J. Pharm. Pharmac., 1970, **22**, 745–752 *Received March* 26, 1970

Corticosteroid-collagen interaction *in vitro*: fibril formation from collagen solutions

D. R. COOPER, I. KANFER* AND C. H. PRICE*

Leather Industries Research Institute, Rhodes University, Grahamstown

**Pharmacy Department, Rhodes University, Grahamstown,*

Republic of South Africa

The disodium phosphate ester salts of betamethasone, prednisolone and hydrocortisone dissolved in a fibril initiating buffer accelerated the precipitation of fibrils from saline solutions of acid-soluble collagen at pH 7·0, when compared with the rate for the initiating buffer alone at the same pH. Without the initiating buffer these corticosteroids also precipitated collagen fibrils, but at a slower rate than when the initiating buffer was present. A relation has been found between the substitution of active groups on the basic steroid nucleus and the rate of collagen fibril formation from solution. Electron microscope examination of the fibrils precipitated by these corticosteroids showed that they corresponded to normal collagen fibrils in appearance and in having the characteristic repeat period of 60–70 nm.

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 & Kirk, 1958; Bensusan & Hoyt, 1958; Gross, 1958b; Bensusan, 1960; Bensusan & Scanu, 1960; Convy & Wynn, 1967; Bowden, Chapman & Wynn, 1968; Wasteson & 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 & Hoyt, 1958; Wood & Keech, 1960; Wood, 1960a).

We have examined the effect *in vitro* of several water-soluble corticosteroids on fibril formation from solutions of soluble collagen. The relatively minor changes in active groups substituted on the basic steroid nucleus in these compounds allows a systematic correlation of the effect of this substitution on fibril formation.

EXPERIMENTAL

Preparation of acid-soluble collagen

Two preparations of acid-soluble collagen from calf-skin were used. The first (P1) was obtained according to Cooper & Davidson (1965), the second (P4) by the method of Piez, Eigner & Lewis (1963). The acid-soluble collagen was purified by phosphate precipitation (Gross, 1958a), and the purity checked by amino-acid analysis, chromatography and ultracentrifugation (Cooper & Davidson, 1965; Davidson & Cooper, 1967).

Preparation of collagen solutions

The freeze-dried acid-soluble collagen was dissolved in physiological saline to give a final concentration of 1·2 to 1·4 mg/ml and a pH of 4·2. Clarification of collagen solutions was by centrifugation at 5° for 1 h in an M.S.E. centrifuge at a maximum of 32 000 *g*, and in one case at 5° for 1 h in a Spinco model L2–65B ultracentrifuge at a maximum of 107 000 *g*.

Corticosteroids

Prednisolone disodium phosphate [batch EPY (C) 7/7; 11β,17α,21-trihydroxypregna-1,4-diene 3,20-dione 21-(disodium phosphate)] and hydrocortisone disodium phosphate [batch EPY (C) 7/6; 11β,17α,21-trihydroxypregna-4-ene-3,20-dione 21-(disodium phosphate)] were obtained from Glaxo Laboratories Limited, England, and betamethasone disodium phosphate [batch DOH–M–13–1; 9α-fluoro-11β,17α, 21-trihydroxy-16β-methyl-pregna-1,4-diene-3,20-dione 21-(disodium phosphate)] from the Schering Corporation, South Africa. All these compounds were of B.P. purity.

Methods of following fibril precipitation

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

Modified Bensusan and Hoyt Method (*Method* 1). Two matched cuvettes (1 cm path length) were placed for 30 min in the sample and reference beams of the spectrophotometer with the cell-housing being kept at 25°. The collagen solution was removed from a refrigerator at 4° and kept in a water bath at 25° for 30 min. 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 min at 25°. At zero time, 1 min after their removal from the water bath, 3 ml of the collagen solution was added to 3·4 ml of the initiating buffer in a glass stoppered test tube which was inverted ten times during 1 min. The mixture was then placed in a water bath at 25° for 4 min and an aliquot of 3·2 ml then transferred to the sample cuvette kept in the spectrophotometer, this operation being done in 1 min. The recorder connected to the spectrophotometer was started exactly 2 min 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°. The change in extinction with time was recorded. All the operations were done in an air-conditioned room at 25°.

