1986

Collagen fibril formation dexamethasone.pdf

George W. Dombi, *University of Rhode Island*
Brian H. Halsall

Connective Tissue Research, 1986, Vol. 15, pp. 257–268
0300-8207/86/1504–0257/$20.00/0

Printed in the United States of America

COLLAGEN FIBRIL FORMATION IN THE PRESENCE OF DEXAMETHASONE DISODIUM PHOSPHATE

GEORGE W. DOMBI

Department of Chemistry, University of Cincinnati, Cincinnati, Ohio 45221

(Received June 28, 1985; accepted December 2, 1985)

Dexamethasone disodium phosphate was found to inhibit *in vitro* fibrillogenesis in a buffered collagen solution that otherwise formed *in vivo* like fibrils. A nonlinear relationship was observed between the steroid salt concentration and the kinetic parameter half transition time. Full fibril inhibition occurred at dexamethasone phosphate concentrations above 15 *mM*. At lower concentrations, sample buffers that also contained inorganic phosphate were not different from the control in activation energy of 224.3 ± 29.3 kJ/mol (53.6 ± 7.0 kcal/mol). The idea is advanced that the soluble steroid salt associates directly to the collagen and prevents the formation of lateral, hydrophobic interactions between adjacent collagen aggregates.

INTRODUCTION

Natural corticosteroids and their synthetic analogs are widely used therapeutically for their anti-inflammatory and immunosuppressant effects (review, Garber *et al.*, 1981). Because of this, steroids are sometimes used in the treatment of rheumatoid arthritis where an autoimmune response develops with the induction of both humoral and cellular antibodies against collagen (Solinger and Hess, 1982).

The generally irreversible joint damage is due to the infiltration of the tissue by immunocompetent cells which initiate or promote collagen damage by enzymatic degradation (Gardner, 1978). Steroids are used because they reduce the secretion of proteases, including collagenase (Werb, 1978) and stabilize lysosomal membranes thereby blocking the release of hydrolytic enzymes (Weissman and Thomas, 1984).

Prolonged administration of steroids is not without clinical side effects (Kjellstrand, 1975). These include thinning of the skin (Kirby and Munro, 1976) and retardation of wound healing (Lenco *et al.*, 1975). In addition, steroids inhibit cell division and reduce net secretion of collagen by fibroblasts (Uitto *et al.*, 1972). These effects are apparently mediated by steroid receptors in the cytoplasm and nuclei of fibroblasts (Nacht and Garzon, 1974).

Both the anti-inflammatory actions and side effects of steroids are considered to be cell mediated responses. A question exists concerning the effects of steroids on collagen directly. To address that question, this paper describes the influence of a soluble steroid, dexamethasone disodium phosphate, on the rate of collagen fibril formation by monitoring changes in solution turbidity and fibril morphology. The

possibility that the steroid effect is due to disruption of hydropholic interactions is discussed.

METHODS AND MATERIALS

All materials were reagent grade or better. Dexamethasone disodium phosphate, a gift from Merck Chemical Co. (Rahway, NJ), was of the highest quality as measured by thin layer chromatography and was used without further purification.

Collagen preparation

Acid soluble, Type I collagen was isolated from the tail tendons of four week old Holtzman rats by modifying established procedures (Chandrakasans *et al.*, 1976; Williams *et al.*, 1978). All procedures were conducted at 4°C (Dombi, 1984). Tendons were excised and washed in cold 50 *mM* Tris buffer containing the following protease inhibitors: 10 *mM* N-ethylmaleimide, 5 *mM* benzamide HCl and 1 *mM* phenylmethylsulfonyl fluoride. Collagen was extracted by stirring the tendons overnight in 0.5 *M* acetic acid, 2 μg/ml pepstatin A, 2 μg/ml leupeptin at a ratio of one gram of wet tendon per 100 ml of solution. Further purification continued as per Chandrakasan *et al.* (1976) including the use of the "3–4% salt-cut" fractionation step. In order to further minimize the presence of nonspecific aggregates, the purified collagen was centrifuged at 20,000 xg for 60 min. the collagen was adjusted to a final concentration of 0.2 mg/ml with 5 *mM* acetic acid using the specific rotation value $[\alpha]^{rt}_{313} = -2300$ deg ml/(dm g) (Williams *et al.*, 1978). This stock solution was stored at 4°C.

The relative content of collagen components (α, β, γ) was determined by sodium dodecyl sulfate (SDS)-polyacrylamide electrophoresis after heat denaturation at 60°C for 10 min (Laemmli, 1970). The ration of band intensity, based on densitometry of the gels, was 2.6 to 1 between α and β bands and 6 to 1 for α and γ bands.

