anhydrous DMF (150 ml). The bulk of the solvent was removed in a rotary evaporator and the residue was dissolved in cold H_2O (1 l.). The product was precipitated by the addition of AcOH and recrystallized (EtOH) to give bright orange crystals, 17.46 g (81%), mp 214–216°.

Ethyl Cyano(1-ethyl-7-methyl-1,8-naphthyrid-4(1H)-ylidene)-acetate (9).—Compound **3** (9.0 g, 0.035 mole), EtI (22.0 g, 0.14 mole), anhydrous K_2CO_3 (10.9 g, 0.08 mole), and anhydrous DMF (75 ml) were boiled under reflux for 6 hr. The bulk of the solvent was removed in a rotary evaporator and the residue was diluted with H_2O (100 ml). The product was recovered by filtration and washed with ice-cold EtOH, followed by H_2O. Recrystallization from EtOH gave bright yellow-green crystals, 7.8 g (79%), mp 182–183°.

The compounds listed in Table I were prepared by procedures similar to those described in the previous two examples.

1-Ethyl-7-methyl-1,8-naphthyrid-4(1H)-ylideneacetamide (9a).—Ethyl cyano(1-ethyl-7-methyl-1,8-naphthyrid-4(1H)-ylidene)acetate (11) (8.0 g) and 70% v/v of H_2SO_4 (80 ml) were heated at 70°, with stirring, for 90 min during which time CO_2 was evolved. The solution was poured onto ice and neutralized with NH_4OH and the precipitated product was recovered by filtration. Two recrystallizations from EtOH gave khaki needles, 3.46 g (54%). The compound slowly decomposes when heated above 180°.

The compounds listed in Tables II and III were prepared in a manner similar to that described above.

Biological Tests.—The methods used for determining the anti-inflammatory activity of the compounds were those previously reported by Cashin and Jackson,[1] and included the rat paw edema test, using carrageenin as the phlogistic agent, and the uv erythema test in guinea pigs.

Results and Discussion

The antiinflammatory results of the intermediate substituted cyanoacetates, the substituted acetamides, and the pyrido[3,4-*c*]quinoline-2,4-diones are recorded in Tables I–III.

Of the cyanoacetates, only ethyl cyano-4(1H)-quinolylideneacetate (**1**) showed any activity. Of the two simple acetamides prepared and tested, 4-quinolylacetamide (**1a**) showed fair activity in the carrageenin test, whereas 7-methyl-1,8-naphthyrid-4-ylacetamide (**3a**) showed no activity. Neither compound showed any activity in the uv erythema test.

The most active compound prepared, 1-ethyl-7-methyl-1,8-naphthyrid-4(1H)-ylideneacetamide (**9a**), showed fair activity in both tests. Replacement of the ethyl group by other alkyl or a substituted-alkyl group resulted in loss of activity. The same appears true of the pyrido[3,4-*c*]quinoline-2,4-diones where, although only three compounds have been prepared and tested, the ethyl derivative **7a** was the only derivative showing activity.

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Retardation of Collagen Fibril Formation by Unsaturated Fatty Acids

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Long-chain unsaturated fatty acids retarded collagen fibrogenesis. When collagen was precipitated by warming neutral tropocollagen solutions to 37°, oleic, linoleic, palmitoleic, linolenic, and arachidonic acids at levels down to 1 μM increased the nucleation period and decreased the fibril growth rate. C_{12}–C_{22} saturated fatty acids, as well as dipalmitoyl lechithin, dipalmitoyl cephalin, and sodium lauryl sulfate, failed to alter the collagen precipitation.

The participation of collagen fibrosis at some stage in pathological and healing processes makes it essential to understand the nature of collagen precipitation from solution; a crucial aspect of this is the influence of pharmacological agents. This paper reports a significant influence on this phase transformation by long-chain unsaturated fatty acids.

