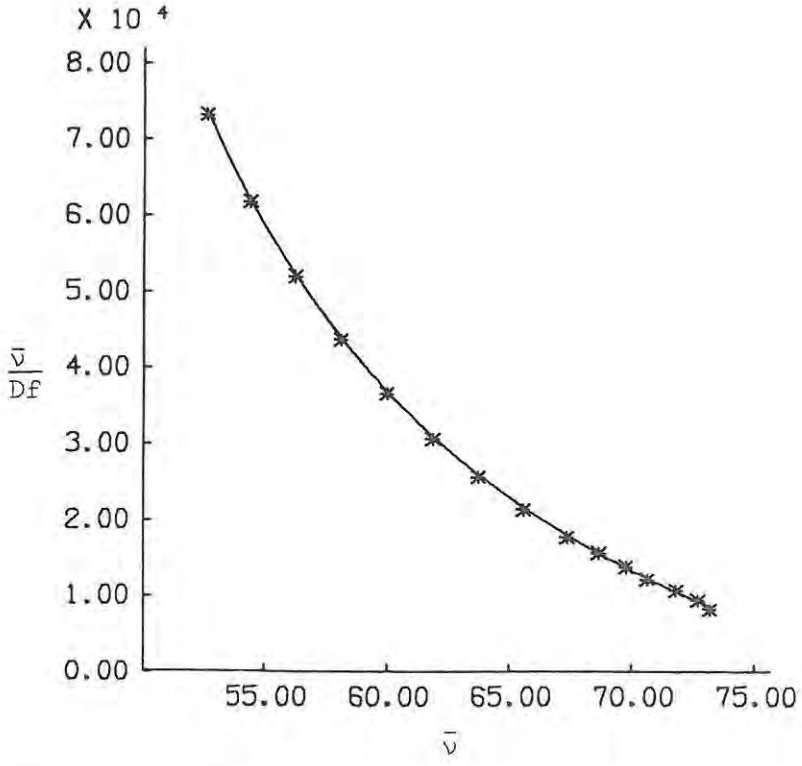


Fig. 28B. Scatchard plot of the binding of betamethasone disodium phosphate to collagen at pH 7.0 and 20°C. The solid curve was computed from the binding parameters.

collagen investigations show small differences between the control and binding runs. These observations, in the light of the conclusions of Meyer and Guttman (1970), imply that the present corticosteroid-collagen interactions should also be of a weak nature. The values obtained for the n's and k's, however, indicate a relatively strong interaction (Table XIII), hence, on initial examination of the Dt versus t plots, erroneous conclusions may be arrived at if no regard to the concentration, size and molecular structure of the protein under investigation is made.

The concentration of collagen was kept below 0.1% during the present series of experiments. Higher concentrations are associated with an undesirable increase in viscosity as well as the risk of protein-protein interactions resulting in the formation of dimers and other higher polymers (von Hippel and Wong, 1962). The possibility of precipitation would thus also be enhanced.

It should also be borne in mind that collagen has a far greater number of possible interacting sites located on the surface of the molecule (Ramachandran, 1967) compared to BSA where analagous binding sites may be 'buried' within the molecule. Also, the size of the collagen macromolecule is more than four times that of BSA.

The values obtained for the binding parameters at pH 3.00 indicate that roughly 70-80 primary binding sites are involved. The values for n_2 and k_2 implicate a second class of binding sites of relatively high capacity and low affinity. When the corresponding n and k values obtained from the study at pH 7.00 are compared, a significant reduction of primary binding sites are seen to have occurred concurrently with an increase in the value for k_1. Similarly, the number of secondary sites were reduced. The k_2 value was also found to be higher than the corresponding k_2 value at pH 3.00. The influence of pH on the number of available binding sites reflects the involvement of ionic interactions. At low pH, the corticosteroid is present in solution mainly as the monoanion (Flynn and Lamb, 1970) whereas collagen has a

TABLE XIII

Summary of binding constants for corticosteroid-collagen interactions

Corticosteroid	n_1	k_1 (litre/mol)	n_2	k_2 (litre/mol)	ΔG (kJ/mol)	pH
BDP	78.55	1.19×10^4	3.90×10^2	4.84×10^1	-22.90	3.00
	48.47	1.96×10^4	3.19×10^1	4.19×10^2	-24.10	7.00
DDP	70.56	6.62×10^4	9.93×10^2	8.70	-27.27	3.00
	43.36	1.19×10^5	2.06×10^1	1.09×10^3	-28.50	7.00
PDP	70.85	2.29×10^5	3.19×10^3	1.92	-30.11	3.00
	31.49	7.93×10^4	3.19×10^4	0.62	-27.50	7.00
HDP	97.06	2.63×10^5	1.83×10^4	1.34	-30.44	3.00
	54.73	2.29×10^3	1.18×10^3	2.10×10^1	-18.87	7.00

Abbreviations:- BDP: betamethasone disodium phosphate; DDP: dexamethasone disodium phosphate; PDP: Prednisolone disodium phosphate; HDP: Hydrocortisone disodium phosphate.

high proportion of cationic sites available at this pH. The reduction in the number of available binding sites observed at neutral pH may be rationalized in terms of a reduction in the number of cationic groups on the collagen molecule at pH 7.00 as well as a possible increase in the repulsive forces due to residual charge effects on the bound corticosteroid molecule which exists predominantly as the dianion at pH 7.00 (Flynn and Lamb, 1970).

It has also been shown that a variation of pH on collagen in solution within the range under consideration produces little or no effect on the stability of the molecule (Burge and Hynes, 1959; Dick and Nordwig, 1966). Furthermore, in the light of the recent report of the effect of pH on the thermal stability of collagen (Russell, 1974) it appears extremely unlikely that cooperative interactions (Steinhardt and Reynolds, 1969) may be involved, particularly as the present studies were conducted at a relatively low

temperature of 20°C. In considering the involvement of class 2 binding sites, inspection of the Scatchard plot at pH 3.0 indicates that under the prevailing experimental conditions of dialysis, the range of data for $\bar{\nu}$ extended only as far as a value of approximately 165. This implies that insufficient data was available to accurately determine the constants for the second class of binding sites. In spite of this implication it is still reasonable to assume that high capacity, low affinity binding sites exist. In addition, the extremely good fit obtained for 2 classes of binding sites allows a better estimate of the binding parameters for primary binding sites to be obtained. A linear fit for estimates of n_1 and k_1, would not take into account the contribution from the involvement of the second class of binding sites implicated by the curvature of the Scatchard plot. The incomplete characterization of secondary binding sites is probably due to the fact that complete saturation of all the binding sites had not been attained in spite of the high ratio of corticosteroid-collagen concentrations employed during these studies. Klotz (1950; 1953), Karush and Sonnenberg (1949), and Rosenberg and Klotz (1960) have emphasized that the value for Σn_i, representing the maximum number of small molecules bound by a protein, cannot be obtained without extrapolation of the experimental data.

The binding study of betamethasone disodium phosphate at pH 7.0 produced a Scatchard plot in which the highest value for $\bar{\nu}$ was just over 70. The binding parameters obtained form this data indicated that $\Sigma n_i \approx 80$. Although the range of $\bar{\nu}$ values attained during this particular study seemed to indicate that a better representation of class 2 binding sites was obtained, the n_2 value would still appear to be slightly erroneous due to incomplete saturation of this particular class of sites. As a result of these observations, the values obtained for secondary binding constants are treated semi-quantitatively.

In view of the fact that the disodium phosphate ester derivatives of several corticosteroids were used in these studies, it may be assumed that these steroids exist as organic anions in solution. When charged ions are bound by a protein, the first bound ion should tend to reduce the affinity of the protein for the second and subsequent ions because of electrostatic repulsion between these species of like charge. This effect has not, however, been detected with univalent anions and albumin (Klotz, 1953). Corrections for electrostatic factors have been used by Scatchard et al. (1950) in the treatment of chloride and thiocyanate binding by albumin but inspection of the data indicates that agreement within experimental error could be obtained with equations omitting electrostatic factors. It has been shown by Klotz and Walker (1947) that electrostatic repulsions between successively bound bivalent, trivalent and multivalent ions appear to be more significant. Karush and Sonnenberg (1949) were also unable to resolve their data by introducing electrostatic correction factors, a further indication of the dubious validity of this treatment. Steinhardt and Reynolds (1969) further indicated that the application of such corrections are probably not valid as efforts to apply electrostatic corrections often lead to anomalous deviations from the simple stoichiometric law of mass action behaviour, while the use of uncorrected data does not lead to these anomalies. In addition, consideration must be given to the presence of counterions in solution which may bring about a reduction in the calculated net charge (Steinhardt and Reynolds, 1969). A further complication regarding the use of electrostatic correction factors lies in the fact that collagen, unlike the spherical globular proteins, has a rod-like molecular structure.

In view of this structural difference, the usual mathematical models available for this purpose cannot, strictly speaking, be applied to collagen.

In the light of the aforementioned discussion, no corrections for electrostatic factors were attempted during the present investigations. This omission was further considered to be justified on the basis that all the studies were conducted under identical conditions of solution environment (excluding pH, the adjustment of which was carried out as previously described in order to minimize dilution) and thus any possible electrostatic factors would remain constant for all the systems studied. This allows comparisons to be made under almost identical solution background conditions.

ii) Binding of dexamethasone disodium phosphate

The results of the dynamic dialysis studies are shown in Figs. 29A & 30A. in which the loss of dexamethasone disodium phosphate in the absence and presence of 0.844mg/ml collagen is plotted as a function of time at pH 3.00 and pH 7.00 respectively. The corresponding Scatchard plots derived from these experiments are illustrated in Figs. 29B and 30B respectively. The results at different pH's follow the identical trend observed in the investigations of the binding of betamethasone disodium phosphate to collagen. When the pH was decreased from 7 to 3, approximately thirty additional primary binding sites appeared to be involved. This may be reconciled with the fact that collagen has a larger number of cationic sites available at pH 3.00 and thus should be more receptive to the corticosteroid anion at the lower pH. The decrease in binding affinity reflected by the positive difference in the ΔG parameters as a result of the pH variation (Tables XIII & XIV) are only of the order of 1.2 and 1.4kJ/mol respectively. The significance of these small values are rather doubtful.

The values for n and k as well as ΔG from the present study are summarized in Table XIII and depict that approximately the same number of

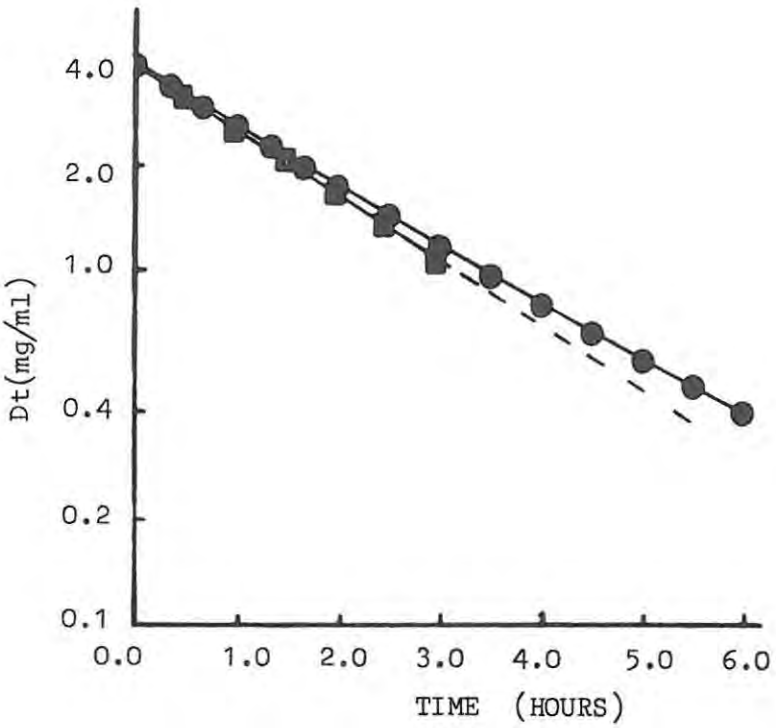


Fig. 29A. The loss of dexamethasone disodium phosphate from inside a dialysis sac at pH 3.0 and 20°C. ■ , control; ●, in the presence of 0.084% collagen.

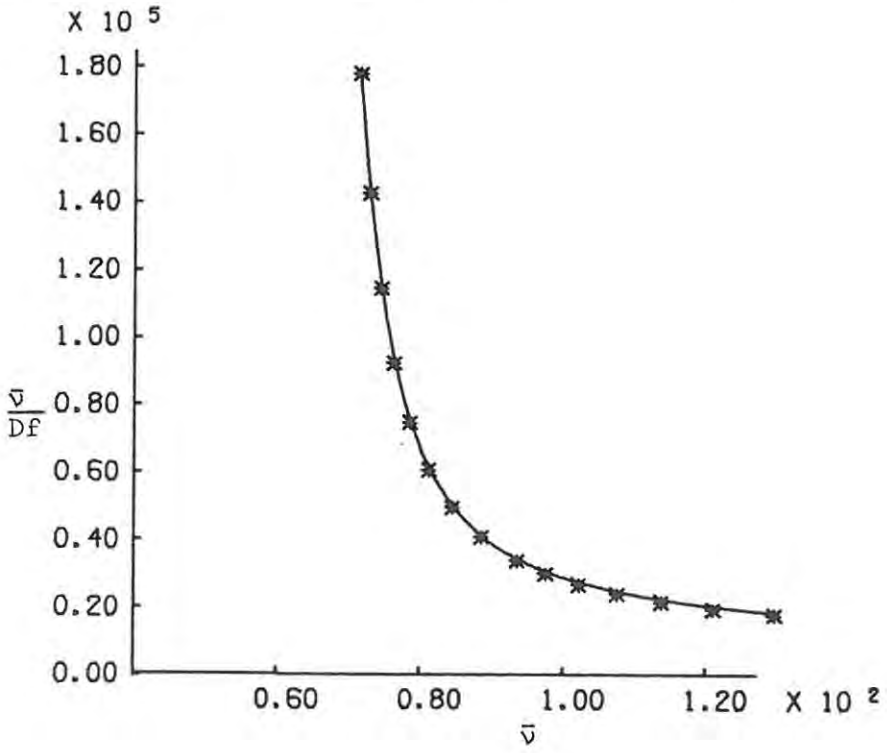


Fig. 29B. Scatchard plot of the binding of dexamethasone disodium phosphate to collagen at pH 3.0 and 20°C. The solid curve was computed from the binding parameters.