Fibril formation

Native-type fibrils were formed in a control buffer by mixing 1 ml of stock collagen solution with 1 ml of a double strength salt solution at 4°C to give final concentrations of 30 *mM* N-(tris(hydroxymethyl)methyl-2-amino) ethane sulfuric acid (TES), 30 *mM* Na_2HPO_4, 0.2 *mM* NaCl, 0.1 mg/ml collagen, 2.5 *mM* acetic acid, pH 7.0–7.2 (Williams *et al.*, 1978). Under these conditions, 30 *mM* phosphates is considered optimal for *in vivo* like fibril formation. Sample buffers were of the same composition with 0–30 *mM* Na_2HPO_4 and 0.01–30 *mM* disodium dexamethasone phosphate. Fibril formation was begun by mixing the collagen solution with formation buffer just prior to use. Aliquots of the mixture were loaded in triplicate into a 300 μl microcell at 4°C and rapidly heated to either 22, 24 or 26°C inside a Du-8 spectrophotometer. Turbidity was recorded up to 18 hours by monitoring the optical density at 350 nm. Reblanking was done automatically every 10 minutes against air. The choice of 350 nm was a compromise

TABLE I

Activation Energies

Sample	Buffer	Ea ± 1 SD (kJ/mol)
1	30 *mM* Na_2HPO_4 (control)	224.3 ± 29.3
2	30 *mM* Na_2HPO + 10 *mM* Dex PO_4	229.7 ± 58.6
3	20 *mM* Na_2HPO_4 + 10 *mM* Dex PO_4	225.1 ± 17.2
4	0 *mM* Na_2HPO_4 + 10 *mM* Dex PO_4	284.7 ± 2.5*
5	30 *mM* tris	239.3 ± 24.3
6	30 *mM* Na_2HPO_4	242.7

*Significantly different than sample 1 control $p < 0.05$. Samples 1–4 from this work. Sample 5 from Evans and Drouven (1983). Sample 6 from Williams *et al.* (1978).

between turbidity sensitivity, which increases at lower wavelengths, and interference between the broad dexamethasone absorbance bands centered at and below 290 nm. The resultant fibril formation curves were plotted from the numerical print out of absorbance units recorded at 10 min intervals. The curves represent typical runs and were analyzed by reference to pseudokinetic parameter $t_{1/2}$, the time to half maximum turbidity change.

Electron microscope

At the conclusion of the observation period, 50 µl of sample was retrieved and placed on a 400-mesh carbon-coated copper grid for 1–2 minutes. The grid was slowly drained with filter paper then floated inverted on a 500 µl drop of 1% sodium phosphotungstate, pH 7.2 for 10 minutes. After air drying, the grid was examined in a transmission electron microscope at an acceleration voltage of 80 kV. The mean fibril diameters ± one standard deviation were determined from micrographs present here. Photos were taken at random from a densely populated region of each EM grid.

RESULTS

Fibril formation

Two types of experiments were conducted. In the first, the inorganic phosphate concentration was 30 *mM* and dexamethasone phosphate was added up to 30 *mM*. In the second, the dexamethasone phosphate and inorganic phosphate concentrations were totalled to 30 *mM* with gradual replacement of the inorganic phosphate by the steroid phosphate.