Experimental Section

The unsaturated fatty acids included in this study, the position of their double bonds, and the number of their carbon atoms are palmitoleic, Δ^9-C_{16}; oleic, Δ^9-C_{18}; linoleic, $\Delta^{9,12}$-C_{18}; linolenic, $\Delta^{9,12,15}$-C_{18}; and arachidonic, $\Delta^{5,8,11,14}$-C_{20}. All of the unsaturated fatty acids except arachidonic acid were purchased from Applied Science Laboratories, Inc., State College, Pa., and were stated to be 99–100% pure. Arachidonic acid (90–95% purity), C_4–C_{22} saturated fatty acids (99–100% purity), and related compounds were purchased from the Hormel Institute, Austin, Minn.; tlc showed no oxidation contaminants in the arachidonic acid. Reduced DL-6,8-thioctic acid was purchased from Sigma Chemical Co. The unsaturated fatty acids were stored in the dark at 1° as 0.1 and 0.4% solutions in 95% EtOH.

At all but the lowest fatty acid concentrations, the presence of a solubilizing agent was necessary. For this purpose, we used EtOH at 10% except in several specified experiments in which the levels were lower. Qualitatively, the influence of EtOH alone varied with its concentration, as first observed by Bensusan;[1] with increasing EtOH concentration fibrogenesis was accelerated, then inhibited, and finally accelerated again. Therefore, collagen–ethanol controls were always run.

Collagen was isolated from the tail tendon of 6–8-week-old male Sprague–Dawley rats weighing 200–235 g. The isolation, extraction, and fibril precipitation were carried out as described previously.[2] Solutions were buffered in 0.13 *M* potassium phosphate, pH 7.4. Turbidity readings were made every minute for 10 min and every 2–5 min thereafter during a period of 20–40 min. As Wood[3] and other investigators have shown, the precipitation reaction comprises two stages: a lag period, during which nucleation occurs, and a fibril growth period. The over-all rate reflects both, and often long nucleation periods are accompanied by slow growth rates. The ultimate absorbance value, that value which no longer changes with time, is independent of both the lag time and the growth rate. It does, however, mark the time at which precipitation has become essentially complete.

The growth rate can be expressed either as the slope of the linear portion of the sigmoidal turbidity–time curve or by $T_{0.5}$, the half-growth time. The slope is a valuable indicator of the growth rate when at least one of the precipitations in a series

(1) H. B. Bensusan, *J. Am. Chem. Soc.*, **82**, 4995 (1960).
(2) N. H. Grant and H. E. Alburn, *Biochemistry*, **4**, 1271 (1965).
(3) G. C. Wood, *Intern. Rev. Connective Tissue Res.*, **2**, 1 (1964).

is so slow that the curve fails to level off during the experimental period. The sum of lag time and the half-growth time gives an estimate of the over-all rate; dividing this sum by the corresponding value in the experimental control gives a value T/T_c, which is an index of acceleration (<0.90), retardation (>1.10), or little or no effect (0.90–1.10).

Results

In the concentration range of 10^{-6}–10^{-4} M, C_{12}–C_{20} unsaturated fatty acids inhibited the precipitation of collagen fibrils.

Figure 1 shows that 10^{-4} M linolenic acid decreased the growth rates by 53 and 72% in 1.5 and 2% EtOH systems. In the system containing 5% EtOH, in which the nucleation period was already prolonged, the addition of linolenic acid almost abolished fibril formation.

Figures 2–4 show the inhibition by linolenic, oleic, and arachidonic acids at several concentrations. In each case there occurred a prolongation of the nucleation period; with 56.7 μM oleic acid (Figure 3) and 52.6 μM arachidonic acid (Figure 4) a marked decrease in fibril growth rate accompanied this prolongation.

A series of experiments with arachidonic acid (50 μM) demonstrated that the retardation of fibrogenesis was independent of the precipitation temperature (30, 35, 37°) and of the EtOH concentration (0.2–10%).

The saturated fatty acids having 14–22 carbon atoms did not retard fibril formation and sometimes accelerated it. This is illustrated by the T/T_c values in Table I, all but one of which were less than 1.0.