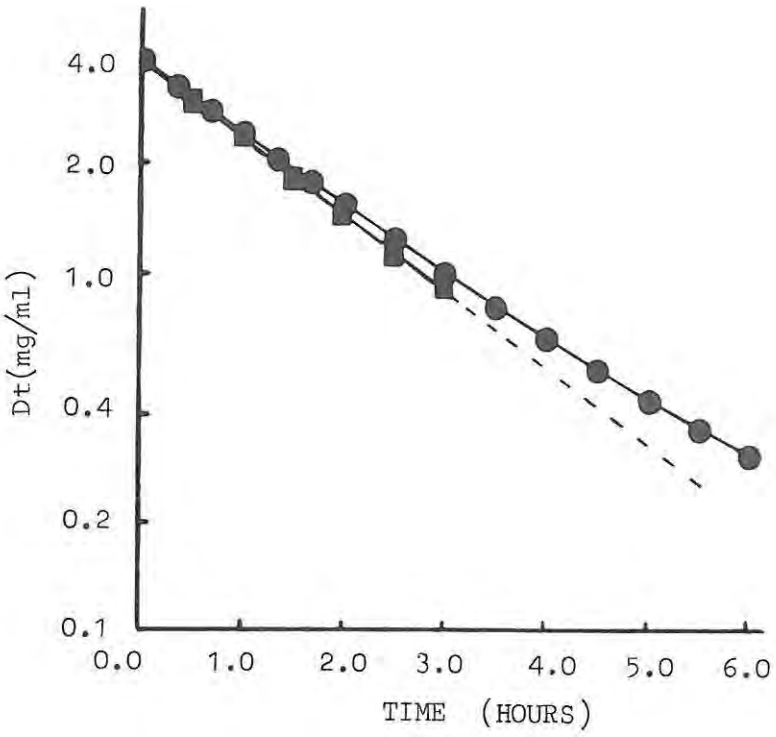


Fig. 30A. The loss of dexamethasone disodium phosphate from inside a dialysis sac at pH 7.0 and 20°C. ■ , control; ●, in the presence of 0.084% collagen.

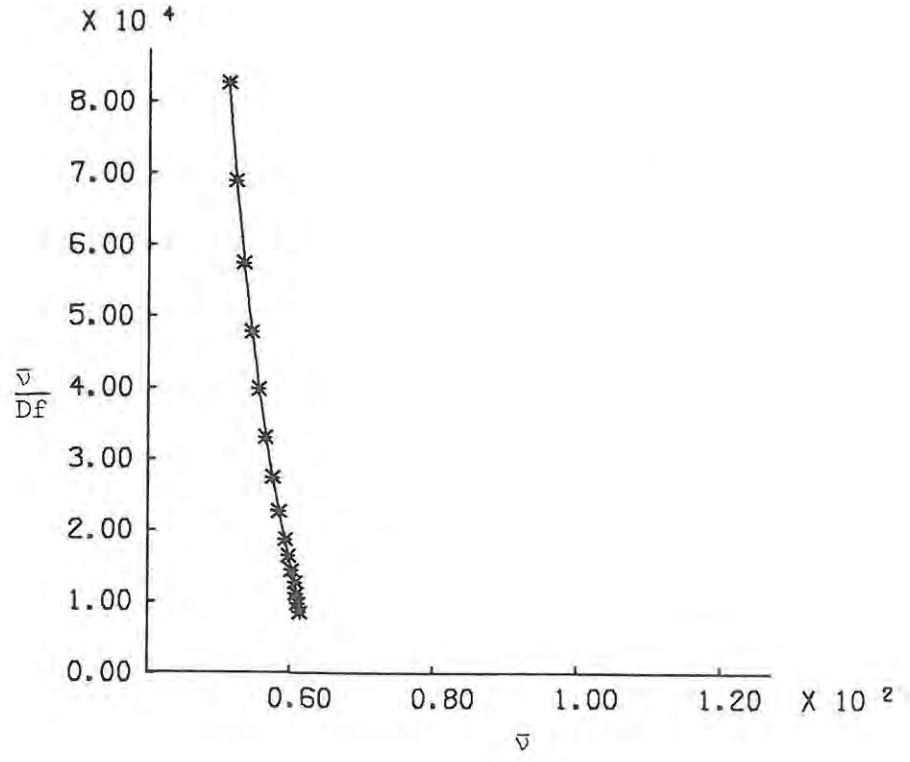


Fig. 30B. Scatchard plot of the binding of dexamethasone disodium phosphate to collagen at pH 7.0 and 20°C. The solid curve was computed from the binding parameters.

primary sites may be involved with respect to betamethasone disodium phosphate at the relevant pH. The binding affinity of dexamethasone disodium phosphate for these sites is slightly higher than the corresponding association constants for the betamethasone disodium phosphate interaction. The larger values for k_1's are reflected in the ΔG values for these systems (Tables XIII and XIV).

The significant difference between these epimeric corticosteroids lies in the configuration of the 16 - CH_3 group. The variation in the binding constants at either pH may thus be attributed to this stereochemical difference. Once again, however, the values obtained for the class 2 binding parameters are not taken into consideration for the same reasons as previously outlined. The larger number of sites found for the betamethasone disodium phosphate-collagen interaction does not completely agree with the concept that protein molecules interact with the rear side (α-face - Plates 2 - 6) of Δ^4- 3 -ketosteroids (Westphal, 1961; 1971). However, the increased affinity for these sites appears to be more consistent with the above concept provided that the relatively small differences in the n_1 values between the epimer-collagen interactions are assumed to be within the experimental error of the available data and hence regarded as being insignificant. This increase in binding affinity may be attributed to the electron repelling 16-methyl group whose effect is purported to be markedly stronger when the configuration is alpha (Westphal, 1964). Moreover, it is possible that the particular orientation of a substituent may produce severe steric interference with neighbouring substituents resulting in a distortion of the planarity of the molecule and thus account for the difference in binding behaviour (Ringold, 1961). The degree of planarity of the steroid has been emphasized as an important factor in the affinity for various steroids (Langer et al., 1959) by serum albumin.

Many results seem to show that multiple binding sites, involving the whole steroid molecule, are responsible for interactions between steroids

and proteins. This is suggested by numerous instances of a distinct influence of structural features on binding to proteins (Eik-Nes et al., 1954; Langer et al., 1959; Westphal and Ashley, 1958; 1959).

iii) Binding of prednisolone disodium phosphate

Dynamic dialysis examination of binding was conducted in the usual manner at 20° ±0.1°C, in 0.05M phosphate buffer containing 0.15M NaCl at pH 3.00 and then pH 7.00. The results are summarized in Figs. 31A and 32A which depict the kinetics of disappearance of prednisolone disodium phosphate from a 0.844mg/ml and a 0.805mg/ml solution of collagen at pH 3.00 and 7.00 respectively. The relevant control systems are also illustrated in these figures. The corresponding Scatchard plots were derived from the kinetic data as previously described and are shown in Figs. 31B and 32B. In view of the curvature of the Scatchard plots, the binding parameters were again calculated on the basis that more than one class of binding sites may be involved at both pH's.

The number of primary binding sites involved followed the same trend as that exhibited by both betamethasone and dexamethasone at pH 3.00 and 7.00 respectively. The value for k_1 at pH 7.00, however, appeared slightly lower than the corresponding k_1 value at pH 3.00 but this reduction in affinity is not readily explicable on the basis of the present and previously reported data. This trend reversal was, however, found to be still larger with the hydrocortisone disodium phosphate-collagen interaction at pH 3.00 (vide infra). Regarding class 2 binding sites, the extremely large estimates obtained for n_2 imply that the secondary binding sites are of very high capacity. The very small values for k_2 reflect that these sites are of extremely low binding affinity.

From an inspection of Table XIII, which includes the values of the binding constants for all present systems studied, it is seen that the number of primary binding sites computed for the prednisolone system was

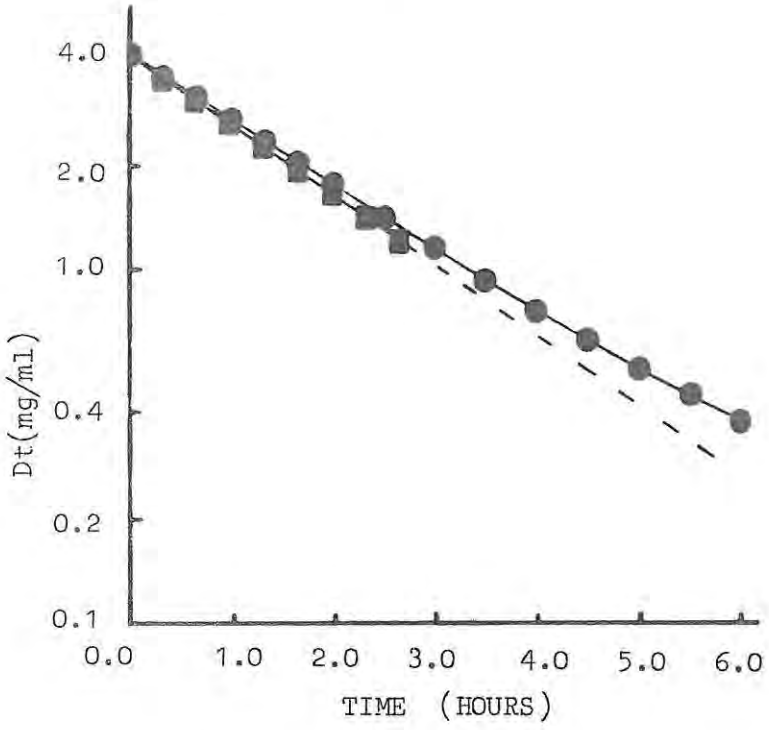


Fig. 31A. The loss of prednisolone disodium phosphate from inside a dialysis sac at pH 3.0 and 20°C. ■, control; ●, in the presence of 0.084% collagen.

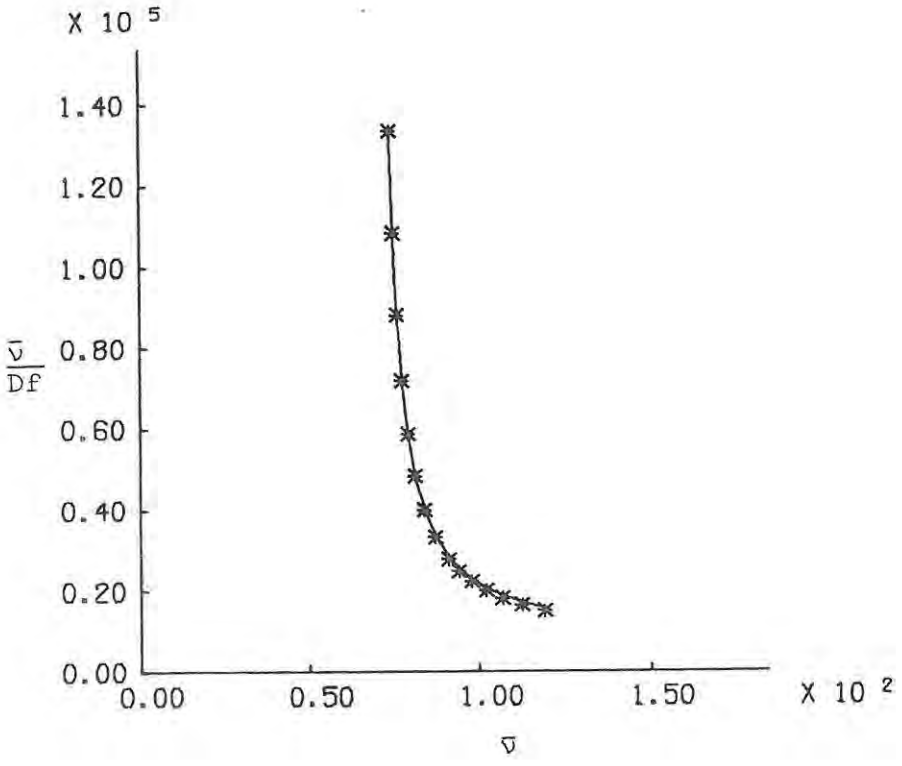


Fig. 31B. Scatchard plot of the binding of prednisolone disodium phosphate to collagen at pH 3.0 and 20°C. The solid curve was computed from the binding parameters.

