STUDIES ON THE MODE OF ACTION OF LATHYROGENIC COMPOUNDS

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(Received for publication, March 21, 1962)

PLATES 10 AND 11

Osteolathyrism is an experimental disease which may be produced in a variety of animals by treating them with one of various compounds; the resulting lesions include skeletal deformity and aortic aneurysm. One of the major effects appears to be a weakening of intermolecular collagen bonding which manifests as an increase in the fragility of various collagenous tissues and in the extractibility of salt-soluble collagen from these tissues (1).

Interest in this condition lies in its use as an experimental model with which to study the extracellular behaviour of fibrous tissue, as well as a possible means for the delay or prevention of its deposition. At present data concerning the functional groups in normal collagen which are responsible for its polymerisation, are incomplete; the present study explores the possibility that lathyrogenic agents may act on certain of these functional groups thus impairing normal polymerisation.

Theoretically considered, lathyrogens must act at one or more of three points during the formation of collagen—on the fibroblast, during synthesis; extracellularly during polymerisation; or finally on the fully matured fibril. Electron microscopic studies on the connective tissue in the healing ear chamber in rabbits treated with a powerful lathyrogen, β-aminopropionitrile (BAPN), have shown that in such a relatively immobile system BAPN does not prevent the deposition of fibroblasts and collagen which morphologically, at least, appear normal (2). Attempts to solubilise fully matured collagen fibrils by incubating normal chick embryo bones with lathyrogens *in vitro*, have failed (3). Finally, it is well established that lathyrogens exert their maximal effect in young, growing animals, rather than in mature animals. It thus seems likely that lathyrogens are most effective in collagen-synthesising systems during the stage of polymerisation.

Recent studies in the lathyritic rat have indicated that there is a defect in the cross-linking of lathyritic collagen at an *intra*molecular level (4). The

* This work was aided by a personal grant from the Medical Research Council and by a grant-in-aid from the Muscular Dystrophy Associations of America, Inc.

present study presents evidence that the defect presumed to be present in chick embryo collagen, may be "repaired" by the *in vitro* addition of certain organic carbonyl groups; the hypothesis is presented that lathyrogenic agents act by blocking carbonyl groups of unknown nature which are normally present in collagen and which are essential to its normal maturation and tensile properties.

Methods and Materials

Production of Lathyrism in the Chick Embryo.—Fertile embryos were injected at 14 days of incubation through a pinhole in the shell onto the chorio-allantoic membrane with 0.054 mM of the lathyrogenic compound dissolved in 0.1 ml of distilled water. 2 days later the survivors were harvested as follows: the tibiae and femora were dissected in the cold and the bones freed of muscle and ligament; pooled bones from 12 embryos were minced with fine scissors and extracted for 24 hours with constant shaking at 5°C and 2 volumes (*v/w*) of cold 1 M NaCl buffered with phosphate, pH 7.6, ionic strength 0.02. The extracts were separated from the residues by centrifugation in a Spinco model L preparatory ultracentrifuge at 20,000 RPM for 30 minutes at 5°C, and the clear supernatant filtered in the cold through a fine sintered glass funnel. The relative viscosity of the extracts which gave an index of the amount of collagen in solution (5) was determined in an Ostwald viscometer at 5°C.

Production of Lathyrism in Guinea Pigs.—Weanling guinea pigs were injected intraperitoneally daily with the lathyrogenic compound dissolved in 1 ml of sterile distilled water; they were treated for a minimal period of 2 weeks, and weighed on alternate days, since weight loss has been found to be a useful index of toxicity. The dosage for BAPN was 1 gm per kg of body weight, and for isonicotinic acid hydrazide (INAH) it was 0.1 gm per kg, since higher dosages resulted in the animal's death. Semicarbazide was found to be acutely toxic and was therefore not used.

Purification of Guinea Pig Collagen.—The method used was that of Gross (6); the animals were killed with nembutal intraperitoneally, shaved and carefully skinned, leaving behind subcutaneous adipose tissue; the skins were spread over the flat surface of an inverted cold ice tray and the remaining fat and epidermis scraped away; the dermis was then finely minced in a precooled mincing machine and the fragments extracted for 24 hours in the cold in 2 volumes (*v/w*) of 0.45 M NaCl with constant shaking. The supernate was separated from the residue by centrifugation in the Spinco model L preparatory ultracentrifuge at 20,000 RPM for 30 minutes and the cleared supernate filtered in the cold through a fine sintered glass funnel.

The collagen was purified by the TCA-ethanol method (6); wherever possible the purified collagen solution was used immediately, but where temporary storage was essential, collagen was lyophilised and stored at 5°C, solution being effected with 0.5 M acetic acid, using a cooled glass homogeniser (6).

As a partial check on the efficacy of the TCA-ethanol procedure, the purified collagens, dissolved in phosphate buffer, pH 7.6, ionic strength 0.45, were examined in the Spinco analytical ultracentrifuge at 59,800 RPM at 5°C;[1] the presence of a single, hypersharp, slowly moving peak indicated that most of the non-collagenous material had been removed (6).