TABLE I

EFFECTS OF C_{14}–C_{22} SATURATED FATTY ACIDS ON FIBRIL FORMATION

Fatty acid	Concn, μM	T/T_c
Myristic (C_{14})	50	0.82
Myristic (C_{14})	44	0.93
Myristic (C_{14})	18	0.88
Myristic (C_{14})	10	0.98
Myristic (C_{14})	5	1.01
Myristic (C_{14})	1	0.96
Palmitic (C_{16})	78	0.92
Palmitic (C_{16})	50	0.76
Stearic (C_{18})	50	0.76
Arachidic (C_{20})	64	0.84
Arachidic (C_{20})	50	0.62
Behenic (C_{22})	59	0.94

A relationship between the fatty acid effect and the ethanol concentration emerged in the series of short-chain saturated fatty acids (C_4–C_{10}) (Table II). At ethanol levels of 7% or lower these fatty acids accelerated the fibrogenesis, while at ethanol levels of 8% or greater they retarded it.

TABLE II

EFFECTS OF SHORT-CHAIN FATTY ACIDS AND ETHANOL CONCENTRATION ON FIBRIL FORMATION[a]

Fatty Acid	T/T_c at % ethanol: 2	5	6	7	8	9	10
Butyric (C_4)	0.85	<1	<1	0.88	1.16	1.32	1.34
Caproic (C_6)	0.82	<1	<1	0.83	>1	>1	>1
Caprylic (C_8)	0.77	<1	<1	0.88	1.19	1.50	>1
Capric (C_{10})	0.78	<1	<1	0.94	>1	1.66	>1

[a] All of the fatty acids were 50 μM. <1 and >1 indicate failure of the control and fatty acid system, respectively, to level off during the experiment.

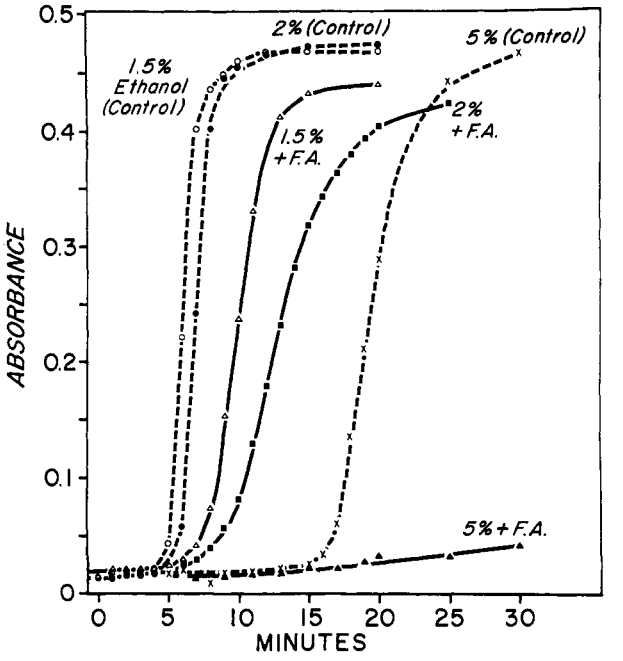

Figure 1.—Retardation of fibrogenesis by 100 μM linolenic acid at several ethanol concentrations.