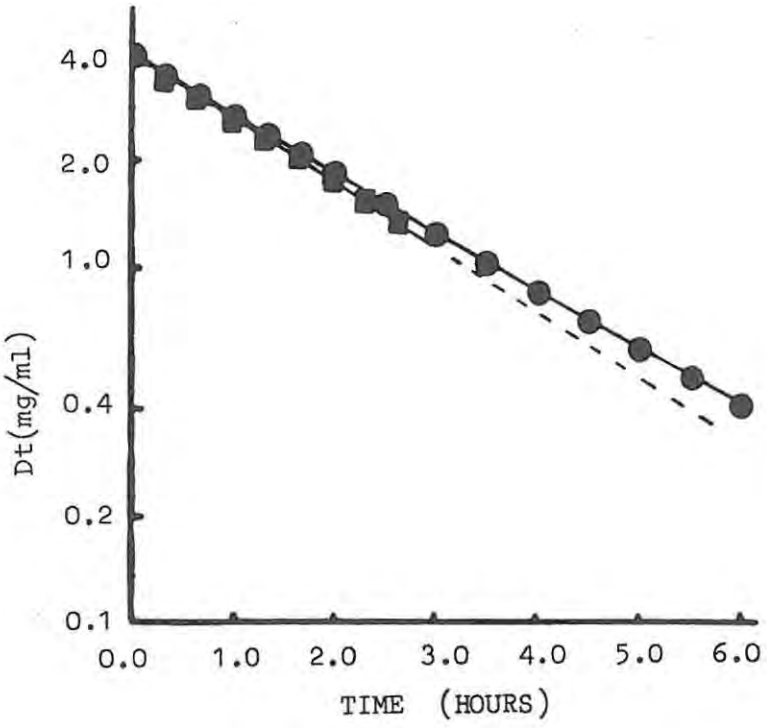


Fig. 32A. The loss of prednisolone disodium phosphate from inside a dialysis sac at pH 7.0 and 20°C. ■ , control; ●, in the presence of 0.081% collagen.

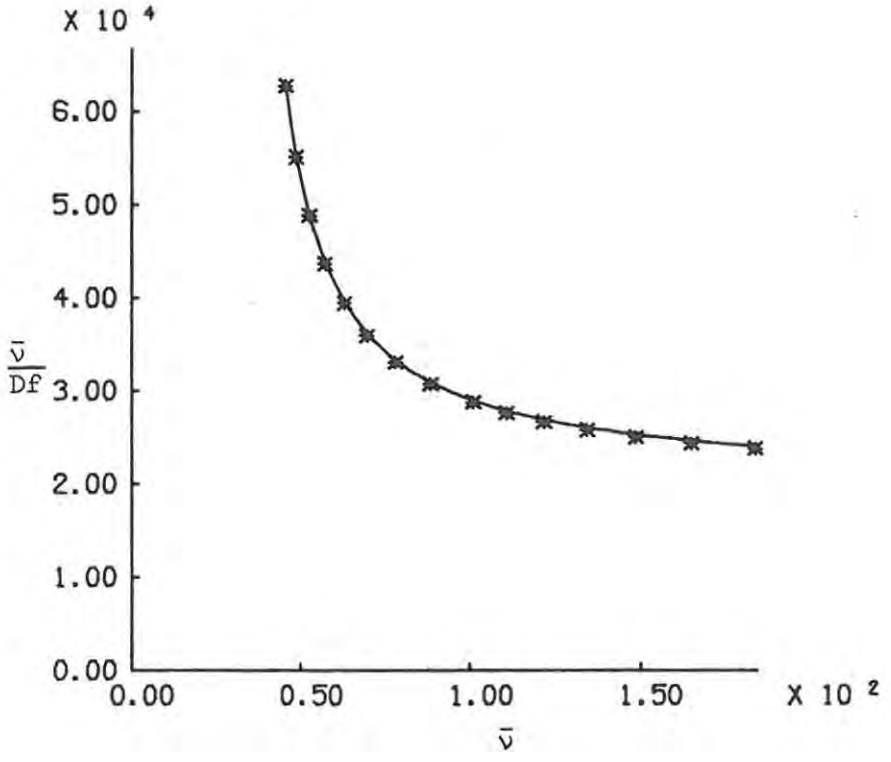


Fig. 32B. Scatchard plot of the binding of prednisolone disodium phosphate to collagen at pH 7.0 and 20°C. The solid curve was computed from the binding parameters.

approximately the same as that at pH 3.00 for betamethasone and dexamethasone disodium phosphate. The value of n_1 at pH 7.00, however, was lower than the corresponding values found at the same pH in the case of the former two corticosteroids.

The differences between this corticosteroid and the previously studied epimers is shown in Fig. 7. Plates 2,3 and 5 should also be inspected for this purpose. The alteration in binding behaviour may thus be attributed to the absence of both the 9α-fluorine substituent as well as the 16-methyl group. The observation of a consistent pH trend, once again implicates the role of ionic interactions, in the form of corticosteroid anion-protein-cationic-site interaction.

It has been shown by Westphal (1961) during his studies with transcortin, that the introduction of a 9α-fluoro group decreases the affinity for the binding site in transcortin. This may imply that a hydrogen bonding mechanism via the 11β-hydroxy group does not contribute to the interaction as it has been reported by Fried and Borman (1958) that the introduction of the 9α-fluoro substituent strengthens the acidity of the 11β-hydroxy group thereby making the proton of the hydroxyl more available for stronger hydrogen bonding between the corticosteroid and receptor. Westphal (1971) has also reported that the introduction of electron-attracting substituents weaken the interaction with human serum albumin. Recent reports regarding the effect of the 9α-fluoro substituent on the molecular geometry of the corticosteroid molecule indicate that a gradual bowing towards the α side in ring A occurs (Weeks et al.,1973; Kollman et al.,1973). This observation parallels the variation in anti-inflammatory activity observed between cortisol, 6α-fluorocortisol, 9α-fluorocortisol, and 6α-methylprednisolone. Although a relatively slight increase in k_1 was obtained during the present study at pH 3.00 with prednisolone disodium phosphate it would, indeed, be premature to attribute this to the conformation effect in question. The relevance of this geometric

distortion may, however, become more apparent when additional evidence regarding the nature of corticosteroid-collagen interactions is provided.

The part played by the 16-methyl group is not readily apparent. The present results indicate that removal of this electron-donating group may contribute to enhanced binding activity between corticosteroids and collagen at pH 3.00. This is somewhat analagous to the findings of Westphal (1961) who reported that removal of an electron-donating group from a β-position has an effect comparable to the removal of an electronegative group from the rear side. These findings were based on studies with β-(axial)-methyl groups at C_6 and C_{10}.

iv) Binding of hydrocortisone disodium phosphate

The dynamic dialysis data depicting the loss of hydrocortisone disodium phosphate at pH 3.00 is shown in Fig. 33A whereas the study at pH 7.00 is illustrated in Fig.34A. The studies were, once again, conducted in 0.05M phosphate containing 0.15M NaCl at 20° ±0.1°C using 0.844mg/ml collagen in the former and 0.805mg/ml in the latter study. Fig. 33B & 34B illustrate the relevant Scatchard plots generated from the computer analysis of the data presented in Figs.33A & 33B, respectively. The binding parameters obtained from these Scatchard plots are shown in Table XIII.

The results obtained for the values of n_1 at the different pH's follow the previously observed phenomenon that a lowering of pH causes an increase in the number of primary binding sites. In view of this apparent consistent trend, the likelihood that cationic sites on the collagen molecule are involved in ionic interactions with the corticosteroid anions is enhanced. Once again, the existence of a high capacity, low affinity second class of binding sites is implied from the estimates obtained for n_2 and k_2.

The observation that approximately 30 additional sites at pH 3.00 are involved in the hydrocortisone disodium phosphate-collagen interaction may be attributed to the absence of the Δ^1-double bond when this corticosteroid

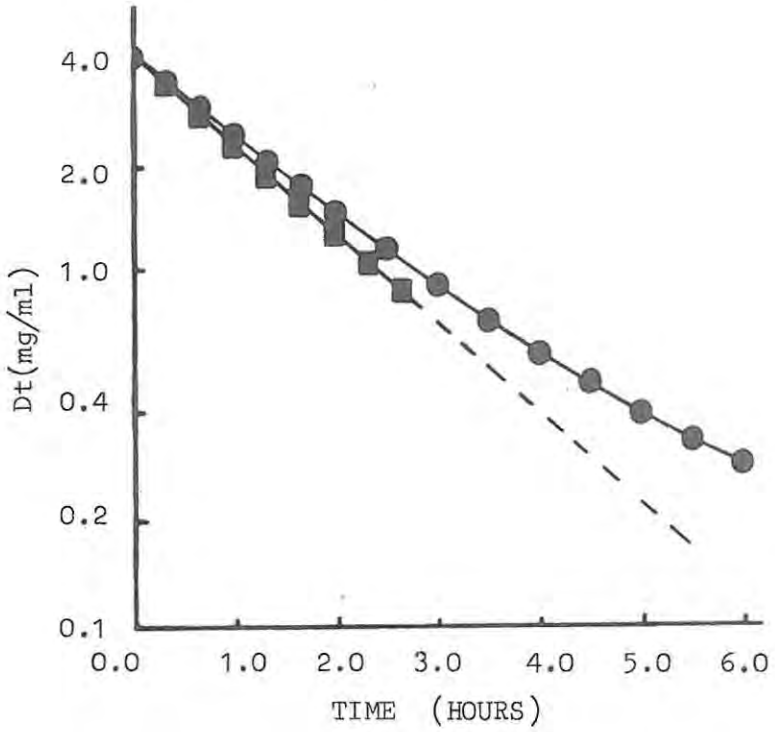


Fig. 33A. The loss of hydrocortisone disodium phosphate from inside a dialysis sac at pH 3.0 and 20°C. ■ , control; ● , in the presence of 0.084% collagen.

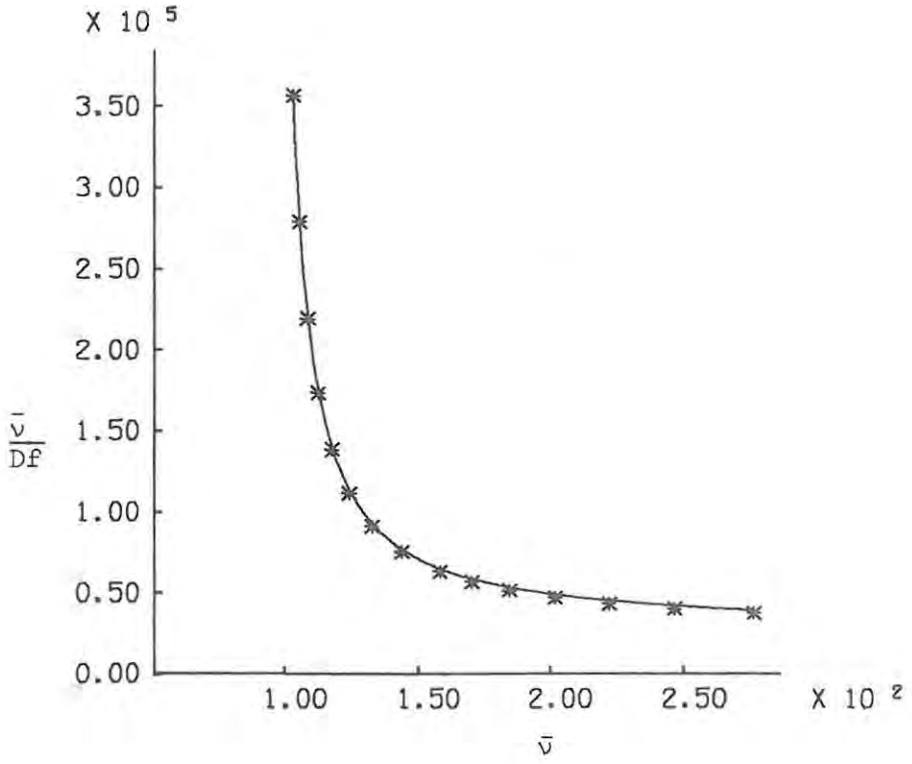


Fig. 33B. Scatchard plot of the binding of hydrocortisone disodium phosphate to collagen at pH 3.0 and 20°C. The solid curve was computed from the binding parameters.

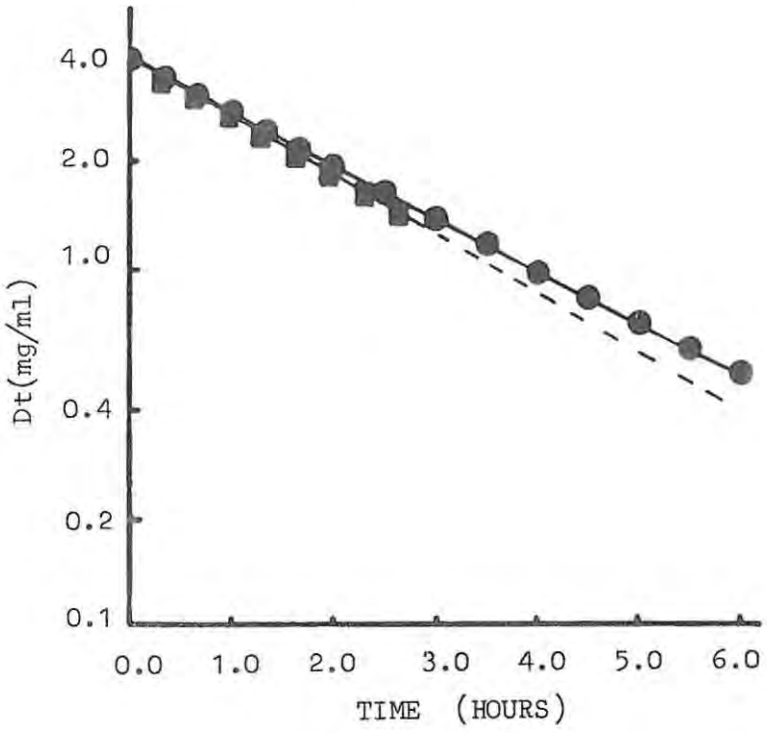


Fig. 34A. The loss of hydrocortisone disodium phosphate from inside a dialysis sac at pH 7.0 and 20°C. ■, control; ●, in the presence of 0.081% collagen.