Electron Microscopic Observations.—Segment-long-spacing collagen (SLS) was prepared by mixing equal volumes of an 0.5 M acetic acid solution of collagen with adenosine triphosphate, sodium salt, 5 mg/ml distilled water, and allowing them to sediment in the cold overnight (7); the following day the precipitate was treated for 20 minutes with a 1 per cent aqueous phosphotungstic acid solution to stain the fibres, which were then washed with distilled water and

[1] The ultracentrifugal analyses were kindly performed by Dr. W. E. van Heyningen.

placed on a formvar-covered grid and dried *in vacuo*; the grids were examined in a Philips electron microscope.[2]

Experimental

It has been previously shown that the lathyrogenic effect of INAH in the chick embryo may be reversed *in vivo* by certain organic aldehydes (3); the mechanism postulated for this has been by the formation of a Schiff base between the terminal amine of the hydrazide group and the aldehyde. The following experiment attempts to clarify this effect using an *in vitro* system.

Fresh tibiae and femora were obtained from 16-day-old chick embryos which had been treated 2 days earlier with 0.054 mM of INAH/egg; aliquots of these lathyrogenic bones were incubated in the whole state with a series of organic carbonyl and non-carbonyl compounds, dissolved in 2 volumes (*v/w*) of Krebs-Ringer phosphate buffer to give a final concentration of 0.04 M/ml. The mixtures were shaken at 37°C for 3 hours; controls were employed using bones and buffer alone. The bones were then washed in fresh buffer, minced with fine scissors, and extracted with cold 1 M NaCl as described; the relative viscosity of the extracts was measured, giving an index of the amount of extractible collagen.

Results

The procedure of incubating bones with buffer had very little effect on collagen extractibility; non-carbonyl compounds such as pyridoxine, nicotinamide and 3-acetylpyridine also had no effect, but carbonyl compounds produced a diminution in the amount of extractible collagen ranging from a slight to a complete reversal (Table I); in general aldehydes were more potent than ketones in reversing the INAH effect.

A more detailed study of the reversal of the INAH effect by carbonyl compounds was performed, using D-L-glyceraldehyde as the reversal agent; the effect appears to depend upon the time of incubation (Text-fig. 1), the concentration of D-L-glyceraldehyde (Text-fig. 2), and the temperature of incubation, within the range tested (Text-fig. 3); it is independent of the pH of the incubating medium (Text-fig. 4) or of the optical form of glyceraldehyde used (Text-fig. 5); furthermore, the glyceraldehyde effect was unaltered by pretreating the bones with potassium cyanide and sodium iodoacetate singly or together, in order to poison the oxidative and glycolytic metabolism of the bone cells.

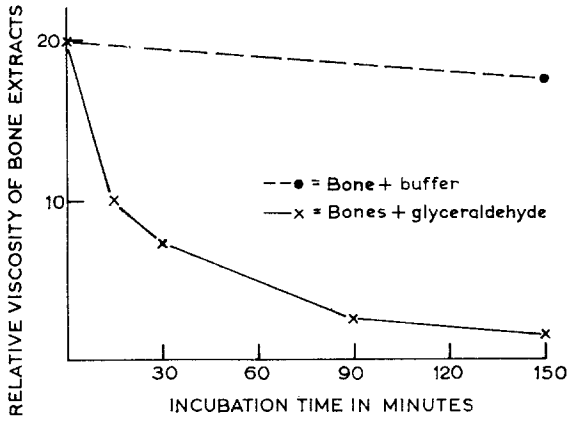
Lathyrogenic agents have been previously classified on a structural basis into 4 groups—organic nitriles, ureides, hydrazides, and hydrazines (8); it was desired to know whether D-1-glyceraldehyde would reverse the extractibility of collagen induced in chick embryos by examples of the above groups such as BAPN, semicarbazide, and hydrazine hydrate. As previously described, for INAH, 14-day-old chick embryos were treated with 0.054 mM of these lathyrogens and 2 days later aliquots of the freshly dissected lathyritic bones were

[2] The electron microscopic examinations were kindly performed by my colleagues, particularly Drs. J. Casley-Smith and D. Kay.

incubated under similar conditions with D-L-glyceraldehyde. It was seen that as with INAH, glyceraldehyde completely reversed the lathyrogenic effect produced by BAPN, semicarbazide, and hydrazine hydrate.