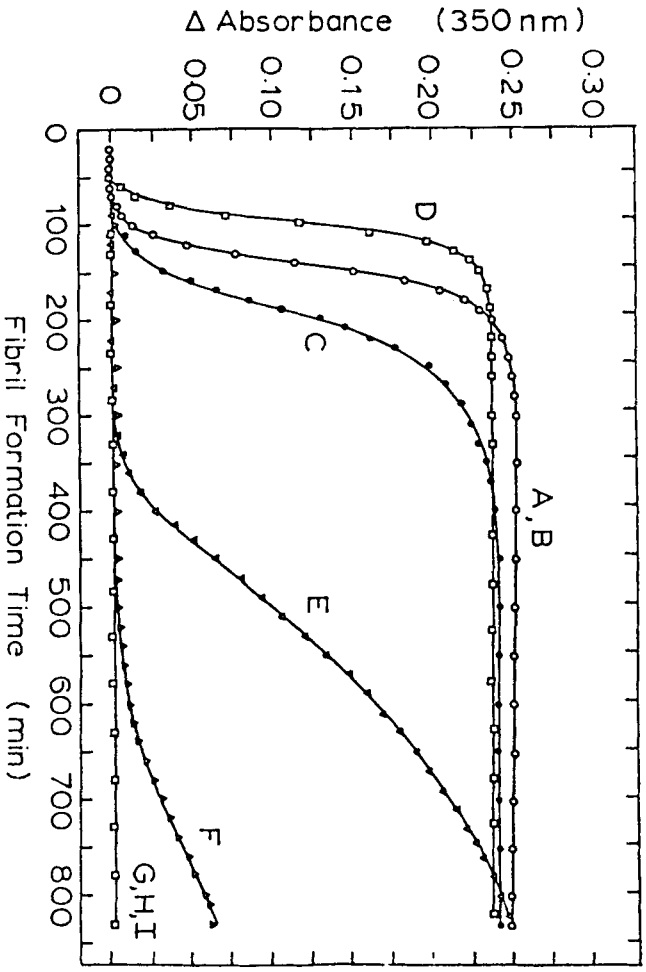

FIGURE 1 Dexamethasone phosphate effect on fibril formation. Turbidity was monitored at 350 mm. Cuvette temperature was maintained at 26°C. All solutions contained 30 *mM* Na_2HPO_4, 200 *mM* NaCl, 30 mM TES, 2.5 *mM* CH_3COOH and 0.1 mg/ml collagen at pH 7.0–7.2. Dexamethasone phosphate was added to concentrations of 0 *mM* (A), 0.01 *mM* (B), 0.1 *mM* (C), 1 *mM* (D), 10 *mM* (E), 15 *mM* (F), 20 *mM* (G), 25 *mM* (H) and 30 *mM* (I).

The extent of fibril formation at variable dexamethasone phosphate concentration at 26°C with 30 *mM* inorganic phosphate is seen in Fig. 1. Line A, represents the turbidity trace of fibril formation in the control buffer containing 0 *mM* dexamethasone phosphate. This line is characterized by a $t_{1/2}$ value of about 150 minutes. Line B, 0.01 *mM* is indistinguishable from the control. Except for line D, 1.0 *mM*, increasing the concentrations of dexamethasone phosphate shows a retardation of fibril formation.

Figure 2 shows the effect of replacing sodium phosphate by steroid phosphate. A trend towards longer fibril formation times was evident with near total inhibition at full replacement. The full change in turbidity was increased to about 30% over the control value.

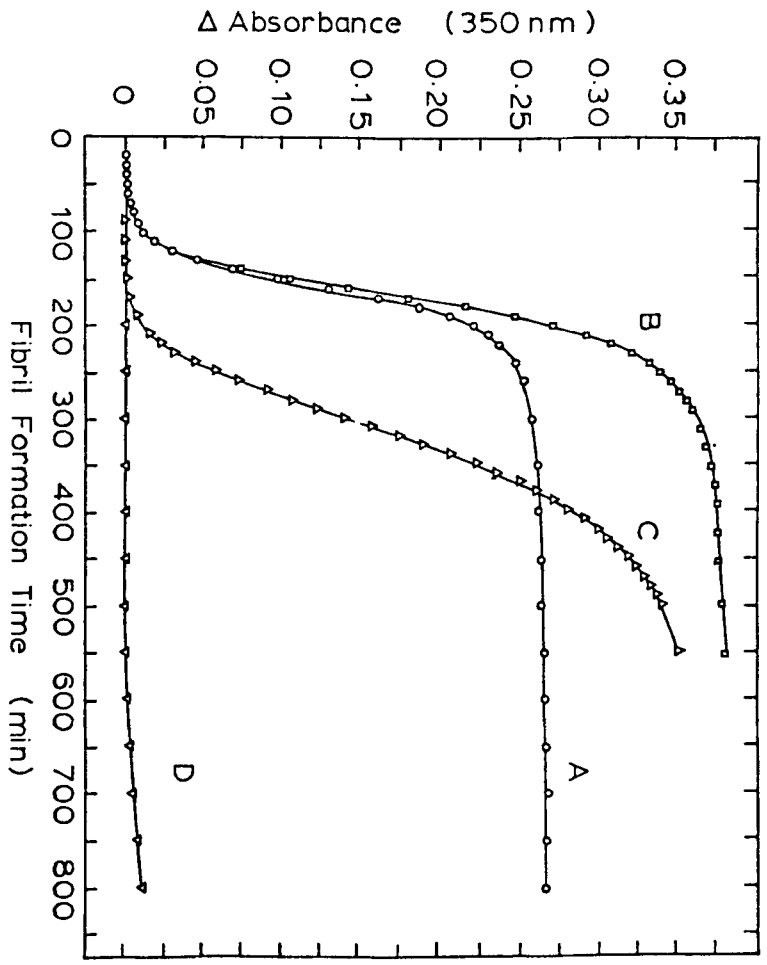

FIGURE 2 Constant phosphate effect on fibril formation. Turbidity was monitored at 350 nm. Cuvette temperature was maintained at 26°C. All solutions contained 200 *mM* NaCl, 30 *mM* TES, 2.5 *mM* CH_3COOH, and 0.1 mg/ml collagen at pH 7.0–7.2. Phosphate concentrations were mixtures of sodium phosphate and dexamethasone phosphates Solutions contained: 30 *mM* Na_2PHO_4, 0 *mM* Na_2 Dex PO_4 (A); 20 *mM* Na_2HPO_4, 10 *mM* Na_2 Dex PO_4 (B); 10 *mM* Na_2HPO_4, 20 *mM* Na_2 Dex PO_4 (C); 0 *mM* Na_2HPO_4, 30 *mM* Na_2 Dex PO_4 (D).