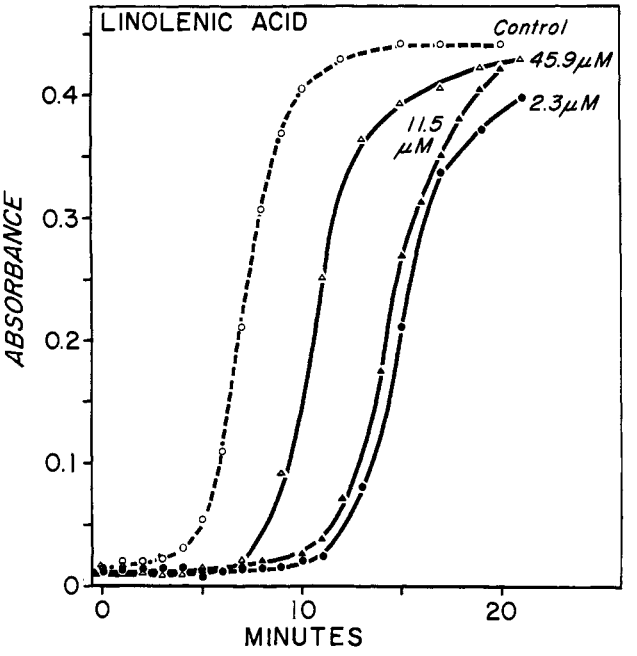

Figure 2.—Retardation of fibrogenesis by several concentrations of linolenic acid.

Various odd-numbered fatty acids, hydroxy fatty acids, thio fatty acids, fatty alcohols, and esters were tested. None of these retarded the fibrogenesis, and only two gave any indication of acceleration (Table III).

One respect in which *cis* unsaturated fatty acids differ from saturated fatty acids is that they are kinked. Kinking in structures attracted to bonding regions of tropocollagen chains might be capable of preventing the close enough approach of a neighboring chain for additional attractions and bonding to develop. We attempted to test this by comparing *cis*- and *trans*-vaccenic acid (11-octadecenoic acid), but the solubilities were so limited that it was necessary to use 10 μM solu-

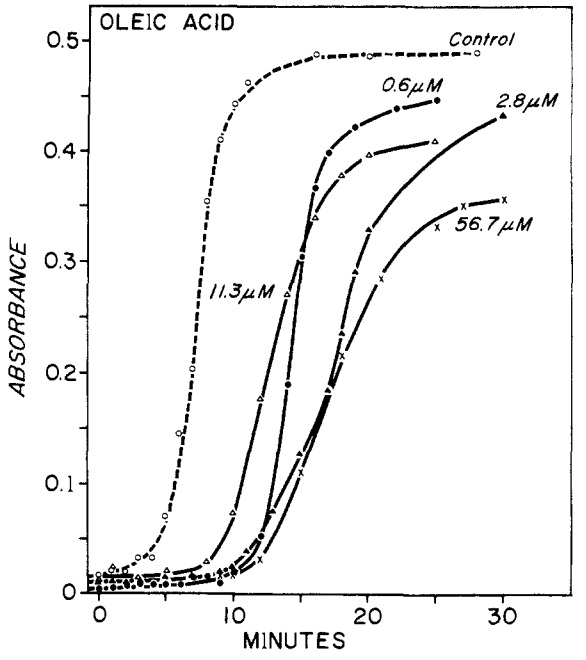

Figure 3.—Retardation of fibrogenesis by several concentrations of oleic acid.

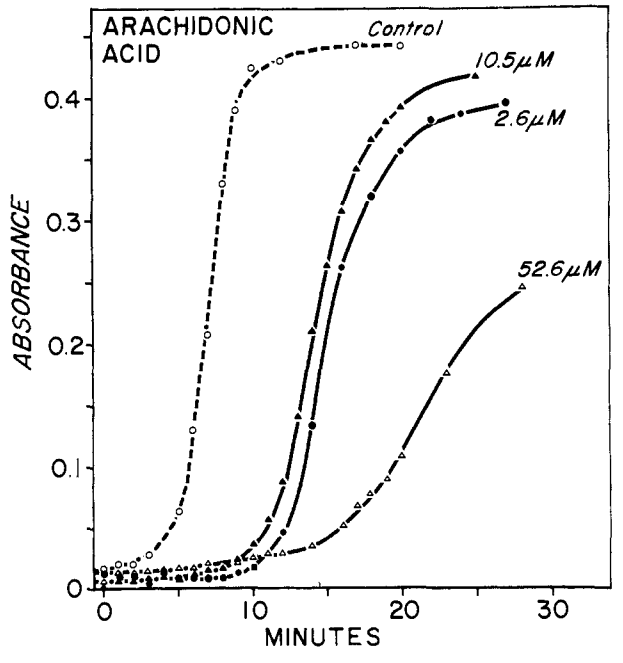

Figure 4.—Retardation of fibrogenesis by several concentrations of arachidonic acid.

tions. At this fatty acid level, there was no difference between *cis* and *trans* with 10% ethanol (T/T_c = 0.93 and 0.91); a small difference with 5% ethanol (T/T_c = 0.82 for *cis* and 0.95 for *trans*) lies in the direction opposite to that expected if kinking were important.