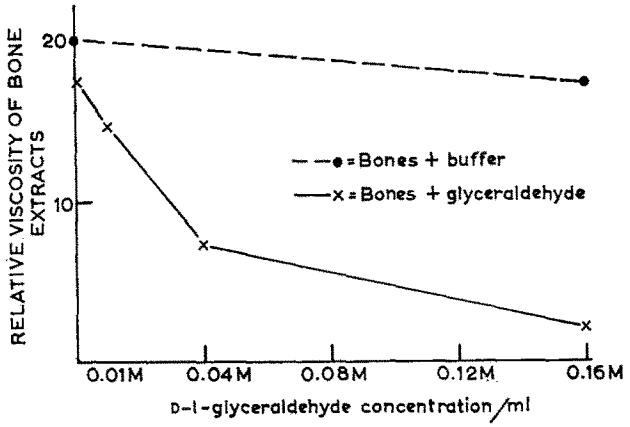
TABLE I

	Carbonyl compound incubated with bones from INAH-treated embryos; bones subsequently extracted with 1 M NaCl	Relative viscosity of 1 M NaCl bone extracts
Controls	Bones extracted without prior incubation	21.6
	Bones extracted after incubation with buffer alone	18.6
Aldehyde compounds	Bones extracted after incubation with buffer and pyridoxal	15.7
	" " " " " " and acetaldehyde	11.5
	" " " " " " and salicylaldehyde	7.1
	" " " " " " and D-1-glyceraldehyde	1.5
	" " " " " " and formaldehyde	1.1
Ketone compounds	Bones extracted after incubation with buffer and sodium pyruvate	15.2
	" " " " " " and acetone	13.9
	" " " " " " and α-ketoglutaric acid	9.8

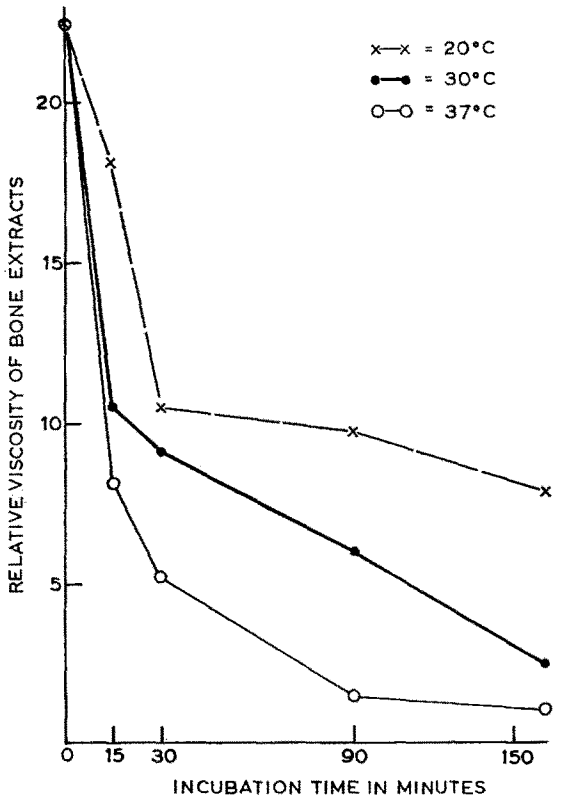


TEXT-FIG. 1. Effect of time of incubation of D-L-glyceraldehyde (0.04 M/ml at 37°C) on extractibility of collagen from bones of 16-day chick embryos injected 2 days earlier with 7.4 mg. INAH.

It is thus evident that the fragility induced in chick embryo bones by treating the embryo *in vivo* with lathyrogens, and which is manifest by the ease of extractibility of collagen from these bones, is reversed *in vitro* by the addition of carbonyl compounds, and that this reversal is probably independent of the cells and due to simple chemical union, probably Schiff base formation. Chemical evidence exists that the collagen molecule contains free aldehyde groups (9); furthermore the 4 groups shown to be lathyrogenic in the chick embryo

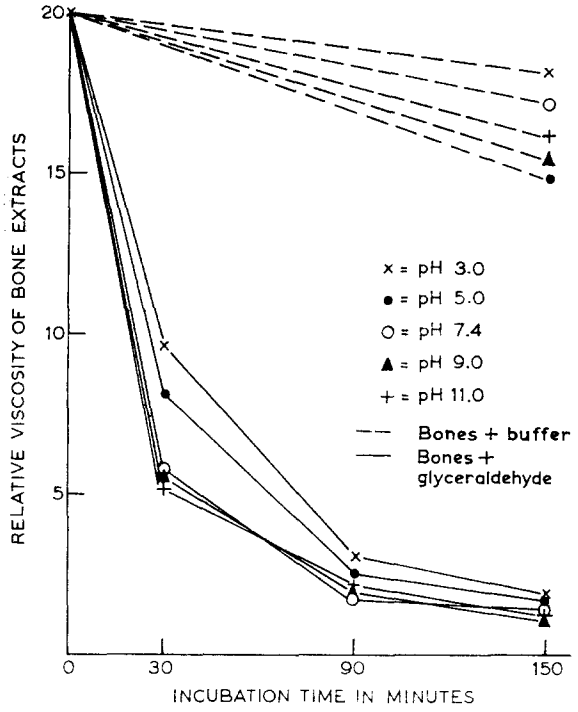


TEXT-FIG. 2. Effect of concentration of D-L-glyceraldehyde on extractibility of collagen from bones of 16-day chick embryos injected 2 days earlier with 7.4 mg INAH and incubated for 30 minutes at 37°C.