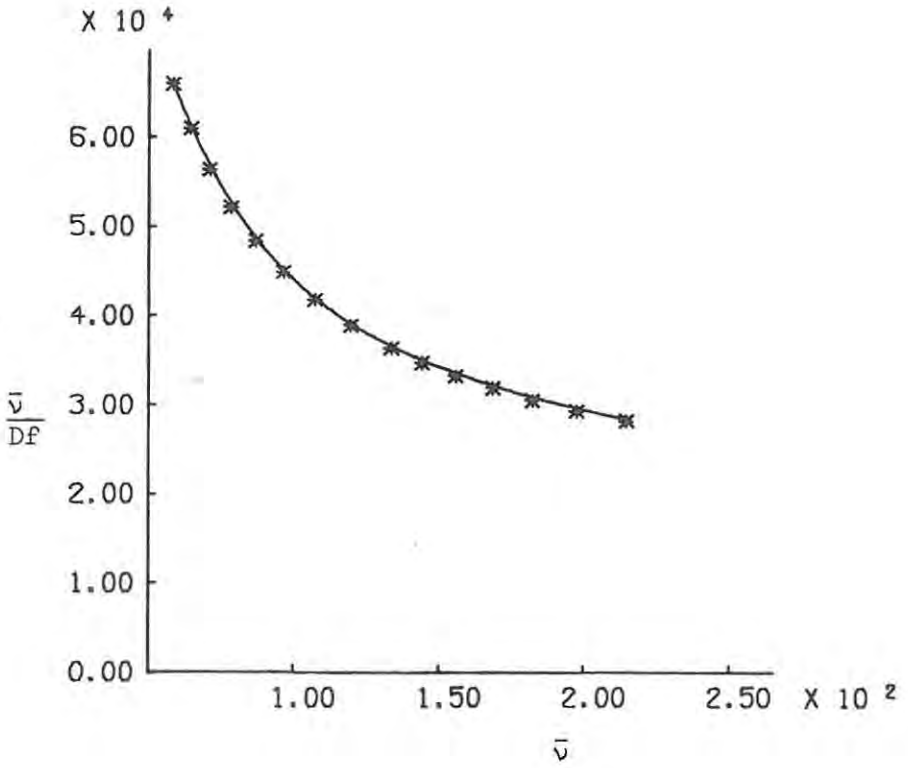


Fig. 34B. Scatchard plot of the binding of hydrocortisone disodium phosphate to collagen at pH 7.0 and 20°C. The solid curve was computed from the binding parameters.

is compared directly with prednisolone disodium phosphate. Westphal (1961) has reported that the introduction of a Δ^1-double bond decreases the interaction with human serum albumin (particularly if the new double bond is conjugated to the keto group in ring A) and the present findings are in agreement with this observation. The affinity for these sites appear to be roughly equivalent in both prednisolone and hydrocortisone disodium phosphate at pH 3.00, implying the same type of interactions are involved in both instances. Hydrogenation of the Δ^1-double bond in prednisolone to produce hydrocortisone disodium phosphate is accompanied by a decrease in polarity as well as distortion of the previously planar A ring. This fact implicates the possible role of hydrophobic interactions in these corticosteroid-collagen interactions.

The value of k_1 at pH 7.00 is considerably lower than the corresponding k_1 values found with the other corticosteroids studied. It is difficult to reconcile this observation in terms of the evidence available from the present studies. Whereas the k_1 values for betamethasone and dexamethasone increased at pH 7.00, these values decreased at the same pH when prednisolone and hydrocortisone were investigated.

Although the present investigation was concerned with the molecular interactions between collagen and charged corticosteroid ester salts, and in spite of the available evidence which indicates that the interactions are mediated by electrostatic bonding, it cannot be assumed that this type of mechanism is exclusive. There is evidence, both from these studies and from the literature, that the neutral portion of the corticosteroid molecule participates in the binding process. Both polar and apolar interaction may occur in the same complex. Foster (1960) suggested that correlation between the number of anions bound and the number of cationic sites on serum albumin was fortuitous, suggesting that the hydrophobic nature of the anion plays an important role in the binding to proteins. Davis (1946) also reported

that some aliphatic amino acids have a high affinity for the non-polar portion of anionic molecules, further implicating the role of hydrophobic bonds in these interactions. The importance of both electrostatic attraction and hydrophobic interaction has been generally accepted for the binding of long-chain fatty acids to serum albumin (Spector et al.,1969). The possibility of hydrogen bonds and dipole-dipole interactions also cannot be discounted.

It is apparent, however, from Westphal's observations that the rear surface of the steroid molecule plays a distinctive role in interactions with protein structures (Westphal, 1961; 1971). The α-substituents which form the rear side plane, including the oxygen function at C_3 (Fig. 7) appear to constitute points of contact with the surface of the protein. A better appreciation of the spatial requirements of the substituents in the corticosteroid molecule is obtained with space-filling models (Plates 2,3,4,5 and 6). It appears likely that the two angular methyl groups, C - 18 and C - 19 (Fig. 7), shield the β-surface (upper) of the corticosteroid molecule from close interaction with approaching molecules. This becomes evident in the side view (Plates 2,3,4,5 and 6) which show the whole corticosteroid molecule as possessing a convex shape, with the upper side forming a curved surface. The substituents on the α-face (rear side) appear to be less hindered and therefore more apt to interact. A close fit of the corticosteroid and protein surfaces in terms of hydrogen bonds and/or Van der Waal's forces, is thus required for efficient interaction (Marcus and Talalay, 1955).

Similar conclusions concerning the stereochemical aspects of steroid-protein interactions were reached by Talalay and Marcus (1956), Daughaday (1958), Langer et al. (1959) and Munck et al. (1957). The degree of planarity of the steroid molecule has also been emphasized as an important factor in these interactions (Langer et al.,1959).

Many reports seem to indicate that multiple binding sites, involving

the whole steroid molecule, are responsible for interactions between steroids and proteins. The simplest interpretation, however, is a geometrical concept of complimentariness as assumed in other instances of drug-receptor interactions (Goldstein et al., 1969).

Consideration of the steroid molecule alone indicates the possible occurrence of a large amount of intramolecular diaxial interactions (Eliel et al., 1966) while electronic shifts such as inductive effects and electronic field effects (Kirk and Hartshorn, 1968) may play a significant role. Furthermore, steroids may be markedly altered by relatively minor structural changes at sites remote from the reaction centre. These long range effects are regarded as being due primarily to the transmission of conformational strains through the fused ring system (Barton et al.,1957; Kollman et al., 1973). It is thus apparent that corticosteroid-collagen, and all small molecule-protein interactions are probably mediated by contributions from a multitude of possible reaction mechanisms and any attempt to reconcile experimental observations on a mechanistic basis must, of necessity, take into consideration the theoretical possibilities outlined in the aforementioned discussions.

In summary, it appears that ionic interactions are involved in these corticosteroid-collagen interactions as indicated by the pH variation studies. Both betamethasone and dexamethasone disodium phosphates behaved in a similar manner regarding their interaction with collagen. When the pH was decreased from 7 to 3, approximately 30 additional primary binding sites appeared to be involved. This may be reconciled with the fact that collagen has a larger number of cationic sites available at pH 3 and thus should be more receptive to the corticosteroid anion at this low pH. The decrease in binding affinity reflected by the positive difference in the Δ G parameters (Table XIV) as a result of the pH variation are only of the order of 1.2 and 1.4kJ/mol respectively and hence interpretation of this phenomenon remains

unclear. Although the same trend regarding the increased number of primary binding sites at the lower pH was established with both prednisolone and hydrocortisone disodium phosphates, the ΔG differences in both instances were found to be more negative. This indicates that the binding affinity was stronger as the pH decreased and was exaggerated with the less polar hydrocortisone disodium phosphate where the contribution of ΔG was found to be relatively large (-11.6kJ/mol). This could indicate that additional forces become involved and are superimposed on the ionic forces operating at pH 3. On the basis of the data available from the present investigations a specific bonding mechanism cannot be identified although Reynolds et al.(1970) attributed the difference in the free energy of association of decyl sulphate to native bovine serum albumin between pH 6.8 and 4.8 as being due to multiple hydrophobic interactions operating simultaneously with ionic interactions. The ΔG for that particular system being $\approx$ 5kJ/mol.

The orientation of the 16-methyl group in both betamethasone and dexamethasone disodium phosphates appeared to be significant with respect to the binding behaviour of these epimers. In both instances, the number of primary binding sites at each pH remained approximately constant ($\pm$70 sites and pH 3 and $\pm$ 40 sites at pH 7 for each corticosteroid). The binding affinity in each case was stronger with dexamethasone. This is in agreement with the concept that the α-face of the steroid molecule is preferentially involved in interactions with proteins. In addition, the presence of the α-methyl group could impart an additional hydrophobic point of contact on that surface. At each pH, the difference in ΔG between these epimers was found to be $\pm$ 4kJ/mol which, once again, may reflect the contribution from additional weak forces (Reynolds et al., 1970).

Hydrogenation of the double bond in prednisolone to yield hydrocortisone is accompanied by the involvement of roughly 25 - 30 new sites at pH 3.00. The small associated ΔG difference (Table XIV) may, however, be considered

insignificant and within the limits of experimental error. The implications are that the additional sites relate to the appearance of an increased number of cationic sites on the protein at pH 3. Although the increase in the number of primary binding sites when hydrocortisone is compared to prednisolone at pH 7, is similar to that observed at pH 3, the associated difference in ΔG between these corticosteroids at the neutral pH is not readily explained. The positive ΔG increment reflects a weakening of the binding affinity in spite of the fact that hydrocortisone is less polar than prednisolone due to saturation of the double bond between C_1 and C_2.

The difference in the free energy of association between betamethasone and prednisolone at pH 3 reflects an increase in binding strength associated with the removal of both the 9α-fluoro and 16-methyl substituents in the latter. This is in agreement with the observations of Westphal who showed that the presence of an electron-withdrawing group (9α-fluoro) in the steroid molecule causes a reduction in the binding of that steroid to human serum albumin, when the interaction is compared with non-fluorinated steroids. The number of primary binding sites, however, remained constant. In spite of the fact that the presence of an alkyl group was shown to cause an increase in interaction with proteins, it appears that the 9α-fluoro substituent cancels out this effect and preferentially manifests its own binding characteristics. A similar observation was noted when either betamethasone or dexamethasone were compared with hydrocortisone disodium phosphate. When the binding behaviour between dexamethasone and prednisolone are compared at pH 3, the effects on n_1 remain the same. Nevertheless, the difference in ΔG between these two corticosteroids was smaller than the corresponding ΔG difference between betamethasone and prednisolone. This agrees with observations that the presence of an electron-donating group on the α-face of the steroid molecule causes a higher degree of binding when compared with a β-methyl substituent. Hence, the reduction in the binding affinity of betamethasone and dexamethasone

to collagen, when compared with either prednisolone or hydrocortisone at pH 3, is larger in the case when the 16α-methyl group is removed. The effect of the presence of the 9α-fluoro and 16-methyl substituents on the binding behaviour to collagen has thus been demonstrated. The pharmacological importance of these groups substituted into a basic corticosteroid molecule such as hydrocortisone is well known (Fried and Sabo, 1954; Boland, 1958; Liddle and Fox, 1961). The 9α-fluoro substituent appears to have a dramatic effect in that its presence enhances anti-inflammatory activity *in vivo*. This improvement in anti-inflammatory activity is, however, associated with an undesirable increase in salt and water retention properties. The introduction of a 16-methyl group into 9α-fluorinated corticosteroids results in compounds such as betamethasone and dexamethasone which retain the desired anti-inflammatory activity but are devoid of the undesirable salt and water retention characteristics. The exact mechanism through which these above effects are exerted remains unclear although some evidence exists that the presence of the 16-methyl substituent (α or β) hinders reactions on the steroid side chain (Sarett *et al.*, 1963; Taub *et al.*, 1960; Wettstein, 1972). The availability of either a 16-demethyl 9α-fluoro corticosteroid or a 16-methyl 9-defluoro compound would allow the effect of the individual substituents to be investigated with respect to corticosteroid-collagen interactions when further correlations may be obtained. Similarly, because of the very limited supply of paramethasone disodium phosphate (Fig. 7), this particular corticosteroid was only used during the fibril kinetic investigations. Binding studies using paramethasone disodium phosphate would allow the significance of the position of the fluorine atom to be evaluated and compared with the results obtained using betamethasone and dexamethasone disodium phosphates.

Further investigations, such as the effect of temperature, buffer ion, ionic strength and steroid-protein concentration ratio on corticosteroid-collagen interactions appear to be logical extensions of this work which may provide additional useful information regarding the binding forces.

TABLE XIV

Summary of ΔG (kJ/mol) contributions for i = 1*

	BDP (pH = 3.0)	BDP (pH = 7.0)	DDP (pH = 3.0)	DDP (pH = 7.0)	PDP (pH = 3.0)	PDP (pH = 7.0)	HDP (pH = 3.0)	HDP (pH = 7.0)
BDP (pH = 3.0)	-	-1.2	-4.2	-	-7.2	-	-7.5	-
BDP (pH = 7.0)	1.2	-	-	-4.4	-	-3.4	-	5.2
DDP (pH = 3.0)	4.2	-	-	-1.4	-3.0	-	-3.4	-
DDP (pH = 7.0)	-	4.4	1.4	-	-	1.0	-	9.6
PDP (pH = 3.0)	7.2	-	3.0	-	-	2.6	-0.33	-
PDP (pH = 7.0)	-	3.4	-	-1.0	-2.6	-	-	8.6
HDP (pH = 3.0)	7.5	-	3.4	-	0.33	-	-	11.6
HDP (pH = 7.0)	-	-5.2	-	-9.6	-	-8.6	-11.6	-

* Primary binding sites.

Abbreviations:- BDP: betamethasone disodium phosphate; DDP: dexamethasone disodium phosphate; PDP: prednisolone disodium phosphate; HDP: hydrocortisone disodium phosphate.

CHAPTER V

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A. INTRODUCTION

1. The use of empirical equations to describe dynamic dialysis "escape curves".

The introduction of the dynamic dialysis technique to study protein binding (Meyer and Guttman, 1968b;1970) has prompted a number of workers to utilize this approach in view of the apparent advantages inherent in this method (Dearden and Tomlinson, 1970; Goto et al., 1971; Asghar and Roth, 1971; Crooks and Brown, 1973; 1974a & b; Brown and Crooks, 1973 and Tukamoto et al., 1974).