Modified Wood and Keech Method (*Method* 2). An aliquot of 1·5 ml of the collagen solution was placed in a cuvette (1 cm path length) in the spectrophotometer and allowed to stand for 30 min at 20° 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 1·7 ml of the "initiating buffer" kept at 20° for 30 min was added to the cuvette containing 1·5 ml 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°.

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, 2 mg of corticosteroid was dissolved in 1·7 ml of the initiating buffer or physiological saline and added to 1·5 ml of collagen solution containing 1·2 to 1·3 mg/ml.

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 min in 0·1% phosphotungstic acid pH 6·1, followed by washing in distilled water, before final drying. The specimens were examined in a Hitachi HU 11B electron microscope.

RESULTS

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. 1a. All subsequent

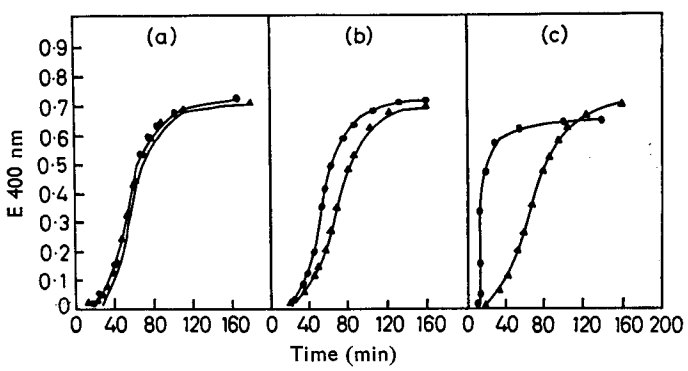

FIG. 1. Effect of varying experimental conditions on fibril precipitation from solutions of acid-soluble collagen in physiological saline using initating buffer at pH 7·04. (a) Duplicate determinations at 25° using filtered solutions, method 1 and preparation P1; (b) Determinations on a filtered solution (●) and a solution centrifuged at 32 000 *g* (▲) using preparation P1 and method 1 at 25°; (c) Determinations on solutions of preparation P1 (▲) and P4 (●) centrifuged at 32 000 *g* and using method 1 at 25°.

graphs or data given in the Tables represent the average of duplicate or triplicate determinations. When solutions of preparation P4 were used, method 1 gave too rapid a reaction compared with preparation P1 (Fig. 1c and Run 3, Table 1) and therefore the modified Wood & Keech method was developed (method 2).

Fig. 1b, corresponding to Run 2 of Table 1, shows that clarification of the collagen solution, after making sure that the collagen had dissolved, had an important bearing on the reaction rate, which corresponds to the findings of Wood & Keech (1960). Centrifugation of the solutions presumably removes collagen molecular aggregates

Table 1. *Rate of precipitation of collagen from physiological saline by initiating buffer.* Experimental conditions: pH of reaction mixture 7·04, I 0·23, 25°

Run No.	Pretreatment of collagen solution	Turbidity method	Collagen preparation	$E\infty$	$t_{0\cdot01}$ (min)	$t_{0\cdot5}$ (min)	S ($\times 10^2$)
1	filtered	1	P1	0·72	19	60	1·54
2A	filtered	1	P1	0·74	19	56	1·54
2B	× 32 000 *g*	1	P1	0·72	21	68	1·22
3A	× 32 000 *g*	1	P1	0·73	21	70	1·13
3B	× 32 000 *g*	1	P4	0·66	11	17	11·92

For definition of $E\infty$, $t_{0\cdot01}$, $t_{0\cdot5}$ and S, see text.

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 later experiments (Table 2, Fig. 2).

All the precipitation curves had a similar sigmoid shape, consisting of a lag period, during which no precipitation was recorded, and a sigmoid portion or growth phase. It was difficult to determine the lag period due to the shape of the curves, but a comparable estimate of this is given by $t_{0\cdot01}$, the time taken for the extinction to rise to the value 0·01. The rate of growth is given by the half-growth time ($t_{0\cdot5}$)being the time taken for the extinction to rise to one-half of its final value. The reactions are also compared by the slope (S) of the linear portion of the sigmoid curve, which is equivalent to the reaction rate (Bensusan & Scanu, 1960). The extinction at the end of the reaction is given by $E\infty$.