TEXT-FIG. 3. Effect of temperature of incubation on extractibility of collagen from bones of 16-day chick embryos treated 2 days earlier with 7.4 mg INAH and incubated with D-L-glyceraldehyde, 0.04 M/ml.

have in common the property of being aldehyde-blocking agents, and of being used as such histochemically (10). In view of this, the following hypothesis has been formulated: that lathyrogenic agents act by blocking carbonyl groups present on the collagen molecule, thus preventing cross-linking essential to normal maturation of the fibril, and that normal cross-linking may be restored



TEXT-FIG. 4. Effect of pH of incubating medium on subsequent extractibility of collagen from bones of 16-day chick embryos treated 2 days earlier with 7.4 mg INAH and incubated with D-L-glyceraldehyde 0.04 M/ml at 37°C.

by the addition of carbonyl groups which act by competing either for the lathyrogen or for functional sites on the collagen molecule.

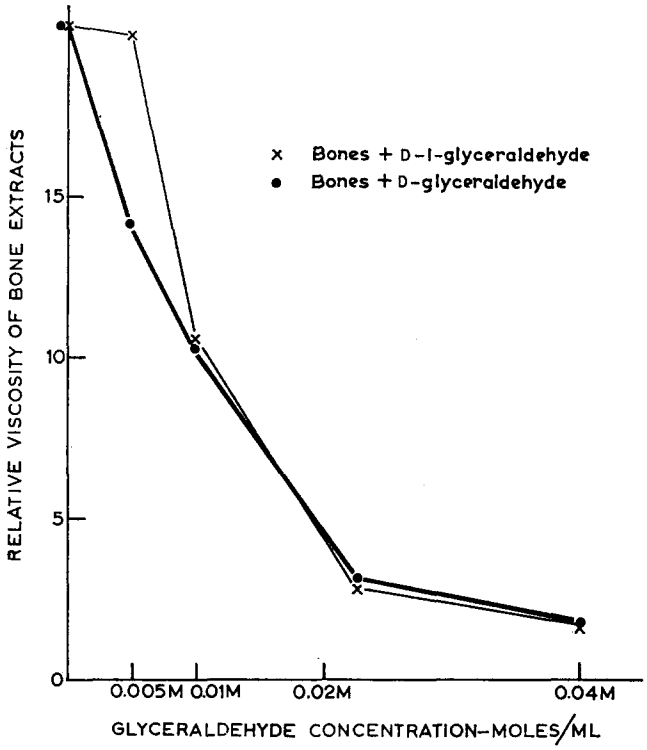
In order to partially test this hypothesis, the following experiments were devised—it is difficult to obtain collagen from normal chick embryo bone (1); since it was desirable to compare the behaviour of normal purified collagen with that of lathyritic animals, guinea pigs, from which normal collagen is easily obtained, were therefore used.

Experimental

It is difficult to assay chemically a few aldehyde groups on a molecule as large and as temperature-labile as collagen without partially denaturing it; in order therefore, to circumvent

this, use was made of the ability of 2,4-dinitrophenylhydrazine (2,4-DNPH) to combine rapidly with aldehyde groups to form 2,4-dinitrophenylhydrazones.

Purified collagen was prepared from normal weanling guinea pigs and from weanling guinea pigs rendered lathyritic with BAPN and INAH. Equal amounts of each of these collagens were dissolved in 0.5 M acetic acid and shaken for 10 minutes at room temperature with ⅛ of the volume of a saturated 0.5 M acetic acid solution of 2,4-DNPH, and left at 37°C for 20 minutes.



TEXT-FIG. 5. Comparison between effect of D-L-, and D- forms of glyceraldehyde in reversing extractibility of collagen from bones of 16-day chick embryos treated 2 days earlier with 7.4 mg INAH; mixtures incubated at 37°C for 150 minutes with shaking.

Uncombined 2,4-DNPH was removed by exhaustive dialysis in the cold for 2 days against several changes of 0.5 M acetic acid, and the collagen solutions examined in the Unicam S.P. 600 spectrophotometer and compared with untreated controls. The solutions were recovered, dialyzed for 24 hours against cold phosphate buffer, and against cold water to precipitate the collagen (6) which was then lyophilised.