Arrhenius plots

The effect of temperature on fibril formation was examined at 22, 24 and 26°C. This range was used since it is known to produce a linear change in $t_{1/2}$ with no discernable difference in fibril appearance (Williams *et al.*, 1978). An Arrhenius plot comparison of fibrils formed in the control buffer and a sample buffer that contained 10 *mM* dexamethasone phosphate is given in Fig. 3. Energy of activation (Ea) values are presented in Table 1 for a number of fibril formation buffers including two from the literature. The only activation energy that is different from the control value of 224.3 kJ/mol (53.6 kcal/mol) is that of 283.7 kJ/mol (67.8 kcal/mol) where fibril formation occurred in a buffer containing no sodium phosphate and 10 *mM* dexamethasone phosphate.

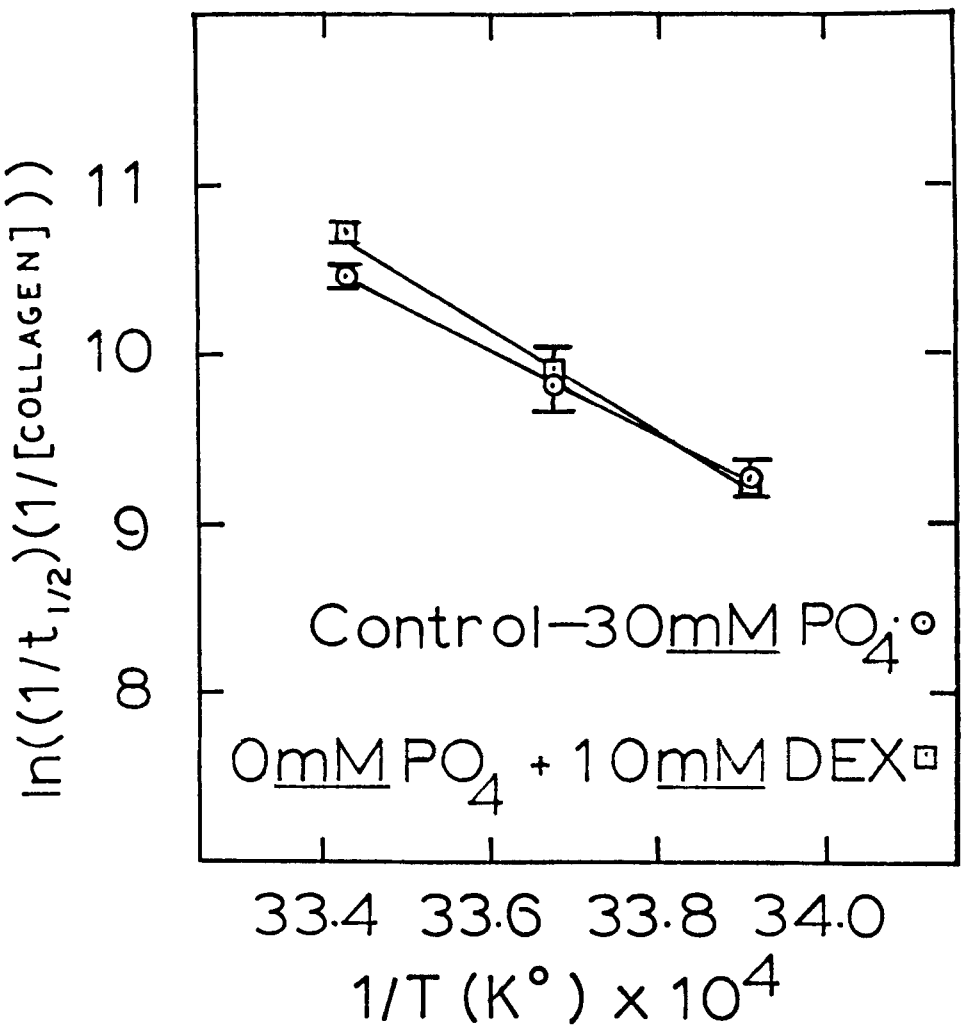

FIGURE 3 Arrhenius plot. Fibril formation was measured at 22, 24 and 26°C. The collagen molar concentration was based on a weight of 300,000 daltons. Both formation buffers contained: 30 *mM* TES, 200 *mM* NaCl and 0.1 mg/ml collagen at pH 7.0–7.2. In addition, the control buffer (0) contained 30 *mM* Na_2HPO_4 and the sample ( ) contained 0 *mM* Na_2HPO_4, 10 *mM* Na_2 Dex PO_4.

Morphology

All fibrils appear native-banded (Fig. 4). The controls formed tactoids with no loose or unravelled portions (C). There is evidence of fusion of separate fibrils resulting in larger fibrils with bands in register.

In the sample containing 30 *mM* Na_2HPO_4, 10 *mM* Dex PO_4 (B) there is also evidence of fusion. There are noticeable areas of loose or unravelled regions particularly near the fibril ends.