The difference in behavior between long-chain saturated and unsaturated fatty acids suggests the possible relevance of a preliminary double bond oxidation. However, despite the established correlation between the degree of unsaturation and autoxidation rate no correlation appeared between the degree of unsaturation and the influence on collagen precipitation.

In a test of whether deliberate air oxidation of the fatty acids would result in further retardation of fibrogenesis, aliquots of arachidonic acid and linolenic acid stock solutions were shaken in illuminated open flasks for 18 hr at 37°. Nucleation periods for the control and untreated arachidonic and linolenic acids (final 50 μM, 2% EtOH) were 4, 6, and 9 min, respectively. Corresponding half-growth times were 1, 4, and 3 min. Aeration of the fatty acids *shortened* the nucleation periods to 5 min for arachidonic and 6 min for linolenic acid while leaving the growth rates unchanged. Similar results were obtained when aeration was carried out by bubbling air through the fatty acid solution for 45 min at room temperature.

Table III

Effects of Various Fatty Acids and Related Compounds on Fibril Formation

Compd (50 μM)	T/T_c
Nonanoic acid	0.93
Undecanoic acid	0.98
Heptadecanoic acid	0.72
12-Hydroxystearic acid	0.83
Thioctic acid (reduced)	0.97
Cholesterol oleate	1.10
Methyl oleate	1.03
Methyl caprylate	1.04
Oleyl alcohol	1.06
Linoleyl alcohol	0.90

Of the numerous agents reported by Matsushita and Ibuki[4] as having prooxidative or antioxidative activities toward unsaturated fatty acids, we tested procaine, a prooxidant, and L-tyrosine and reduced glutathione, both antioxidants, in collagen systems. At 50 μM these additives did not alter the retardation by 50 μM oleic, linolenic, and arachidonic acids. The addition of hydroquinone (1 mM) to systems containing 50 μM arachidonic and linolenic acids also produced no alteration of the fatty acid effect.

We considered the possibility that unsaturated fatty acids retard precipitation of collagen through an effect on the surface tension of the medium. Sodium lauryl sulfate, at 100 and 10 μM in totally aqueous systems and at 10 μM in 0.5 and 2% EtOH, produced no change in the precipitation kinetics. In 5% EtOH, this detergent at 10 μM *reduced* the nucleation period without changing the growth rate. In another experiment, with no added ethanol, dipalmitoyl-DL-α-lecithin and dipalmitoyl-DL-α-cephalin (each at 38 μg/ml) produced no change in the turbidity–time curves. Possession of a high capacity for decreasing surface tension is, therefore, not a sufficient requirement for ability to retard collagen precipitation.

Discussion

This study shows that collagen reacts quite differently with unsaturated and saturated long-chain fatty acids. The retardation of fibrogenesis by unsaturated fatty acids could have relevance to wound healing and atherosclerosis. However, the mechanism of the retardation is not clear. Double-bond peroxidation and kinking do not seem to be primary factors.

The role of a pharmacological agent depends upon the stage at which it acts. The change of tropocollagen to insoluble fibrils on warming is a phase transforma-

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tion; it consists of a nucleation act followed by growth, which is apparently controlled by processes occurring at the particle–solution interface.[5] The endothermic nature of collagen fibrogenesis, as well as tobacco mosaic virus agglomeration, precipitation of denatured proteins, and formation of the mitotic spindle, indicates a fundamental role for hydrophobic bonding.[6] These processes may all involve the expulsion of hydrate water producing the entropy rise necessary to drive the reaction forward.[7] There is, in addition, evidence that fiber growth entails lateral alignment of bonding regions in neighboring molecules by electrostatic and hydrogen bonds.[8–10] Over longer periods, covalent cross-linking leads to the increasingly insoluble network characteristic of mature and aged fibers.