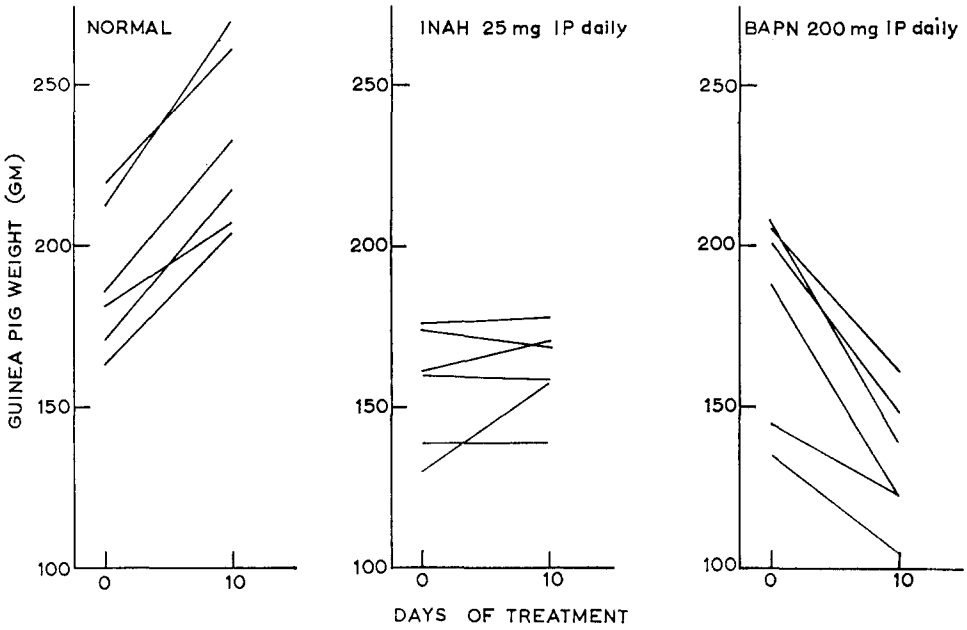
Results

The effect of treating guinea pigs with INAH was to arrest growth, whilst BAPN produced weight loss (Text-fig. 6).

Purified normal and lathyritic collagens show the presence of a peak at 320

mμ, which disappears in all 3 after 2,4-DNPH treatment, to be replaced in the case of the normal only, by a peak at 360 mμ (Text-figs. 7, 8); 360 mμ was shown to be the absorption band of 2,4-DNPH and of the 2,4-dinitrophenylhydrazones of formaldehyde, acetaldehyde, methylbutyraldehyde, and propionaldehyde.[3]

On lyophilising the collagens which had been treated with 2,4-DNPH, the normal collagen was stained yellow to the naked eye, whereas the lathyritic



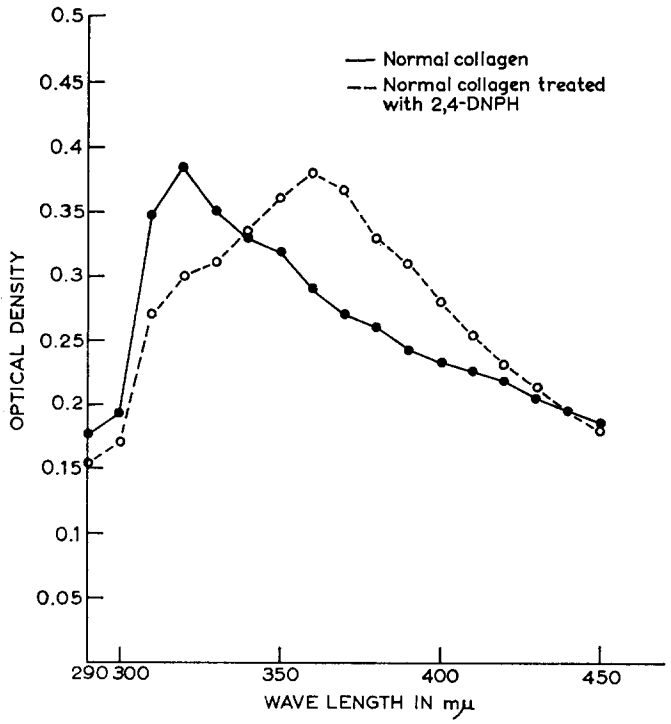
TEXT-FIG. 6. Effect of BAPN and INAH on the weights of weanling guinea pigs treated for 10 days by intraperitoneal injection.

collagens were white, suggesting that normal collagen had fixed the dye, whilst the lathyritic collagens had fixed less of the dye, if any.