It became evident during the present studies utilizing this technique that methods available for obtaining the value of the slope of the curvilinear plot at various values of time could lead to anomalies in the subsequent Scatchard plots. The values of the slopes are necessary to obtain the instantaneous rate at a value of Dt in the presence of protein. In addition, the computational problems associated with the various equations used to fit the dialysis data for the variation of Dt with time, and the evaluation of the subsequent Scatchard plot, necessitated the development of a particular strategy, in each instance, to facilitate data processing.

Meeter (1964) described a number of problems in the analysis of non-linear models and emphasized that errors in interpretation of results, which are often overlooked, may be far more serious than any experimental errors which might have occurred. The question of multiple minima in non-linear least squares was also raised. This particular problem was manifest during the present studies involving the use of a non-linear regression program. Meeter (1964) concluded from his investigations that "the range of problems encountered when one is investigating the statistical theory of non-linear models is so great that one suffers from an embarras du choix".

Meyer and Guttman (1968b) in their original publication suggested that the slopes of the escape curve, in the presence of protein, at various values of time, could be estimated graphically. However, in view of the convenience and accuracy of fitting the data from plots of Dt versus time with the aid

of a digital computer, the latter approach was employed by them. This involved the use of a six-parameter tri-exponential equation ($Dt = \sum_{i=1}^{3} A_i e^{-b_i t}$). From their studies of the binding of polymethylene bisquaternary ions to chondroitin in vitro, Asghar and Roth (1971) pointed out that the graphic evaluation of the instantaneous rates yielded only approximate values.

Dearden and Tomlinson (1970) used the dynamic dialysis technique to study the binding of p-substituted acetanilides to BSA. A modified single exponential equation, which included a constant term ($Dt = Ae^{-Bt} + C$), was employed to fit their dialysis data in the presence of protein (Dearden, 1972).

Crooks and Brown (1973) and Brown and Crooks (1973) fitted their plots of Dt versus time to a fourth order polynomial equation which included the value of the initial concentration of drug in the protein solution.

2. Theoretical calculation of dynamic dialysis data

Kruger-Thiemer (1966) derived equations which describe rates of loss of unbound and total small molecule capable of being bound, from a protein compartment. His derivations, however, were restricted to situations where binding occurred to one class of sites on the protein. Meyer and Guttman (1970) modified these derivations to account for the existence of two classes of sites. Their modification resulted in EQUATION V-1 which describes the rate of disappearance of total concentration of small molecule, Dt, from a protein compartment as a function of an apparent first-order elimination constant, K_e, the concentration of unbound small molecule, Df, the total concentration of protein, Pt, the number of binding sites in each of two classes, n_1 and n_2, and the corresponding dissociation constants, K_1 and K_2.

$$\frac{-d(Dt)}{dt} = \frac{K_e(Dt)}{1 + [n_1(Pt)]/[(Df) + K_1] + [n_2(Pt)]/[(Df) + K_2]} \quad \text{(EQUATION V-1)}$$

The rate of loss of free drug (Df) from the protein compartment is given by:

$$\frac{-d(Df)}{dt} = \frac{K_e(Df)}{1 + [n_1K_1(Pt)]/[(Df + K_1)^2] + [n_2K_2(Pt)]/[(Df + K_2)^2]} \quad \text{(EQUATION V-2)}$$

The relationship between the total and unbound drug concentration is given by:

$$(Dt) = (Df)\left[1 + \frac{n_1(Pt)}{(Df) + K_1} + \frac{n_2(Pt)}{(Df) + K_2}\right] \quad \text{(EQUATION V-3)}$$

It is apparent that an expression for Dt as a function of time cannot be obtained directly by integration of EQUATION V-1 and, therefore, a more circuitous approach must be utilized to generate theoretical kinetic data simulating the dynamic dialysis system. EQUATION V-3 is written in terms of initial total (Dt^o) and initial free (Df^o) drug concentrations. The resulting equation is then re-arranged to yield a cubic expression in Df^o:

$$(Df^o)^3 + (Df^o)^2[K_1 + K_2 + (Pt)(n_1 + n_2) - (Dt^o)] +$$
$$(Df^o)[K_1K_2 + (Pt)(n_1K_2 + n_2K_1) - (Dt^o)(K_1 + K_2)] - (Dt^o)(K_1K_2) = 0 \quad \text{(EQUATION V-4)}$$

N.B. When only one class of binding sites is involved, a quadratic expression in Df^o results. From the integration of EQUATION V-2 between the limits of t = 0 to t, and Df = Df^o to Df, an expression for time may be obtained:

$$t = \frac{1}{K_e}\left\{\ln\frac{(Df^o)}{(Df)} + \frac{n_1(Pt)}{K_1}\left[\ln\frac{(Df^o)(K_1 + Df)}{(Df)(K_1 + Df^o)}\right] + \right.$$
$$\frac{n_2(Pt)}{K_2}\ln\left[\frac{(Df^o)(K_2 + Df)}{(Df)(K_2 + Df^o)}\right] - (Pt)(Df^o - Df) \quad X$$
$$\left.\left[\frac{n_1}{(K_1 + Df^o)(K_1 + Df)} + \frac{n_2}{(K_2 + Df^o)(K_2 + Df)}\right]\right\} \quad \text{(EQUATION V-5)}$$

It is evident from EQUATIONS V-1, V-2 and V-3 that a semi-log plot of Dt versus time, for the escape of unbound drug from a protein-containing compartment, will not be linear.

Thus, for values of n_1, n_2, K_1, K_2, Dt^o and Pt, a value of Df^o may be calculated from EQUATION V-4. Then, assuming various values for Df, the time, t, at which the assumed Df concentrations will be present in the protein compartment can be calculated from EQUATION V-5. Finally, using EQUATION V-3, the values for Dt, corresponding to the times which were determined from EQUATION V-5, are calculated. These computations were carried out with the aid of a BASIC computer program written for a Hewlett-Packard model 2100A computer.

B. EXPERIMENTAL

In view of the fact that a number of empirical equations, which have no clear physical significance, have been utilized by a number of workers to describe the kinetic behaviour observed experimentally, it was necessary to test the fitting of the curvilinear plots of Dt against time to various equations claimed to conform closely to the experimental data.

Least squares fitting of fourth, fifth and higher order polynomial equations as well as of a single exponential equation was used. In addition, fits were attempted using these same functions incorporating the 'method of fourth differences' (Lanczos, 1967). A number of other equations were also used, viz.:

$$Dt = Ae^{-at} + Be^{-bt} + Ce^{-ct} \quad \text{(Guttman, 1970)} \qquad \text{(EQUATION V-6)}$$

$$Dt = Ae^{-Bt} + C \quad \text{(Dearden, 1972)} \qquad \text{(EQUATION V-7)}$$

$$Dt = Dt^o + at + bt^2 + ct^3 + dt^4 \quad \text{(Crooks and Brown, 1973; Brown and Crooks, 1973)} \qquad \text{(EQUATION V-8)}$$

where Dt^o is the initial concentration of drug in the protein solution and a, b, c and d are constants.

$$Dt = Ae^{-at} + Be^{-bt} \qquad \text{(EQUATION V-9)}$$

$$Dt = Ae^{-at} - Be^{-bt} + Ce^{-ct} \qquad \text{(EQUATION V-10)}$$

All curve fitting was done with the aid of Fortran IV computer programs and an ICL 1901A computer. At the commencement of the binding studies, unsuccessful attempts from local sources were made to obtain a non-linear least squares program for fitting data to a tri-exponential equation. Systematic studies using various other functions as well as the graphic procedure to estimate slopes were therefore undertaken but these resulted in anomalous Scatchard plots due to erroneous estimation of the slopes of the curvilinear plots of Dt versus time. Eventually, however, a Fortran listing for 'Least Squares Estimation of Non-Linear Parameters' was kindly supplied by Dr. D.W. Marquardt (Marquardt, 1971). Adaptation of this program was thus undertaken. This involved extensive overlay techniques in order to accommodate the supplied program with the relatively limited computer capacity available (ICL 1901A - 16K computer). The program involved the use of sophisticated convergence criteria coupled with a line-printer plotting facility. Although considerable time was spent on the development and adaptation of this program, the extremely large size of this program (programmed to run on a UNIVAC 1107 computer) together with the relatively small computer capacity available (extensive overlay procedures included) did not permit the program to run to completion. This did not affect the present studies as solution sets of best-fitting parameters were still obtained albeit without completion of the calculations for the various statistical tests for 'goodness of fit'. Values, however, for the sum of the squares of the deviations were calculated by this program. Preliminary binding data was processed with the aid of this program until a more efficient program based on the same algorithm was obtained (Meyer, 1972). After successful adaptation of this latter program, exhaustive tests on numerous

data sets were undertaken utilizing each program independently. The resulting output from each of these abovementioned programs were compared and found to be identical provided the initial parameter estimates were kept the same. In view of the fact that the additional statistical tests employed during the use of Marquardt's program did not appear to influence the final results and because of the longer 'running-time', the program supplied by Meyer (1972) was subsequently used exclusively.

It should be noted that the successful developmenet and adaptation of these programs as well as the processing of binding data was effected by a 'hands on' computer facility.

Finally, theoretical binding data was generated from EQUATIONS V-3, V-4 and V-5 (i.e. data for Dt versus time). The values of n and k used were the same as those reported by Meyer and Guttman (1970) for the binding of phenol red to BSA ($n_1 = 1$; $n_2 = 6$; $k_1 = 1.74 \times 10^5$; $k_2 = 1.97 \times 10^3$).

C. RESULTS AND DISCUSSION

Asghar and Roth (1971) pointed out that the graphic evaluation of the slopes of the curvilinear plot of Dt versus t in order to obtain the instantaneous rate as suggested by Meyer and Guttman (1968b), yielded only approximate values for these parameters. This was confirmed during the initial calibration experiments utilizing this graphical procedure, when the subsequent Scatchard plots for phenol red-BSA deviated considerably when compared with the previously reported results for that system (Meyer and Guttman, 1968b;Rodkey, 1961). Least squares fitting of fourth, fifth and higher order polynomial equations with and without the 'method of fourth differences' as well as a single exponential function (Lanczos, 1967) did not meet with success. These calculations were carried out with the aid of a Hewlett-Packard Model 9100B programmable desk-top calculator. The results indicated that the final Scatchard plot was extremely sensitive to slight

variations in the value of the slopes of the curvilinear plots of Dt versus time which are required to compute the instantaneous rate at a value of Dt. This point has not been emphasized enough nor examined in sufficient detail in previous publications.

1. Application of various empirical equations to theoretical kinetic dialysis data

The results obtained from the independent applications of EQUATIONS V-6, V-7, V-8, V-9 and V-10 to the theoretical binding data are illustrated in the form of Scatchard plots in Figs. 35 - 39, respectively. The solid curves represent the theoretical Scatchard plot based on the published values for n's and k's (Meyer and Guttman, 1968b;1970) while the symbols in each graph represent the binding data ($\bar{\nu}$ and $\bar{\nu}/Df$) obtained from the application of the particular function used to fit the curvilinear plot of Dt versus time and subsequent computation of the slopes of this curve for various values of time.

Inspection of these various Scatchard plots indicates that the use of the tri-exponential equation (EQUATION V-6) resulted in the best representation of the theoretical binding curve for phenol red-BSA (Fig. 37). In spite of the fact that this is an empirical equation, the excellent reproduction of the data indicates the applicability of this equation to fit the binding curves obtained from the dynamic dialysis system. Figs. 36 and 38 suggest that the use of either the bi-exponential (EQUATION V-9) or the tri-exponential (EQUATION V-10) also results in a reasonable approximation of the data. However, in view of the fact that the intercept on the abscissa gives Σn_i whereas the ordinate intercept gives $\Sigma n_i k_i$, subsequent application of this data (values of $\bar{\nu}/Df$ versus $\bar{\nu}$) to the hyperbolic regression program for estimates of n's and k's leads to erroneous values for these binding parameters. This is due to the deviations at the ends of the Scatchard curves. Fig. 35 also indicates that erroneous results may be obtained when the data from the curvilinear plot of Dt versus time is fitted