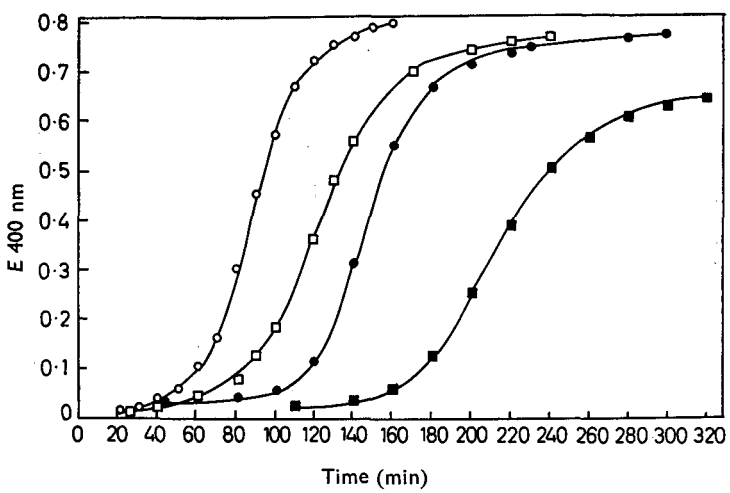

FIG. 2. Fibril precipitation from solutions of acid-soluble collagen (P1) in physiological saline using initiating buffer alone (pH 7·04) and initiating buffer containing betamethasone disodium phosphate (pH 6·95) and method 1 at 25°; □, solution centrifuged at 32 000 *g* and precipitated with initiating buffer alone; ○, solution centrifuged at 32 000 *g* and precipitated with initiating buffer containing betamethasone disodium phosphate; ■, solution centrifuged at 107 000 *g* and precipitated with initiating buffer alone; ●, solution centrifuged at 107 000 *g* and precipitated with initiating buffer containing betamethasone disodium phosphate.

Table 2. *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 P1, reaction temperature 25°

Run No.	Initiating solution	Pretreatment of collagen solution	$E\infty$	$t_{0\cdot01}$ (min)	$t_{0\cdot5}$ (min)	S ($\times 10^2$)
4A	IB	filtered	0·84	23	69	1·45
4B	IB + BDP	filtered	0·94	18	56	2·05
5A	IB	filtered	0·74	19	56	1·51
5B	IB + BDP at half conc.	filtered	0·82	18	52	2·12
6A	IB	× 32 000 *g*	0·78	37	124	0·96
6B	IB + BDP	× 32 000 *g*	0·86	33	98	1·48
6C	IB	× 107 000 *g*	0·66	114	228	0·77
6D	IB + BDP	× 107 000 *g*	0·86	50	152	1·43

For definition of $E\infty$, $t_{0\cdot01}$, $t_{0\cdot5}$ and S, see text. Abbreviations: IB, initiating buffer, BDP, betamethasone disodium phosphate.

The action of corticosteroids in initiating buffer on fibril formation

In the initial study of the effect on fibril formation of the corticosteroids, the drugs were dissolved in the initiating buffer, and the rate of fibril formation compared with that for initiating buffer alone. The action of betamethasone disodium phosphate on fibrillogenesis is clearly illustrated in Fig. 2, accelerated formation of fibrils occurring after addition of the corticosteroid to the initiating buffer. The reaction constants (Table 2), also show an acceleration of fibril formation, while the extinction value $E\infty$ 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 constants were not as great (Run 5, Table 2). Betamethasone disodium phosphate also accelerated fibril formation with a different collagen preparation (P4) and the second method of following fibril formation (Run 7, Table 3). In all the above studies the pH of the reaction mixture was 7·04 (initiating buffer only) and 6·95 (betamethasone disodium phosphate dissolved in initiating buffer).

Prednisolone disodium phosphate dissolved in the initiating buffer (Run 8, Table 3) also accelerated fibril formation. The pH of the reaction mixture being 6·95. To confirm that the three corticosteroids used had different effects on fibril formation when dissolved in the initiating buffer, duplicate determinations on each were made with the same collagen solution on successive days. These were reproducible and the reaction constants (Run 9, Table 3) illustrate the differences.