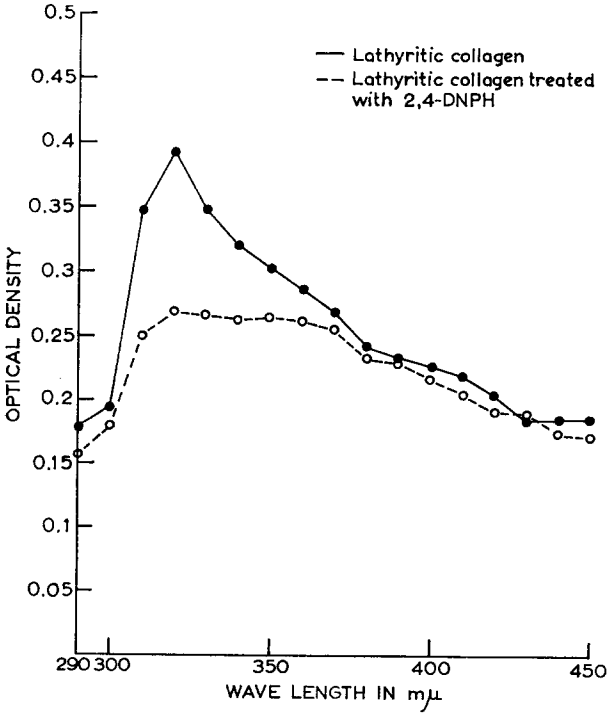
Experimental

Collagen is formed from a unit building block, the tropocollagen particle, which is a polar molecule about 2800 A in length and which, under the influence of different chemical and physicochemical environments, will reform to various electron microscopically characteristic forms of collagen (11); one of these forms, the segment-long-spacing (SLS), is prepared by the addition of adenosine triphosphate to an acetic acid collagen solution. It was considered to be a useful functional index of molecular structure, to see whether lathyrogenic collagen retained the ability to form SLS collagen. Purified collagen was obtained from normal, BAPN-, and INAH-treated guinea pigs and SLS collagen prepared from all three, and examined in the electron microscope.

[3] The generous gifts of Dr. E. P. Abraham, F.R.S.



TEXT-FIG. 7. Spectral absorption curves of acetic acid solutions of normal, guinea pig collagen before and after treatment with 2,4-dinitrophenylhydrazine; examined with visible light.



TEXT-FIG. 8. Spectral absorption curves of acetic acid solutions of collagen from guinea pigs treated with BAPN; before and after treatment of collagen with 2,4-dinitrophenylhydrazine; examined with visible light.

Results

All three collagens were apparently able to form normal looking SLS collagen; however, measurements of the segments at their narrowest widths, indicated that normal SLS measured 800 A ± 600 A, INAH-SLS measured 700 A ± 280 A, and BAPN-SLS measured 430 A ± 70 A (Figs. 1 a, 1 b).

Experimental

The two foregoing experiments suggested the possibility of impairing the 2,4-DNPH uptake and SLS fibre formation of normal guinea pig collagen by blocking free carbonyl groups; a 0.5 M acetic acid solution of normal, purified guinea pig collagen was allowed to react for 30 minutes at 37°C with a 0.5 M acetic acid solution of INAH (5 mg/ml), and the excess INAH was dialysed away overnight against large volumes of 0.5 M acetic acid in the cold. The uptake of 2,4-DNPH and the formation of SLS collagen were then examined as previously described and compared with that of normal collagen which had been similarly treated with the exception of the INAH step.

Results

INAH-pretreatment of normal collagen appeared to diminish its uptake of 2,4-DNPH by half; SLS collagen formation, whilst still occurring, was delayed greatly (Fig. 2), the resultant fibres being less than half the width of the control fibres (Figs. 3 a, 3 b).

DISCUSSION

The hypothesis which has been presented rests on three points—that collagen possesses free carbonyl groups; that in lathyrism, cross-linkage of collagen is impaired; and finally, that lathyrogens block carbonyl groups.

Landucci *et al.* (9) have provided chemical evidence for the presence of free aldehyde groups on the collagen molecule, and in support of this the present study demonstrates the uptake of 2,4-DNPH by normal collagen with the shift of the absorption band to what could be the 2,4-dinitrophenylhydrazone absorption band. On the other hand, Gustavson has found no evidence indicating the role of carbohydrate in the stabilisation of collagen (12).

The view that lathyrogens impair intermolecular cross-linking of collagen is based on the tissue fragility and increased collagen extractibility found in lathyritic chick embryos (1); more recently, it has been shown that the collagen in lathyritic rats suffers from an *intra*molecular failure of cross-linking (4).

The evidence that lathyrogens block carbonyl groups on the collagen molecule may be summarised as follows: first, the diminished uptake of 2,4-DNPH by lathyritic collagen; second, the 4 groups shown to be lathyrogenic are also aldehyde-blocking agents *in vitro;* and third, the *in vitro* reversal by carbonyl groups of the collagen-solubilising effect of various lathyrogens on the chick embryo bone *in vivo.*

The electron microscopic evidence may be summed up as follows: lathyritic collagen forms normal appearing SLS collagen which is, however, thinner than normal; furthermore, pretreatment of normal collagen with INAH results in a

thinner form of SLS collagen. Now, if SLS collagen does consist of laterally aggregated tropocollagen particles in register as proposed by Schmitt, Gross, and Highberger (11), then a blockage of lateral linkages could well result in thinner SLS collagen formation; in fact, the skin of chick embryos made lathyritic with BAPN does show great variation in the cross-section diameter of the collagen fibres as seen in the electron microscope (13), a finding which is explicable by the foregoing.