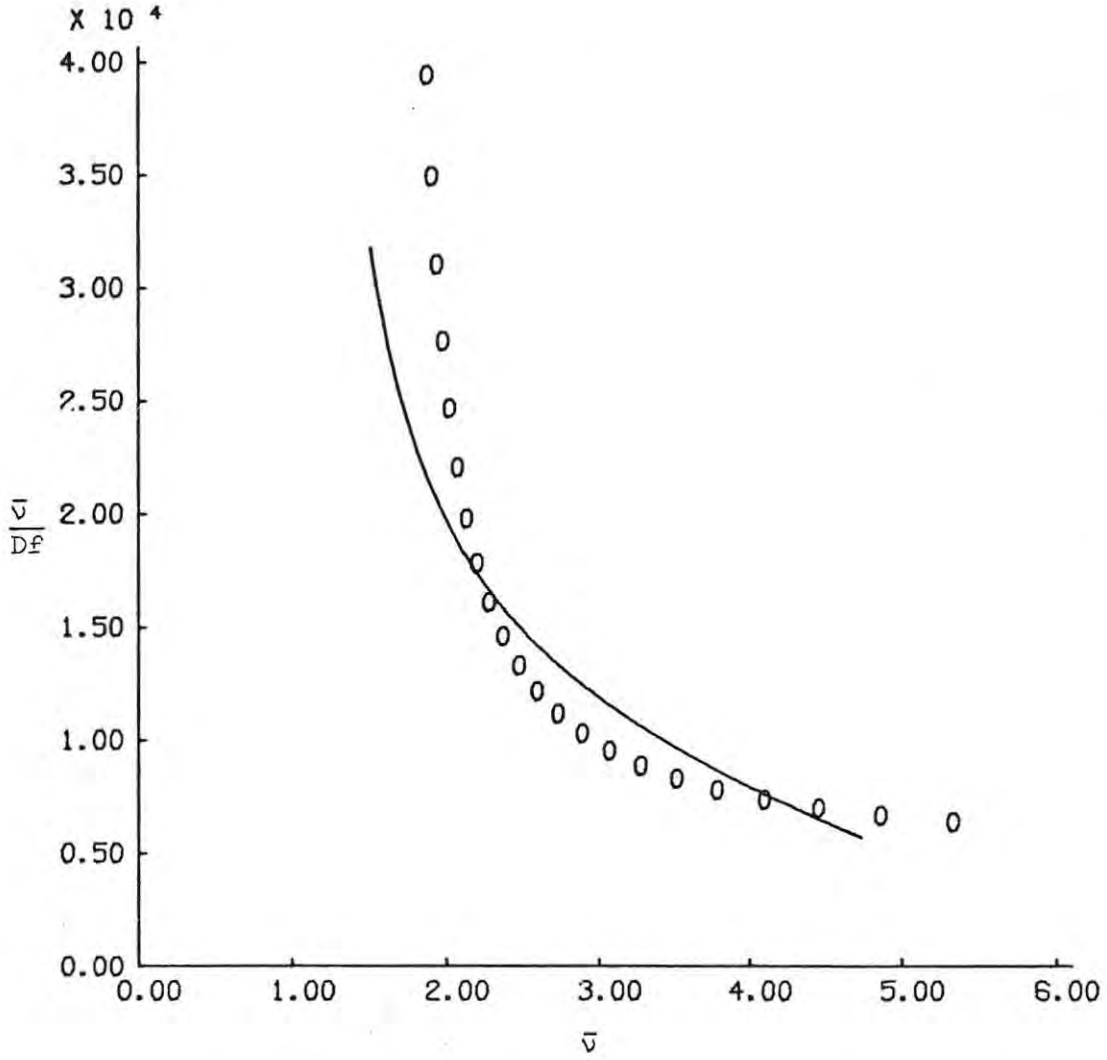

Fig 35. Scatchard plot of the binding data (O) obtained from fitting theoretical values of Dt and time to EQUATION V-7 ($Dt = Ae^{-Bt} + C$). The solid curve was generated from the binding parameters, $n_1 = 1$; $n_2 = 6$; $k_1 = 1.74 \times 10^5$; $k_2 = 1.97 \times 10^3$.

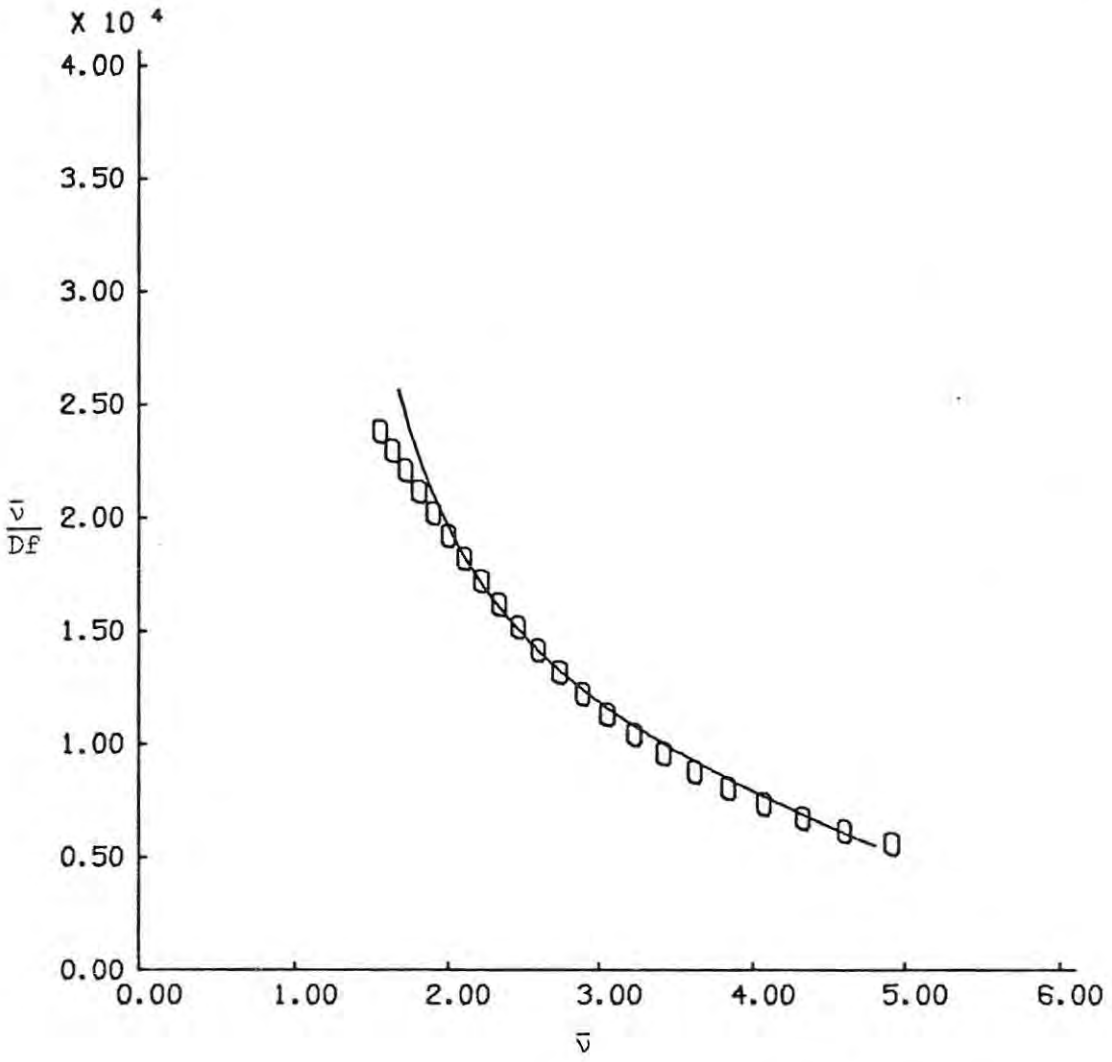

Fig. 36. Scatchard plot of the binding data (O) obtained from fitting theoretical values of Dt and time to EQUATION V - 9 ($Dt = Ae^{-at} + Be^{-bt}$). The solid curve was generated from the binding parameters, $n_1 = 1$; $n_2 = 6$; $k_1 = 1.74 \times 10^5$; $k_2 = 1.97 \times 10^3$.

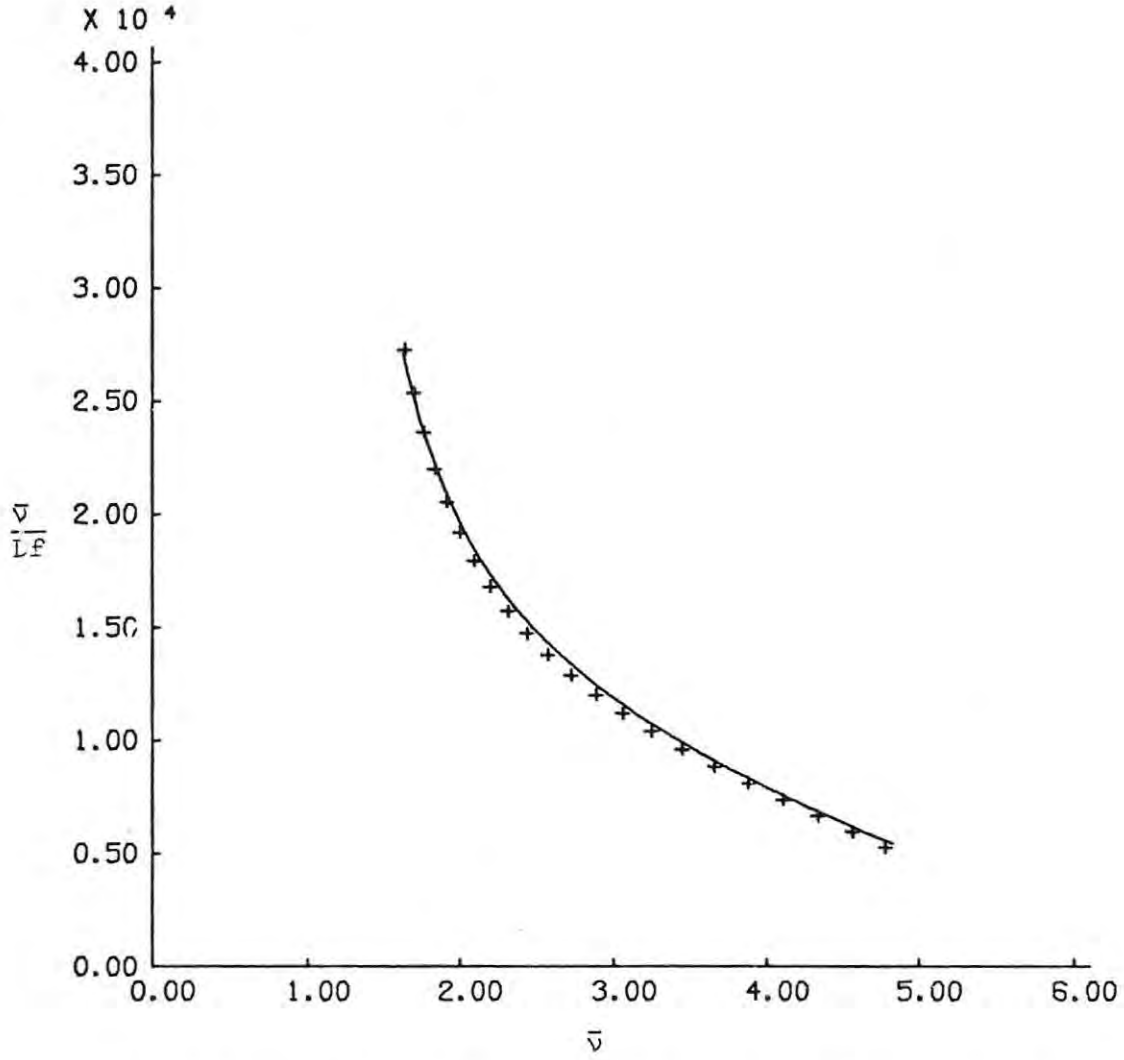

Fig. 37. Scatchard plot of the binding data (+) obtained from fitting theoretical values of Dt and time to EQUATION V - 6 ($Dt = Ae^{-at} + Be^{-bt} + Ce^{-ct}$). The solid curve was generated from the binding parameters, $n_1 = 1$; $n_2 = 6$; $k_1 = 1.74 \times 10^5$; $k_2 = 1.97 \times 10^3$.

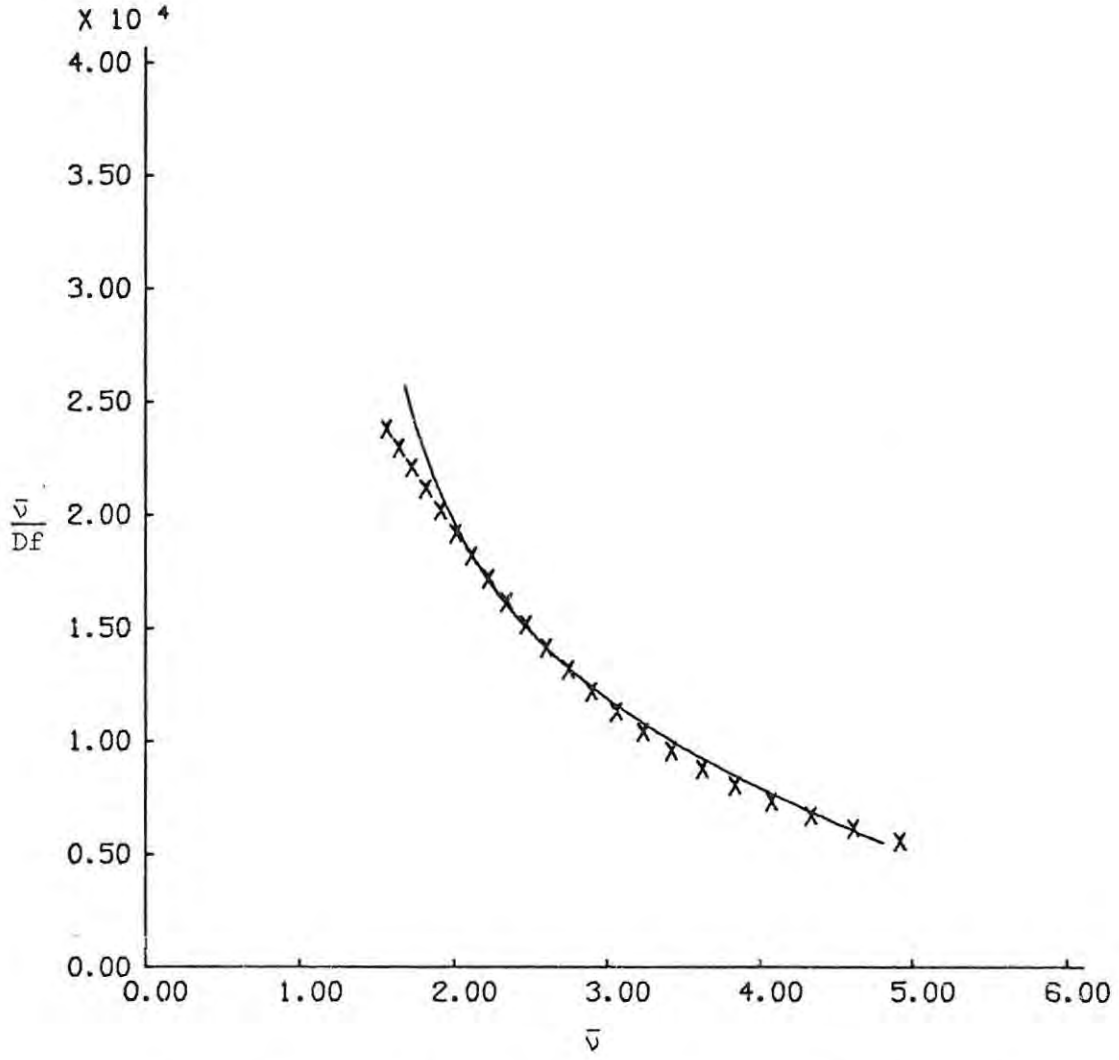

Fig. 38. Scatchard plot of the binding data (X) obtained from fitting theoretical values of Dt and time to EQUATION V - 10 ($Dt = Ae^{-at} - Be^{-bt} + Ce^{-ct}$). The solid curve was generated from the binding parameters, $n_1 = 1$; $n_2 = 6$; $k_1 = 1.74 \times 10^5$; $k_2 = 1.97 \times 10^3$.