In the sample containing 20 *mM* Na_2HPO_4, 10 *mM* Dex PO_4 (A) there is no evidence of loose or unravelled regions as in the controls. The fusion of small

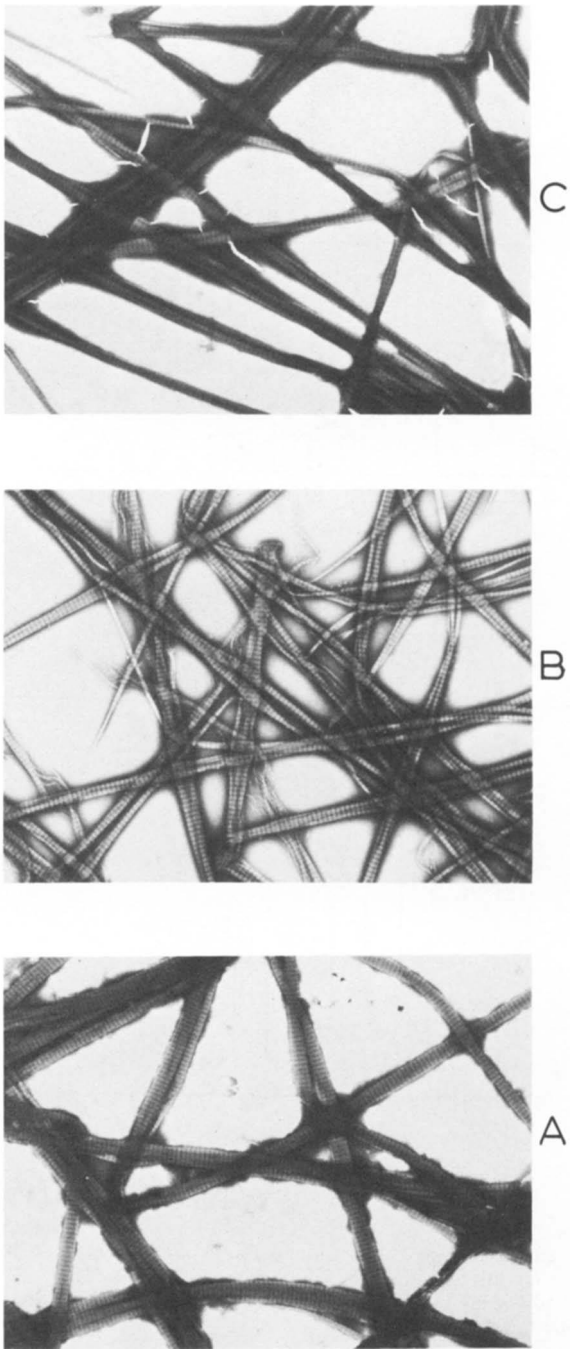

FIGURE 4 Electronmicrographs of negatively stained collagen fibrills. Magnification × 12,000. (A) fibrils formed in a buffer containing 20 *mM* disodium phosphate, 10 *mM* dexamethasone phosphate. Fibrils appear larger than controls due mainly to fusion of smaller fibrils. (B) fibrils formed in a buffer containing 30 *mM* disodium phosphate, 10 *mM* dexamethasone phosphate. Poorly packed regions are seen. (C) a native-banded fibrils formed in the control buffer containing 30 *mM* disodium phosphate.