The nature of the carbonyl-containing compound is unknown; Gustavson has pointed out (12) that if, in fact, aldehydes are present in collagen to the extent of one per unit, molecular weight 200,000, their effect on the stability of collagen should be of prime importance. The final word on this matter must await the isolation and characterisation of such aldehyde-containing compounds as well as a clearer understanding of the structure of the collagen molecule.

SUMMARY

The lathyrogenic effect of INAH in the chick embryo may be measured by the increase in the extractibility of collagen from the bones with 1 M NaCl. Incubation of these bones *in vitro* with carbonyl compounds diminishes the amount of extractible collagen; with D-L-glyceraldehyde the reversal of the INAH effect is complete. This reversal effect is dependent on the time and temperature of incubation and on the quantity of D-L-glyceraldehyde, but is independent of the pH of the incubating medium, the optical form of the glyceraldehyde, or the metabolism of the cells; this suggests that it depends on a simple chemical combination. D-L-glyceraldehyde also reverses completely the extractibility of collagen from the bones of embryos rendered lathyrogenic with BAPN, semicarbazide, and hydrazine hydrate.

The hypothesis has been advanced "that lathyrogenic agents act by blocking carbonyl groups on the collagen molecule, thus preventing cross-linking essential to normal maturation; normal maturation may be restored by the addition of carbonyl groups which act by competing either for the lathyrogen or for functional sites on the collagen molecule."

In support of this hypothesis, it has been shown that purified lathyritic guinea pig collagen takes up lesser amounts of 2,4-dinitrophenylhydrazine—a compound which combines with carbonyl groups—than does normal collagen; it has been shown that lathyritic collagen still possesses the ability to form segment-long-spacing (SLS) collagen, but that these fibres are much thinner than normal; this is due perhaps to blockade of groups essential for lateral cross-linking of the tropocollagen unit.

It has also been shown that normal, purified guinea pig collagen which has been pretreated with INAH, takes up lesser amounts of 2,4-dinitrophenylhydrazine and forms much thinner SLS fibres than the untreated controls.

The author wishes to thank Professor Sir Howard Florey, P.R.S., for encouragement, Miss Valerie Petts for invaluable technical assistance, and Miss Christine Court and Mr. Stanley

Buckingham for reproduction of graphs and photographs. The gifts of BAPN by Abbott Laboratories of North Chicago, and of INAH by Roche Products, Llelwyn Garden City, England, are gratefully acknowledged.

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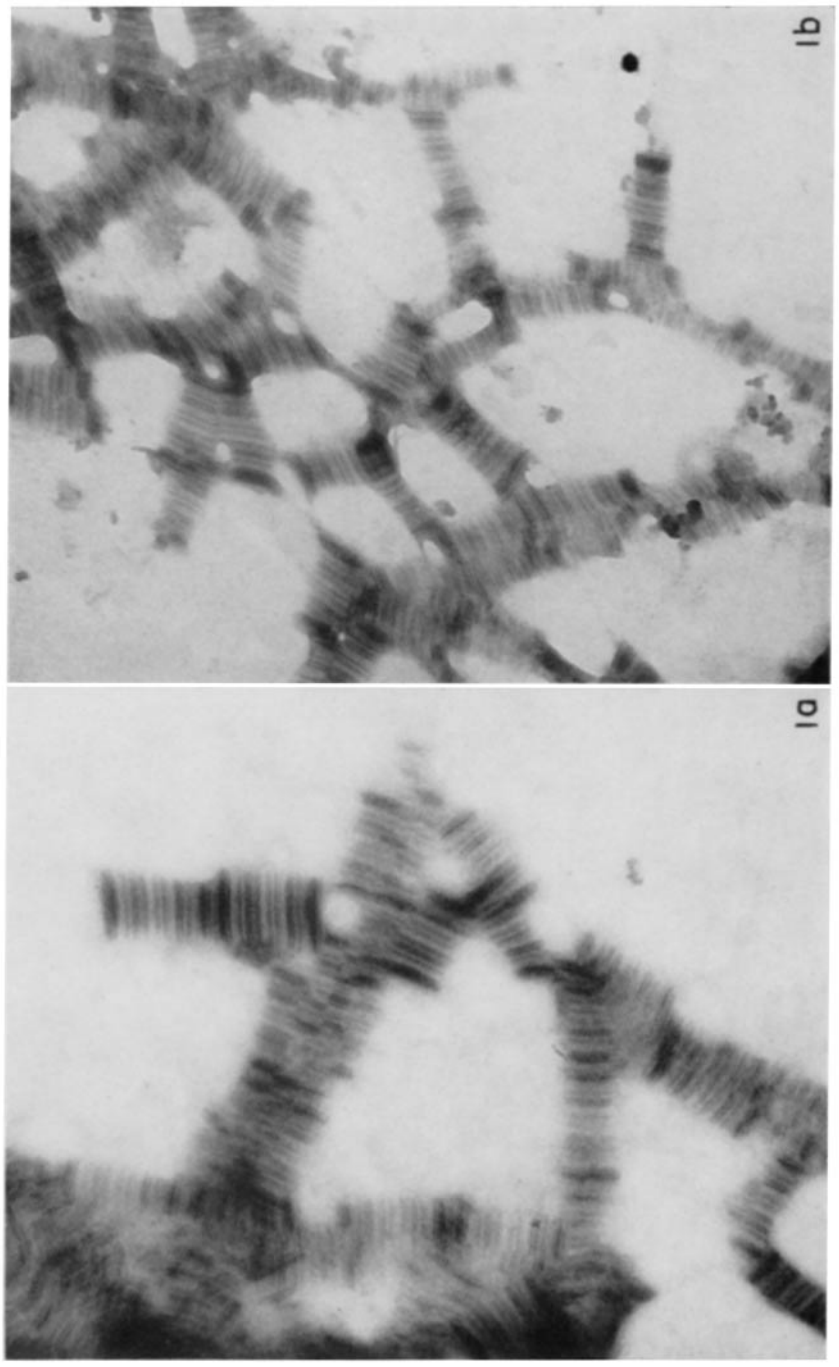
EXPLANATION OF PLATES