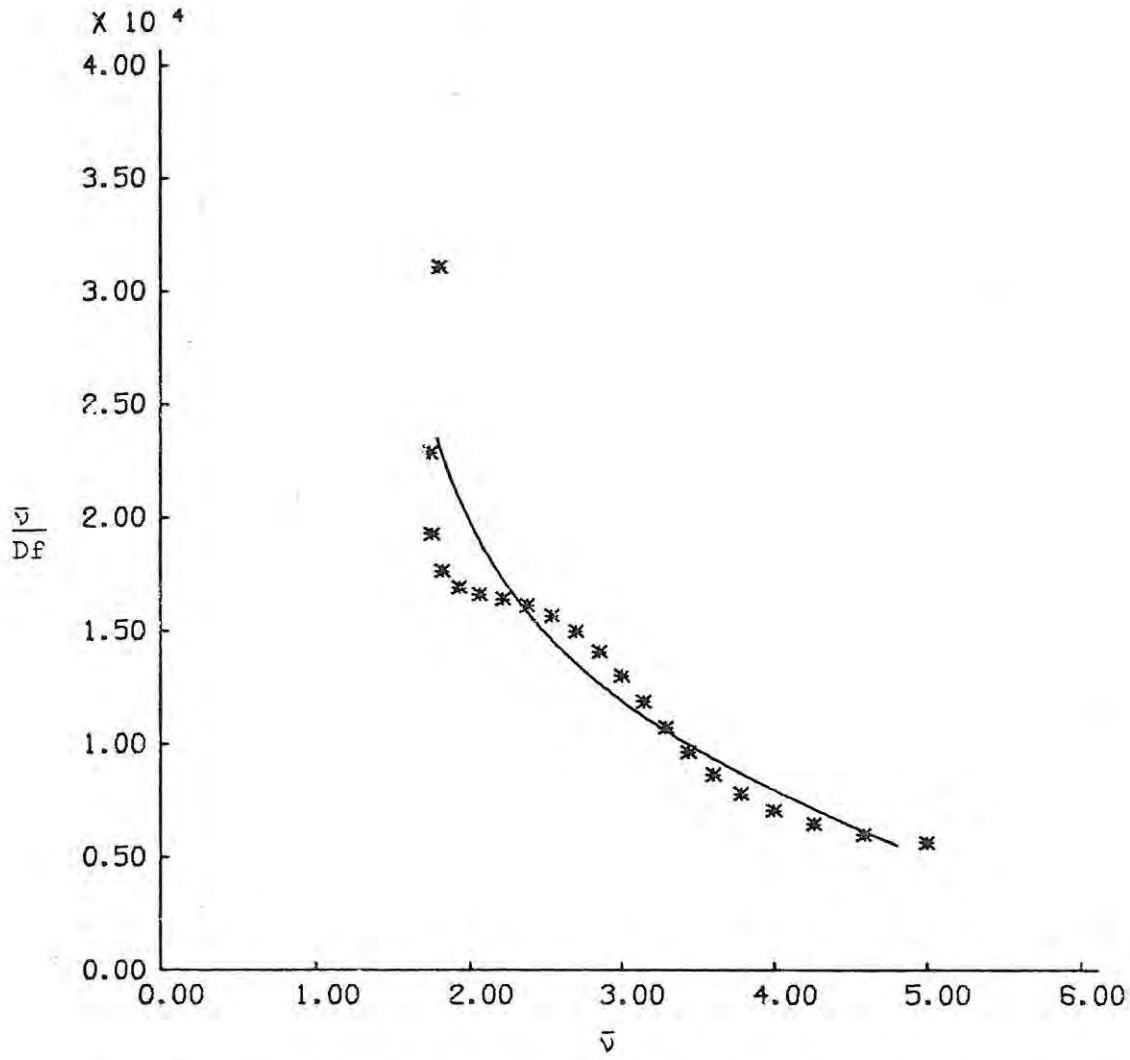

Fig. 39. Scatchard plot of the binding data (*) obtained from fitting theoretical values of Dt and time to EQUATION V - 8 ($Dt = Dt^{o} + at + bt^{2} + ct^{3} + dt^{4}$). The solid curve was generated from the binding parameters, $n_1 = 1$; $n_2 = 6$; $k_1 = 1.74 \times 10^{5}$; $k_2 = 1.97 \times 10^{3}$.

to EQUATION V-7. When EQUATION V-8 was used, a relatively poor representation of the data was obtained. Two computer programs were used for the polynomial fitting, viz. direct method and the method using using orthogonal polynomials (Hamming, 1973). Fig. 39 depicts the Scatchard plot obtained from the latter method. Using the direct method, the sum of the squares of the deviations were found to be significantly greater than the method involving orthogonal polynomials and these results were thus not further considered. Table XV lists the 'goodness of fit', with respect to the application of the various empirical equations, on the basis of the respective sum of the squares of the deviations. It is evident from this table that the 'best fit' was obtained using the tri-exponential function (EQUATION V-6).

The excellent agreement of the Scatchard plot obtained when EQUATION V-6 was used confirms the validity of the treatment of all the kinetic dialysis data described in Chapter IV.

TABLE XV

Summary of the sum of squares of the deviations (SSQ. OF DEV.) from the fitting of kinetic dialysis data to various empirical equations

Function	Equation No.	SSQ. OF DEV.	Fig. No.
$Dt = Ae^{-Bt} + C$	V-7	5.11×10^{-5}	35
$Dt = Ae^{-at} + Be^{-bt}$	V-9	3.36×10^{-6}	36
$Dt = Ae^{-at} + Be^{-bt} + Ce^{-ct}$	V-6	1.42×10^{-6}	37
$Dt = Ae^{-at} - Be^{-bt} + Ce^{-ct}$	V-10	3.36×10^{-6}	38
$Dt = Dt^{o} + at + bt^{2} + ct^{3} + dt^{4}$	V-8	1.04×10^{-5}	39

2. Non-linear curve fitting by least squares

When a parameter changes value slightly, the computed model curve also changes. Based on such changes (i.e. the partial derivatives of the model with respect to the parameters) the object of the exercise is to compute parameter changes that approach a local solution (minimum) at a reasonable rate.

Using least squares as a 'goodness of fit' criterion, the sum of squares of the deviations can be expressed as a function of the model parameters. This function represents, geometrically, a surface in the parameter space. The objective of the procedure is to find the lowest point on this surface. Two fundamental strategies are employed. At first the surface is assumed to be bowl-shaped and a computational procedure is used to calculate an approximate minimum point, (i.e. the bottom of the bowl). When this procedure fails, the procedure is altered to take small steps downhill in order to effect convergence. A compromise between these two strategies usually converges to some local extremum. A solution set (set of best-fitting parameters) for a non-linear problem is a vector around which the sum of squares seems to grow larger. There may be many such points, however, in a single curvilinear plot. When one is in the centre of a 'bowl', one cannot tell if there are other 'bowls' with even deeper centres, hence the occurrence of multiple minima in the curve.

It appeared, during the present studies involving the use of EQUATION V-6 that most of the kinetic curves involved 'multiple minima' (Meyer, 1972). When relatively poor initial estimates of the parameters were used, a minimum was favoured which did not result in the least sum of the squares of the deviations. This was evident when either a poor fit was obtained or when the computer program terminated without a solution being reached. Furthermore, the fact that there is no unique solution set (set of best-fitting parameters) was confirmed when several solution sets to EQUATION V-6 were

obtained with identical data but using different initial estimates. Each set was associated with almost identical sum of squares of the deviations and resulted in almost identical Scatchard plots. Although solution sets for tri-exponential equations having different sign conventions (e.g. EQUATION V-10 and other sign variations in either the coefficients or exponents of the tri-exponential equation) resulted in relatively small sum of squares of the deviations, the subsequent respective Scatchard plots suffered from the same anomalies illustrated in Figs. 35, 36, 38 and 39.

The algorithm upon which the non-linear regression computer program was based, involved a procedure whereby the trials ended and a new iteration began whenever the sum of squares of the deviations ceased to decrease. Moreover, whenever these sum of squares failed to decrease by an arbitrary amount, fixed within the program, the process was terminated.

3. Strategies employed to facilitate curve fitting

As a result of the difficulties encountered as described previously, three procedures were utilized to facilitate the fitting of the kinetic curve. These procedures were designed in order to obtain reasonably good initial parameter estimates.

i) Strategy No. 1

The value for A (EQUATION V-6) was chosen to correspond to the initial concentration of corticosteroid utilized in the particular binding run - $\pm$10% was found to be suitable. The value for the exponent (a) was set at $\pm$10% of the value obtained from the slope of the control run (i.e. negative value). One tenth of the value of A was chosen for B and the exponent (b) was set at one tenth of exponent (a). Finally C and c were given very small values (0.01 - 0.001).

ii) Strategy No. 2

The curvilinear plot of Dt versus t was initially fitted to a single exponential equation. The solution set for this fit was then used as the

initial estimates for EQUATION V-7. The value of C for this equation was kept small. This subsequent solution set was used as the initial estimates for EQUATION V-9, exponent (b) once again, being kept small. Finally, this solution set was used as the initial estimates for EQUATION V-6, the unknown terms, again having small values.

iii) Strategy No. 3

When both strategies 1 and 2 failed, the fit was attempted with numerous first estimates and the various solutions were inspected for the least sum of squares of the deviations. No solution sets were used when the sum of squares of the deviations was greater than 1×10^{-5}.

The necessity of allowing the computer fitting to go through sufficient reiterations to accurately fit the data cannot be overemphasized. In a number of instances, an excellent fit was only accomplished after over 1000 trials by the computer.

iv) Evaluation of n's and k's using a non-linear hyperbolic regression program

Difficulties were also experienced during the computer analysis of the Scatchard data. Once again, the final solution appeared to be influenced by the initial parameter estimates.

The initial estimates of the parameters for this equation were obtained from the shape of the Scatchard plot. These values were obviously rather crude (Klotz, 1973; 1974). In view of the fact that the range of data, obtained experimentally for $\bar{\nu}$, was relatively small in terms of the model for 2 classes of binding sites, additional $\bar{\nu}$ and Df values were extrapolated for extended values of time. Usually, thirty pairs of data points were employed. This data was processed with the aid of a Hewlett Packard model 2100A computer and a Fortran IV program. The parameters obtained in this way were then used as initial estimates in another Fortran IV program, processed with the aid of an ICL 1901A computer. In this instance, the extrapolated values were discarded, and only data pairs obtained experimentally

were utilized. The excellent agreement between the calculated values and the observed values is depicted in all the Scatchard plots in Chapter IV. Figs. 35, 36, 38 and 39 indicate that the procedure involving extrapolation may not be applicable when EQUATIONS V-7, V-8, V-9 or V-10 are used to obtain $\bar{\nu}$ and Df values due to the deviations at the ends of the respective Scatchard plots.

In view of the difficulties encountered with the various curve fitting procedures, it is apparent that non-linear models, strictly speaking, should be used only when there is a theoretical basis for them, or when a non-linear model is most efficient in reproducing a set of data.

Finally, it appears appropriate to mention that over 100 hours of 'computer running time' was used during the processing of experimental data obtained during these dynamic dialyses studies.

The various computer programs employed are summarized in the Appendix.

CHAPTER VI

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GENERAL DISCUSSION

The relationship between chemical structure and biological function of anti-inflammatory corticosteroids has received much attention (Liddle and Fox, 1961; Tolksdorf, 1961; Sarett et al., 1963; Schlagel, 1965; Place et al., 1970). In spite of this data, the primary mechanism whereby the various chemical modifications exert quantitative changes in anti-inflammatory activity has yet to be elucidated.

The initial studies were undertaken in order to determine the effects of several steroidal anti-inflammatory agents on collagen fibrillogenesis in vitro. The fact that these corticosteroids are powerful anti-inflammatory agents and that these drugs precipitate collagen fibrils from collagen solutions suggest that studies of this nature could provide useful information regarding their interaction with this structural protein. Differences in the rates of collagen precipitation in the presence of several corticosteroids have been attributed to the presence or absence of various substituents on the basic corticosteroid nucleus (Fig. 7).

Enhancement factors have been assigned to the various corticosteroid substituents (Sarett et al.,1963). These factors measure the potency increment that a particular function contributes to the anti-inflammatory agent's activity. It is emphasized, however, that these factors permit only a rough prediction of the activity of the various corticosteroids. Moreover, Ringler et al. (1961) observed that the enhancement factors generally tend to decline as one calculates them in relationship to more potent compounds. It is also presumed that each substituent exercises its effect on potency in an independent manner within the accuracy limits of the assays and of the factors.