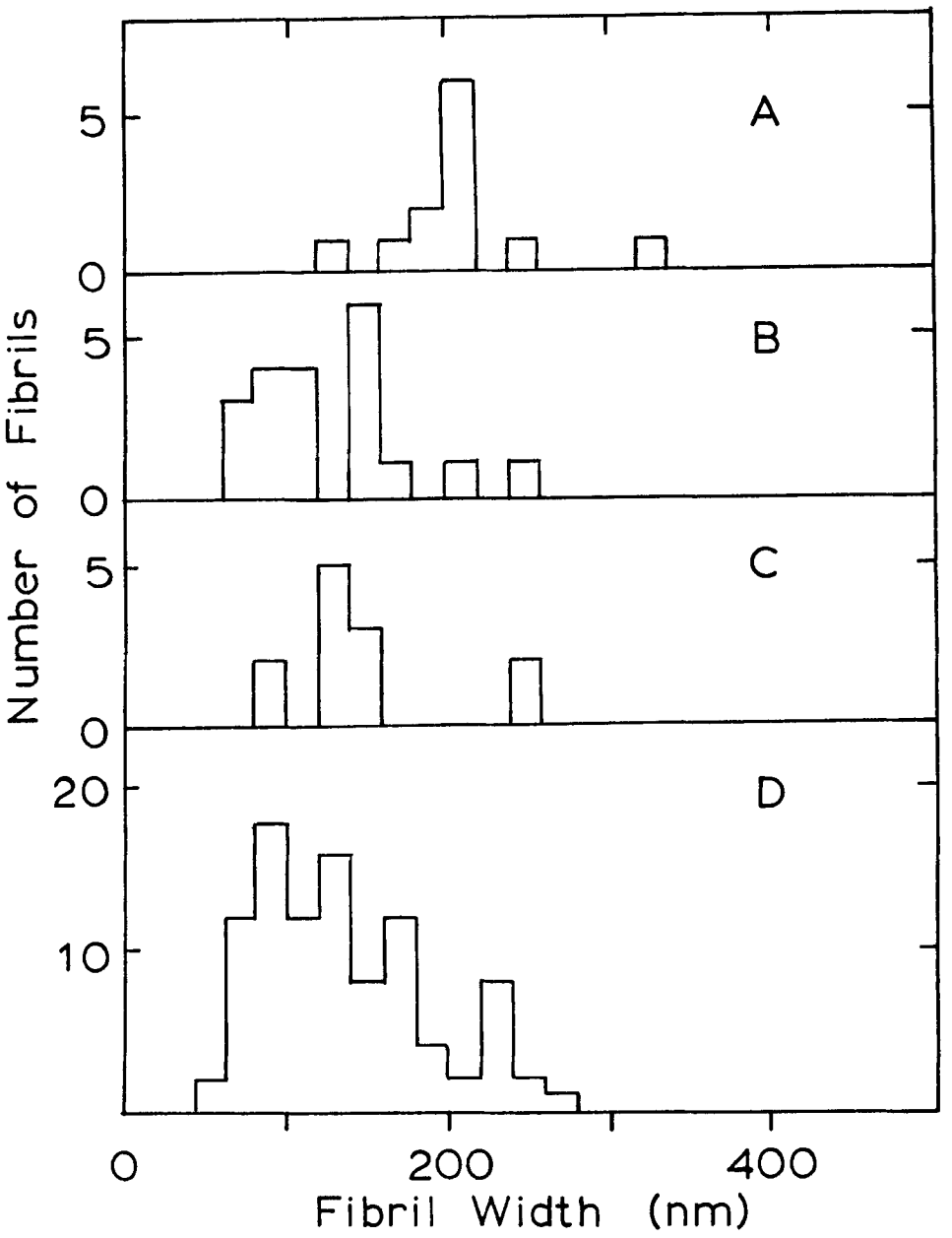

FIGURE 5 Histogram of collagen fibril widths. Fibrils were formed at 26°C with 30 *mM* TES, 200 *mM* NaCl, 0.1 mg/ml collagen, pH 7.0–7.2 plus: A, 20 *mM* Na_2HPO_4, 10 *mM* Na_2 Dex PO_4; B, 30 *mM* Na_2HPO_4, 10 *mM* Na_2 Dex PO_4; C, control, 30 *mM* Na_2HPO_4. Sample D was a literature control data from Bard and Chapman, 1973.

fibrils resulted in the formation of the largest fibrils seen in this study. The fused fibrils were in register. Based on the work of Wood and Keech (1960), it would be expected that if a formation buffer's turbidity change was greater than that in the

control buffer, then the resulting fibrils would be wider than in the controls. This was the case here. Line B on Fig. 2 represents the turbidity trace of the formation buffer that gave the fibrils seen in A of Fig. 4.

Population histograms of fibril widths are presented in Fig. 5. As a method of comparison, the mean width ± 1 standard deviation is determined for each sample. The control fibrils measured 148 ± 52 nm. Those formed in the buffer containing 30 *mM* Na_2HPO_4, 10 *mM* Dex PO_4 were slightly smaller, 127 ± 48 nm, whereas those formed in 20 *mM* Na_2HPO_4, 10 *mM* Dex PO_4 were larger and measured 210 ± 48 nm. These three ranges were similar to values reported by Bard and Chapman (1973), 134 ± 53 nm.

DISCUSSION

When solution turbidity is monitored, the course of collagen fibril formation consists of a lag phase followed by a growth phase that ends in a plateau region (Wood, 1960). During the time course, fibril assembly proceeds by both linear and lateral associative processes which are stabilized predominately by hydrophobic interactions (Hayashi and Nagai, 1972; Williams *et al.*, 1978; Gelman *et al.*, 1979; Silver and Trelstad, 1979; Silver and Birk, 1983). Linear associations predominate during the lag phase. In this phase, turbidity does not change as the collagen molecules assemble to form long filaments. During the growth phase, the filaments aggregate laterally to form banded fibrils. Native banding is a result of collagen molecules in neighboring filaments being aligned in the "quarter-stagger array" (Hodge and Petruska, 1963) which produces the 67 nm distance between bands. Filament aggregation during the growth phase is a entropy-driven process (i.e., hydrophobic process) in which the entropy gain is due to the release of associated water molecules (Cooper, 1970).