Effect of corticosteroids in physiological saline on fibril formation

To show that corticosteroids can initiate fibril formation on their own, the disodium phosphate derivatives of betamethasone, prednisolone and hydrocortisone were dissolved in physiological saline, instead of initiating buffer, and added to the collagen dissolved in the same solvent. The control for each of these reactions was similar initiating buffer to that used previously but made up to give a pH in the reaction mixture of 6·2 which is close to the pH of the reaction mixtures containing the

Table 3. *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 P4; reaction temperature 20°; collagen solutions clarified by centrifugation at 32 000 *g*

Run No.	Initiating solution	$E\infty$	$t_{0.01}$ (min)	$t_{0.5}$ (min)	S ($\times 10^2$)
7A	IB	0·75	12	22	6·13
7B	IB + BDP	0·79	10	18	7·08
8A	IB	0·73	13	24	5·60
8B	IB + PDP	0·79	13	20	7·60
9A	IB	0·93	32	46	1·55
9B	IB + BDP	1·00	26	50	2·47
9C	IB + HDP	0·96	32	48	1·80
9D	IB + PDP	1·01	27	50	2·32

For definition of $E\infty$, $t_{0.01}$, $t_{0.5}$ and S, see text. Abbreviations: IB, initiating buffer; BDP, betamethasone disodium phosphate; PDP, prednisolone disodium phosphate; HDP, hydrocortisone disodium phosphate.

corticosteroids (Table 4). 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 4 and Fig. 3 show that in the absence of the initiating buffer the three corticosteroids induced fibril formation at different rates, all of which were slower than that for initiating buffer alone. Further, the final extinction values ($E\infty$) in the presence of all three of the corticosteroids were significantly greater than the value obtained with the initiating buffer.

Fibril precipitation was also obtained at a tenth of the concentration of betamethasone disodium phosphate used above, though at a very much slower rate ($t_{0.01}$ 180 min, $t_{0.5}$ 1250 min and S 0·0319. Preliminary results indicate 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.

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 64 nm.

Table 4. *Rate of precipitation of collagen from physiological saline by corticosteroids in physiological saline.* Experimental conditions: Collagen preparation P4; turbidity method 2; collagen solutions clarified by centrifugation at 32 000 *g*; 20°

Run No.	Initiating solution	pH of steroid in saline	pH of reaction mixture	$E\infty$	$t_{0.01}$ (min)	$t_{0.5}$ (min)	S ($\times 10^2$)
10A	IB	—	6·2	0·70	20	48	3·08
10B	BDP	7·1	6·3	1·08	41	90	1·76
10C	PDP	7·2	6·3	1·02	54	111	1·33
10D	HDP	7·3	6·4	1·01	55	114	1·33

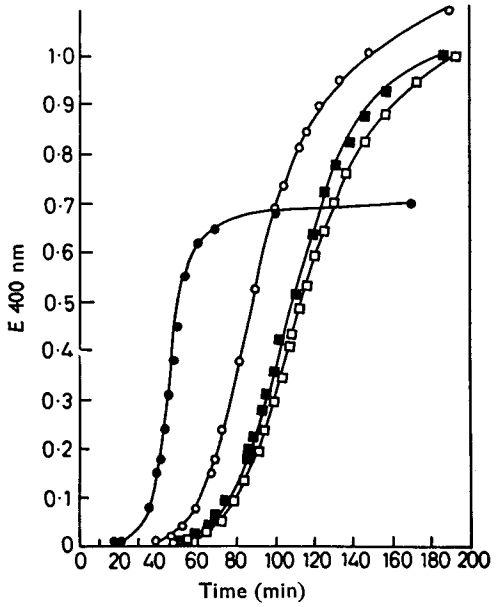

FIG. 3. Fibril precipitation from solutions of acid-soluble collagen (P4) in physiological saline using method 2 at 20°; ●, initiating buffer alone (pH 6·2); ○, betamethasone disodium phosphate in physiological saline (pH 6·3); ■, prednisolone disodium phosphate in physiological saline (pH 6·3); □, hydrocortisone disodium phosphate in physiological saline (pH 6·4).