The following table illustrates the potencies of the relevant corticosteroids calculated on the basis of enhancement factors assigned to the various substituents from rat hydrocortisone potencies and from clinical sources (Sarett et al., 1963).

TABLE XVI

Activity estimates calculated from enhancement factors (Sarett et al., 1963).

Corticosteroid	Anti-inflammatory (Rat)	Anti-inflammatory (Man)
Betamethasone	43	100
Dexamethasone	36	80
Paramethasone	36	20
Prednisolone	3	4
6α-Methylprednisolone	6	5.2
Hydrocortisone	1	1

The order of increasing fibril precipitation rate found experimentally in vitro is hydrocortisone, prednisolone and betamethasone. This order corresponds to the order of increasing anti-inflammatory activity of these drugs as shown in Table XVI. Similar agreement is found between hydrocortisone and 6α-methylprednisolone. When the effect of dexamethasone, paramethasone and betamethasone on fibril precipitation are considered, it is seen that the effect of paramethasone falls between that of betamethasone and dexamethasone. The activity estimates from rat hydrocortisone potency, however, are the same for both paramethasone and dexamethasone, whereas the former was found to increase the rate of collagen precipitation compared to the latter corticosteroid.

The results of the fibril precipitation studies indicated that the various corticosteroids had the ability to precipitate fibrils from collagen solutions at various rates which depended upon the nature of the particular chemical modification of the drug molecule. Even more significant was the fact that these drugs could precipitate collagen on their own (i.e. in the absence of initiating buffer). As a result of these studies, further investigations were undertaken in order to determine the number of binding

sites involved and the corticosteroid affinity for these sites.

The number of sites at pH 3.0 was always found to be higher than those at pH 7.0. This indicates the involvement of electrostatic forces which can be partially reconciled with the anionic nature of the corticosteroid ester phosphates and the presence of charged basic groups on the collagen molecule at acid pH. The values for the number of sites in the first class, obtained from these studies are, however, difficult to reconcile with the number of basic groups present in the collagen molecule under various pH conditions. Recent studies on the amino acid sequence of collagen (Hulmes et al., 1973) have indicated that each of the three chains which comprise the collagen molecule, consist of approximately 1 000 amino acid residues giving a total of about 3 000 amino acid residues per molecule. Although the exact amino acid sequence of the α_2 chain awaits elucidation, it is presumed that the proportions of the various residues in this chain are similar to those in the α_1 chains (Piez et al., 1972; Fietzek et al., 1972). In the light of this information and the evidence provided by Harrington and von Hippel (1961); Tristram and Smith (1963); Piez (1967); Eastoe (1967); Butler et al. (1967); & Traub and Piez (1971), the number of basic groups (arginine, lysine, hydroxylysine and histidine) appears to be between 80 and 100 per 1 000 residues. This indicates that each collagen molecule comprises 240 - 300 basic groups, most of which are charged at pH 3.0 (Bowes and Kenten, 1948; Gustavson, 1956). Hence, the number of class 1 binding sites determined from the dynamic dialysis studies account for approximately one third of the total basic groups (pH 3.0). The results at pH 7.0 are even more difficult to reconcile with the availability of the various reacting groups in collagen. In view of the extremely complex physico-chemical properties of collagen and the probability of the involvement of additional (multiple) non-ionic interactions as previously discussed in the relevant sections of this dissertation, additional information

is required in order to implicate specific reacting sites on the protein molecule. Fig. 40 illustrates the possible reactive sites between the corticosteroids studied and collagen.

In conclusion, it should be noted that although most of the discussions pertaining to the nature of the corticosteroid-collagen interactions, in the light of the present results, have been based on the findings of Westphal (1961; 1971), reports exist which contradict these assumptions. The comprehensive reports on steroid-protein interactions (Westphal, 1961; 1971) emphasizes the importance of the chemistry of the rear side (α-face) of the corticosteroid molecule in interactions with proteins. The reports by Bush (1962; 1967), however, suggest that the upper side (β-face) may be involved. This hypothesis assumes a fit between the tissue receptor and the space-occupying hydrocarbon skeleton of the corticosteroid. The polar groups of the corticosteroid may thus serve as primary (hydrogen-bonding by 11β-hydroxyl group) and secondary attachment points and as contributors to drug specificity.

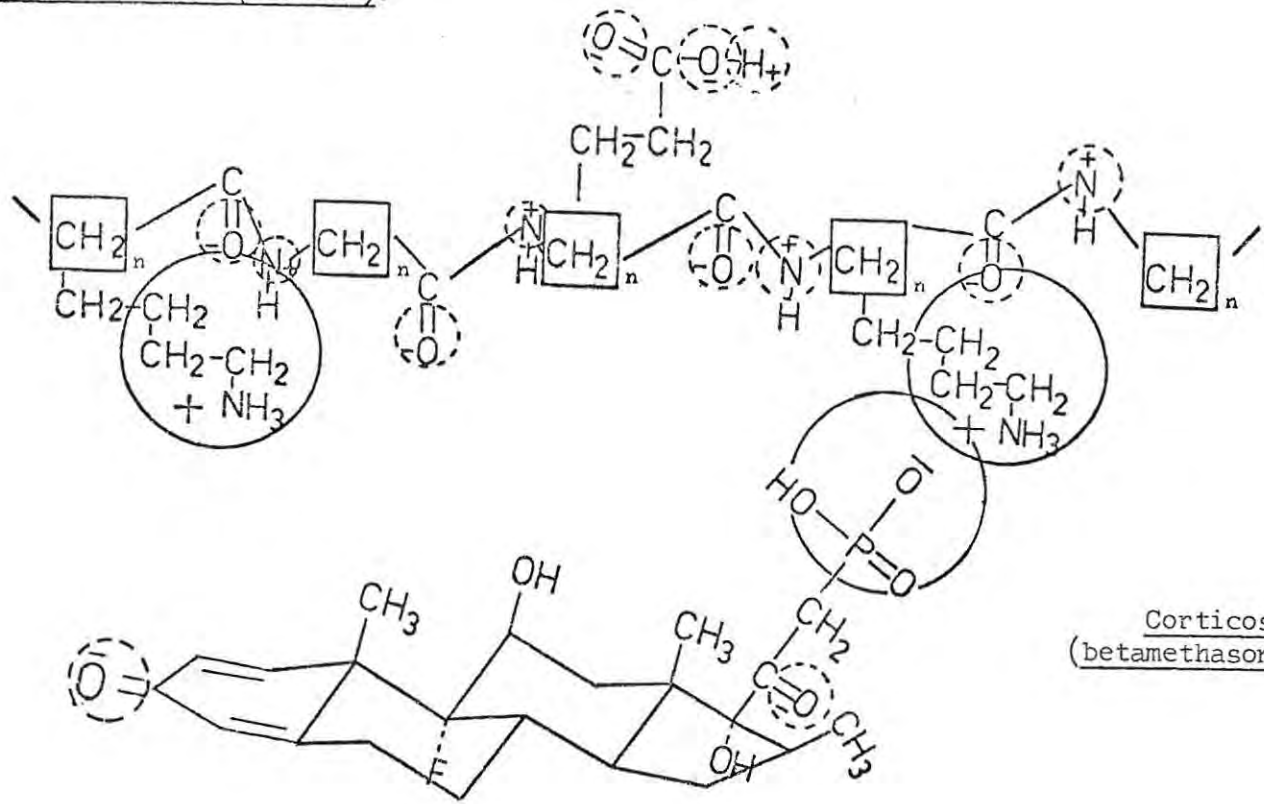
Schlagel (1972) has emphasized that molecular modification of the corticosteroids alters the anti-inflammatory potency as much through changes in intrinsic activity as through alterations of the partition coefficients and the biological availability mechanism. Schlagel's argument is based on biopharmaceutical considerations involving topically applied corticosteroids. Three main ways whereby the various chemical modifications may affect corticosteroid potency have been suggested by Schlagel (1972). These are:

1) Influence on intrinsic activity at the receptor site;

2) influence on the extrinsic factors that determine the relative availability and concentration of the corticosteroid at the site of action; and

3) combination of 2 and 3 above.

Collagen molecule (section).



Corticosteroid molecule
(betamethasone disodium phosphate).

Fig. 40. Diagrammatic representation of a corticosteroid (betamethasone disodium phosphate) molecule approaching the collagen molecule in solution (pH 3). The corticosteroid has an ionic group at the right end of the molecule which exerts a large negative field, illustrated by the big circle. The anionic group can be attracted by a cationic group of the protein molecule. On both molecules are several small circles which depict the presence of weak polar forces. The large non-polar β-surface of the corticosteroid molecule provides a hydrophobic bonding surface. If both molecules make contact, there may be multiple interactions between all these forces. In addition, the presence of the 9α-F atom increases the tendency of hydrogen bond formation with the protein via the 11 - OH group (i.e. the acidity of the 11 - OH is strengthened). In the left part of the corticosteroid molecule there is no electrovalent group which can suppress the weakly polar forces and the two molecules may therefore interact without restriction.

Biopharmaceutical considerations also suggest that chemical modification of the corticosteroid molecule may provide metabolic protection with subsequent manifestation of an increase in anti-inflammatory activity (Glen et al., 1957; Sarett et al., 1963). This fact, however, may be more critical for systemic than for topical potency and more important for determining the ultimate concentration of the active drug that reaches the site of action. This conclusion is partly deduced from the fact that the protection from metabolic inactivation imparts appreciably increased systemic potency in compounds such as prednisolone and dexamethasone when compared with topical application (Schlagel, 1965).

In spite of the conflicting data available regarding the nature of the mechanism of action of corticosteroids and although the complexities of inflammation have not yet been resolved, the numerous results and descriptive accounts of various corticosteroid-protein interactions at the molecular level provide useful guidelines for fruitful discussion and future investigations of the action of these steroidal anti-inflammatory agents.

APPENDIX

1. Program FIBRL (BASIC) - original program written to calculate values of p from E and E_∞, I from these values of p, and a linear least squares regression of I versus time (t) according to EQUATION III-7 from fibril precipitation data. The same program gave the correlation coefficient for the linear least squares regression and computed the values of K_2 and t_c. In addition, the computed values for these parameters were used in the same program to calculate the smooth curve relationship between p and t. The program was run on a Hewlett Packard Model 2100A computer.

2. Program AM02 (FORTRAN IV) - obtained from Dr. P. Terry* and used to plot the data of E versus time and p versus time (experimental and smooth values), obtained from the fibril precipitation studies. A Calcomp 563 graph plotter was used in conjunction with an ICL 1901A computer.

3. Program SPEC - written for and used with a Hewlett Packard Model 9100B programmable desk-top calculator to compute Dt values from spectrophotometric data obtained from the dynamic dialysis studies.

4. Program TRIEXP - 1 (FORTRAN IV) - obtained from Dr. D.W. Marquardt** and modified to run on an ICL 1901A computer. This program was used during the initial dynamic dialysis investigations to fit the Dt and time values to a tri-exponential equation.

5. Program Linear Regression Analysis (Hewlett Packard 9100 series library routine) - used to calculate the slope of the control from the dynamic dialysis studies with the aid of a Hewlett Packard Model 9100B programmable desk-top calculator.

6. Program TRIEXP - 2 (FORTRAN IV) - obtained from Dr. M.C. Meyer*** and

written in FORTRAN II. It was therefore modified to FORTRAN IV and adapted to run on an ICL 1901A computer. This program was subsequently used exclusively to fit Dt and time values from the dynamic dialysis studies to a tri-exponential equation. It was also used to fit theoretical kinetic binding data to EQUATIONS V - 7, V - 9 and V - 10.

7. Programs POLY - D (direct method) and POLY - O (orthogonal polynomials) - (FORTRAN IV) - obtained from Dr. P. Terry* and used to fit theoretical Dt and time values to a fourth order polynomial equation (EQUATION V - 8) with the aid of an ICL 1901A computer.

8. Program DATA (FORTRAN IV) - an original program written to compute the slopes of various empirical equations and then the values of $\bar{\nu}$ and Df with the aid of an ICL 1901A computer.

9. Program AMGRAPH (FORTRAN IV) - obtained from Dr. P. Terry* and incorporated into program DATA in order to plot the Scatchard curves using a Calcomp 563 graph plotter in conjunction with an ICL 1901A computer.

10. Program HYPE - 1 (FORTRAN IV) (Hewlett Packard FORTRAN IV library routine) - used to fit the binding data to EQUATION IV - 10 for 2 classes of binding sites (non-linear hyperbolic regression). Extrapolated values of $\bar{\nu}$ and Df obtained from program DATA were included and the computation carried out on a Hewlett Packard Model 2100A computer. The parameters thus obtained were used as the initial parameter estimates for program HYPE - 2.

11. Program HYPE -2 (FORTRAN IV) - obtained by modifying program TRIEXP - 2 to fit the $\bar{\nu}$ and Df values to EQUATION IV - 10 for 2 classes of binding sites. The initial parameter estimates were obtained from program HYPE - 1 and only experimentally obtained values were used. This program was run on an ICL 1910A computer.

12. Program ESCAP (BASIC) - an original program written to calculate theoretical kinetic binding data with the aid of a Hewlett Packard model 2100A computer.

13. Program FORT (FORTRAN IV) - an original program written to compute binding data from the binding parameters obtained from program HYPE - 2. Program AMGRAPH was incorporated into this program in order to plot the computed smooth curve superimposed on experimental binding data (Scatchard plots) using a Calcomp 563 graph plotter in conjunction with an ICL 1901A computer.

* Dr. P. Terry, (Dept. of Applied Mathematics, Rhodes University, Grahamstown 6140, South Africa.)

** Dr. D.W. Marquardt, (Engineering Service Division, E.I. du Pont de Nemours & Co. (Inc.), Wilmington, Delaware 19898, United States of America.)

*** Dr. M.C. Meyer, (Dept. of Medicinal Chemistry, College of Pharmacy, The University of Tennessee, Memphis 38103, United States of America.)

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