The least studied aspect of the fibrogenesis process is the formation of hydrophobic bonds accompanying hydrate water expulsion. It may be here that unsaturated fatty acids exert their effects.

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The Mitomycin Antibiotics. Synthetic Studies. XXI.[1] Indoloquinone Analogs with Further Variations at C-5

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Indoloquinone carbamate analogs of the mitomycin antibiotics, bearing substituents such as chloro, alkoxy, hydroxy, methylthio, and various amines at the 5 position, were prepared and tested against bacteria. Following the observation of interesting *in vivo* activity for a 5-ethylenimino derivative, analogs containing this group and embodying variants at N-1 and the carbamate nitrogen were also prepared. The *in vitro* activities of the more interesting analogs are reported.

Following the demonstration of interesting antibacterial activity for indoloquinone analogs (*e.g.*, I)[2] of the mitomycin antibiotics, a systematic program of structural variation based on these lead compounds was undertaken. This program has included variants at each position in the pyrrole ring[1,3] and also in the quinone ring.[4]

In the present paper we describe the preparation of analogs of I with further variations at position 5 in the quinone ring. This position is of particular importance since at least two substituents, amino and methoxy, at the corresponding position in the mitomycins confer activity.[5] Furthermore, this position has been suggested as a possible active site in the cross-linking of DNA by the mitomycins.[6]

For the preparation of the 5-chloro analog (IIa), 2,6-dimethyl-1-ethyl-5-hydroxy-4,7-indoloquinone-3-carboxaldehyde[2] (III) was warmed with 2 equiv of $POCl_3$ in dimethylformamide,[7] and the resulting 5-chloro-3-carboxaldehyde (IVa) was converted into IIa by the usual technique[2] (Scheme I). Alkoxy derivatives were prepared by treatment of 5-hydroxyindoloquinone-3-carboxaldehyde (III) with ethyl orthocarbonate or with *n*-hexyl orthocarbonate,[8] followed by conversion of the resulting 5-ethoxy (IVb)[9] and 5-*n*-hexyloxy (IVc) derivatives into hydroxymethyl carbamates IIb and IIc in the usual way.[2] Attempts to prepare 5-methylthio analog IId by a route involving displacement of the 5-methoxy group of V with methyl mercaptide ion were unsuccessful. However, treatment of V with methyl mercaptan and HCl afforded 5-methylthioindoloquinone-3-carboxaldehyde (IVd) in low yield, and conversion[2] of IVd into IId proceeded without difficulty. Several attempts to add methyl mercaptan to the 5-unsubstituted indoloquinone-3-carboxaldehyde (IVe)[4b] gave products of indefinite composition.

Treatment of the lead 5-methoxyindoloquinone carbamate (I) with a variety of amines in methanol afforded an interesting series of 5-amino analogs (IIe–l). These reactions were followed by thin layer chromatography (tlc) and were conducted until there was no evidence of starting material. In this manner a rough qualitative estimate of relative reaction rates for the displacement of the methoxy group of I by the various amines was obtained. The primary amines were most reactive, followed in decreasing order of reactivity by ethyleni-

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(7) This method was developed for hydroxynaphthoquinones by G. R. Allen, Jr. Although the yield was low in the present case, no satisfactory alternate procedure could be found. Reagents such as HCl and $SOCl_2$ caused extensive decomposition of I.
(8) Kindly furnished by R. W. Broschard. The use of orthocarbonates in the preparation of alkylaryl ethers was first reported by B. Smith [*Acta Chem. Scand.*, **10**, 1006 (1956)]. The extension of this method to hydroxyquinones is due to J. B. Patrick, D. B. Cosulich, and R. W. Broschard (private communication).
(9) This experiment was performed by R. H. Roth.