The inhibitory effect on fibril formation by dexamethasone phosphate caused an increase in both lag and half transition time. This concomitant change in both parameters is a common feature in fibril formation experiments. Similar dual changes are seen when collagen concentration, solution temperature or ionic strength are varied (Gross and Kirk, 1958; Wood, 1960; Wood and Keech, 1960). As collagen concentration increases, lag and half time decrease. But not only is the amount of collagen important; its intactness is important. Gelatin (thermally denatured collagen) will not form fibrils. Various salts will also denature collagen. It is known that fibril formation is inhibited by the addition of ionic and non-ionic denaturants: inorganic pyrophosphate (Nemeth-Csoka and Tasnadi, 1981); urea and polyoxyethylene stearate (Honya and Mizunuma, 1974); alkylureas (Suarez *et al.*, 1980) and sodium dodecyl sulfate (Dombi and Halsall, 1985). Those studies support the idea that increasing the length of the hydrocarbon chain increases the potency of the inhibitor. This may explain the differences between the inorganic phosphate anion and the dexamethasone phosphate anion.

It has been reported previously (Williams *et al.*, 1978; Hayashi and Nagai, 1972) that inorganic phosphate plays a specific but undefined role in the formation of *in vivo*-like fibrils. Work here indicates that dexamethasone phosphate is a poor substitute in that role since it inhibits fibril formation at the optimal 30 *mM*

concentration and retards the extend of lateral growth at $10\,mM$. The inability for the like charged steroid phosphate to replace the inorganic phosphate may be due to steric constraints.

Although the high ionic strength of the formation buffers reduces the importance of nonspecific electrostatic interactions, it is possible that inorganic phosphate is involved at specific binding sites that would sequester positive side chain charges on adjacent collagen filaments during lateral growth. The reduction of the net positive charge on collagen would assist in the alignment of neighboring molecules into the native "quarter staggered array" under the direction of the hydrophobic domains along the molecules. Dexamethasone phosphate may similarly sequester positive charges on collagen side chains. But the steroid phosphate would either retard close approach of neighboring collagen molecules due to the bulk of the steroid portion of the molecule, or directly interfere with the role of the hydrophobic regions as they align.

A similar mode of action was postulated for the effect of sodium dodecyl sulfate on fibril formation (Dombi and Halsall, 1985). Dodecyl sulfate was found to inhibit fibril formation at $0.5\,mM$ under similar formation conditions. The greater potency of dodecyl sulfate may be due to the greater conformational mobility of the dodecyl sulfate backbone. It is possible that the rigid steroid backbone of dexamethasone phosphate limits the number of possible binding sites on the collagen molecule.

It was previously suggested that among the sites available to dodecyl sulfate binding were the collagen telopeptide regions (Dombi and Halsall, 1985). The nonhelical terminal regions of the collagen alpha chains (telopeptides) play a critical role in fibrillogenesis by adapting specific conformations prior to association (Camper and Veis, 1977; Helseth *et al.*, 1979). These regions represent only about 2% of the collagen molecule, however, if they are removed or selectively denatured, then fibrillogenesis is inhibited. The action of dedecyl sulfate may be at these sites.

It is not known if such sites are available to dexamethasone phosphate. It has been shown, however, that the association of this steroid salt with type I collagen involves two classes of nonequivalent bind sites with affinities of $K_a \simeq 10^5 M^{-1}$ and $10^3 M^{-1}$ at pH 7.0 (Kanfer, 1977). The higher affinity sites binds about 40 moles of steroid salt per mole of collagen and the lower affinity sites bind about 20. The author did not suggest that the two classes of sites represented two functional domains on the collagen molecule. Rather it was suggested that both ionic and hydrophobic interactions were important in the association of dexamethasone phosphate with type I collagen. Because of the larger number of dexamethasone phosphate binding sites and the repetitive nature of the collagen primary structure, one can speculate that the steroid associates up and down the helical chain in a non-specific manner.

Since the morphology of fibrils formed in the presence of dexamethasone phosphate are similar to controls and since the Arrenhius plots are also similar, there appears to be a threshold level for the action of dexamethasone phosphate. That level is about $10-15\,mM$ under the formation conditions which include $30\,mM$ inorganic phosphate. The action of the steroid salt may be to make the individual collagen molecules more soluble by direct association in a surfactant-

like attachment (i.e., the steroid region of the anion attaches to the hydrophobic regions of the collagen and the polar phosphate group is solvated). At low dexamethasone phosphate levels below 10–15 *m*M, it is possible that the collagen molecules move past each other more easily because dexamethasone attachment increases solubility. This could facilitate the achieving of proper register for the growth phase. As the amount of dexamethasone phosphate increases to 30 *m*M, the individual collagen molecules remain soluble under fibril formation conditions and there is not precipitation to form fibrils. At present it is not known if dexamethasone phosphate actually denatures collagen as SDS can or if the steroid may selectively denature the telopeptides as is the postulated mechanism or SDS fibril inhibition.