DISCUSSION

The results in Tables 2 and 3, and Fig. 2 show conclusively that an increase in fibril precipitation rate was obtained when the disodium phosphates of betamethasone, prednisolone or hydrocortisone were dissolved in the initiating buffer before adding this to the collagen solution. This was independent of the method used to clarify the collagen solution, although centrifugation with increasing centrifugal force reduced the general rate in comparison with filtration, presumably because of the removal of collagen molecular aggregates which would assist the nucleation rate. Wood (1960b) concluded from studies with polyanions that those which accelerated precipitation lowered $E\infty$ whereas in the present case the acceleration of precipitation with the disodium phosphate ester salts of the three steroids was accompanied by a significant increase in $E\infty$. This effect does not appear to be due to a pH factor since the pH of the reaction mixtures containing the corticosteroid (pH 6·92) 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 & Keech, 1960). This was particularly evident when the reaction rate was slowed down by centrifuging the collagen solution at 107 000 *g* (Table 2 and Fig. 2). Further, Wood & 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 corticosteroids to the initiating buffer should have increased the ionic strength slightly, but nevertheless the precipitation rate also increased.

The ability of corticosteroids to induce fibril precipitation was confirmed by experiments in which these were added to the collagen solution after dissolving in physiological saline and not the initiating buffer. These experiments (Fig. 3, Table 4)

involved the phosphate derivatives of betamethasone, prednisolone and hydrocortisone which have relatively slight structural variations. These precipitated fibrils with longer lag and growth phases compared with fibril formation at the same pH using initiating buffer alone. The final extinction value ($E\infty$), which Wood (1960b) has related directly to fibril width, were much increased in the presence of corticosteroid.

The order of increasing anti-inflammatory activity of these compounds *in vivo* is hydrocortisone, prednisolone and betamethasone (Liddle & Fox, 1961; Sarett, Patchett & Steelman, 1963) which corresponds to the substitution of active groups on the basic steroid nucleus, and in turn to the order of increasing fibril precipitation rate found experimentally *in vitro*.

Acknowledgements

One of us (I.K.) wishes to acknowledge research grants from the Council for Scientific and Industrial Research and Rhodes University, South Africa. The authors are grateful to Glaxo Laboratories Ltd., England, Glaxo–Allenburys (S.A.) (Pty) Ltd., South Africa, and the Schering Corporation, South Africa, for supplying the corticosteroids, and to Dr. A. E. Russell for discussion.

REFERENCES

BENSUSAN, H. B. & HOYT, B. L. (1958). *J. Am. Chem. Soc.*, **80**, 719–724.
BENSUSAN, H. B. (1960). *Ibid.*, **82**, 4995–4998.
BENSUSAN, H. B. & SCANU, A. (1960). *Ibid.*, **82**, 4990–4995.
BOWDEN, J. K., CHAPMAN, J. A. & WYNN, C. H. (1968). *Biochim. biophys. Acta*, **154**, 190–195.
CONVY, S. & WYNN, C. H. (1967). *Biochem. J.*, **103**, 413–419.
COOPER, D. R. & DAVIDSON, R. J. (1965). *Ibid.*, **97**, 139–147.
DAVIDSON, R. J. & COOPER, D. R. (1967). *Ibid.*, **105**, 965–969.
GROSS, J. (1958a). *J. exp. Med.*, **107**, 247–258.
GROSS, J. (1958b). *Ibid.*, **108**, 215–226.
GROSS, J. & KIRK, D. (1958). *J. biol. Chem.*, **233**, 355–360.
LIDDLE, G. W. & FOX, M. (1961). *Inflammation and Diseases of Connective Tissue*, pp. 302–327. Editors: Mills, L. C. & Moyer, J. H. Philadelphia & London: W. B. Saunders Co.
PIEZ, K. A., EIGNER, E. A. & LEWIS, M. S. (1963). *Biochemistry*, **2**, 58–66.
SARETT, L. H., PATCHETT, A. A. & STEELMAN, S. L. (1963). *Progress in Drug Research*, **5**, pp. 13–153. Editor: Jucker, E. Basel: Birkhauser Verlag.
WASTESON, A. & OBRINK, B. (1968). *Biochim. biophys. Acta*, **170**, 201–204.
WOOD, G. C. & KEECH, M. K. (1960). *Biochem. J.*, **75**, 588–598.
WOOD, G. C. (1960a). *Ibid.*, **75**, 598–605.
WOOD, G. C. (1960b). *Ibid.*, **75**, 605–612.