Since dexamethasone phosphate can inhibit the *in vitro* formation of fibrils, a cautionary note is added to the therapeutic use of corticosteroids. The concentrations of dexamethasone phosphate used in this study are 0.01–30 *mM*. This range is similar to doses used for injection 4–20 *mM* (Gray *et al.*, 1982). The work in this study was done with type I collagen. The collagen of the synovial cartilage is type II. Both form fibrils although type II fibrils are finer in diameter. More work is needed to determine if the inhibitory effects of dexamethasone phosphate are similar on both and other types of collagen. Also in a purely speculative manner, one can wonder if a delay in the ability of a nascent collagen molecule to join the existing fibrous matrix would render it more susceptible to proteolytic attack *in vivo*. Would the use of steroid salts inhibit the repair processes in the inflammed joint?

The author wishes to thank Mr. Ross Lapera for his help in the electron microscopic examination of the fibrils, also Dr. Sandra Lee and Dr. Karl Piez for their permission to use the facilities at the Institute of Dental Research, National Institutes of Health, Bethesda, Maryland.

REFERENCES

Bard, J. B. L. and Chapman, J. A. (1973) *Nature (New Biology)*, **246**, 83–84.
Chandrakasans, G., Torchia, D. A. and Piez, K. A. (1976) *J. Biol. Chem.* **251**, 6062–6067.
Comper, W. D. and Veis, A. (1977) *Biopolymers* **16**, 2113–2142.
Copper, A. (1970) *Biochem. J.* **118**, 355–365.
Dombi, G. W. (1984) Ph.D. Thesis, University of Cincinnati.
Dombi, G. W. and Halsall, H. B. (1985) *Biochem. J.* **228**, 551–556.
Garber, E. K., Fan, P. T. and Bluestone, R. (1981) *Sem. Arthritis Rheum.* **11**, 231–256.
Gardner, D. I. (1978) in *Capeman's Textbook of the Rheumatic Diseases, 5th Ed.* (Scott, J. T., ed.) pp. 273–317, Churchill-Livingston, Edinburgh.
Gelman, R. A., Williams, B. R. and Piez, K. A. (1979) *J. Biol. Chem.* **254**, 180–186.
Gray, R. G., Tenenbaum, J. and Gottlieb, N. L. (1981) *Sem. Arthritis Rheum.* **10**, 231–254.
Gross, J. and Kirk, D. (1958) *J. Biol. Chem.* **233**, 355–360.
Hayashi, T. and Nagai, Y. (1972) *J. Biochem.* **72**, 749–758.
Helseth, D. L., Lechner, J. H. and Veis, A. (1979) *Biopolymers* **18**, 3005–3014.
Hodge, A. J., Petruska, J. A. (1963) in *Aspects of Protein Structure* (Ramachandran, C. H., ed.) pp. 289–300, Academic Press, New York.
Honya, M. and Mizunuma, H. (1974) *J. Biochem.* **75**, 113–121.
Kanfer, I. (1977) *J. Pharm. Pharmac.* **29**, 314–316.
Kirby, J. D. and Munro, D. D. (1976) *Br. J. Derm.* **94 (s. 12)**, 111–119.
Kjellstrand, C. M. (1975) *Transplant Proc.* **7**, 123–129.
Laemmli, U. K. (1970) *Nature* **227**, 680–685.
Lenco, W., McKnight, M. and Macdonald, A. S. (1975) *Ann. Surg.* **181**, 67–73.

Nacht, S. and Garzon, P. (1974) in *Advances in Steroid Biochemistry and Pharmacology* (Briggs, M. H. and Christie, C. A., eds.) pp. 157–187, Academic Press, New York.
Nemeth-Csoka, M. and Tasnadi, G. (1981) *Exp. Path.* **20**, 58–63.
Solinger, A. M. and Hess, E. V. (1982) *J. Rheum.* **9**, 491–493.
Silver, F. H. and Trelstad, R. L. (1979) *J. Theor. Biol.* **81**, 515–526.
Silver, F. H. and Birk, D. E. (1983) *Coll. Relat. Res.* **3**, 393–405.
Suarez, G., Veliz, M. and Nagel, R. L. (1980) *Arch. Biochem. Biophys.* **205**, 427–429.
Uitto, J., Teir, H. and Mustakalio, K. K. (1980) *Biochem. Pharmacol.* **21**, 2161–2167.
Weissman, G. and Thomas, L. (1964) *Rec. Prog. Hor. Res.* **20**, 215–245.
Werb, Z. (1978) *J. Exp. Med.* **147**, 1695–1712.
Williams, B. R., Gelman, R. A., Poppke, D. C. and Piez, K. A. (1978) *J. Biol. Chem.* **253**, 6578–6585.
Wood, G. C. (1960) *Biochem. J.* **75**, 598–605.
Wood, G. C. and Keech, M. K. (1960) *Biochem. J.* **75**, 588–598.