PLATE 10

FIG. 1 a. SLS fibres prepared from purified normal guinea pig collagen, stained with PTA, and examined in electron microscope. Magnification × 50,000.

FIG. 1 b. SLS fibres prepared from purified guinea pig collagen after treating animal for 14 days with BAPN; segments stained with PTA and examined in electron microscope. These segments, whilst showing the same periodicity, are nevertheless narrower than the normal SLS in Fig. 1 a. Magnification × 50,000.



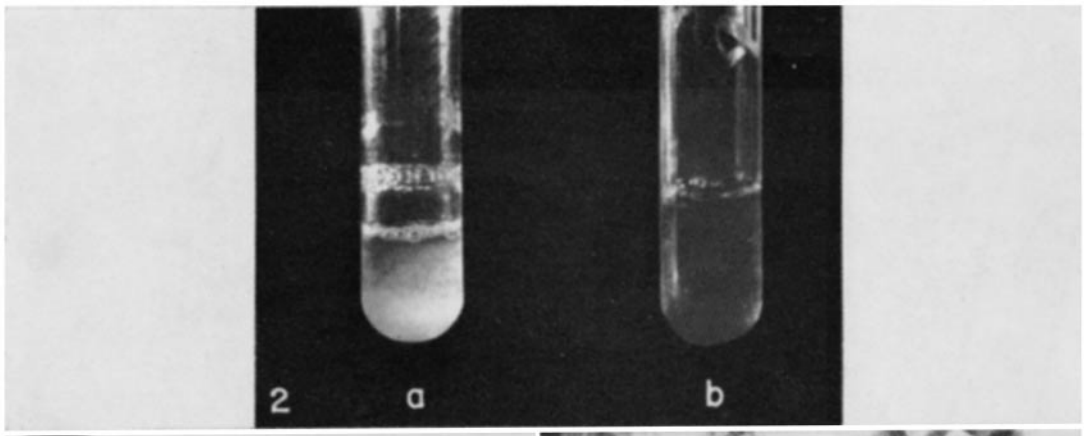
(Levene: Lathyrogenic compounds)

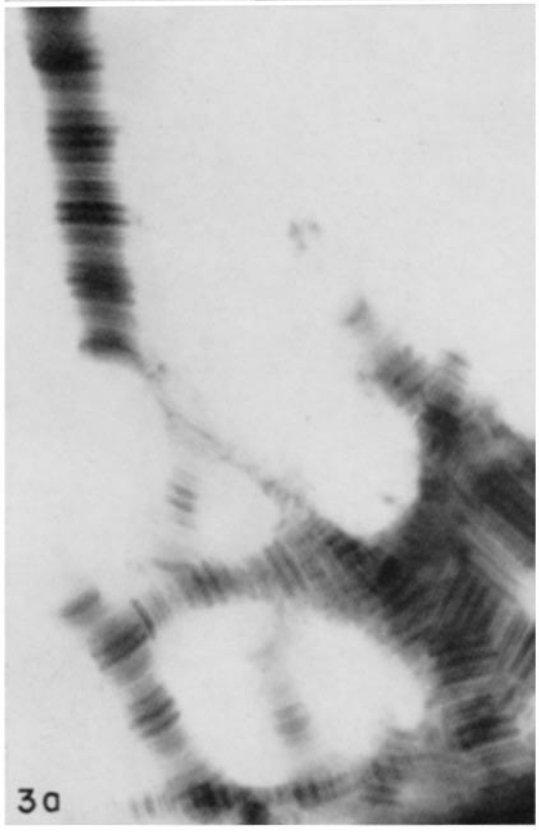
PLATE 11

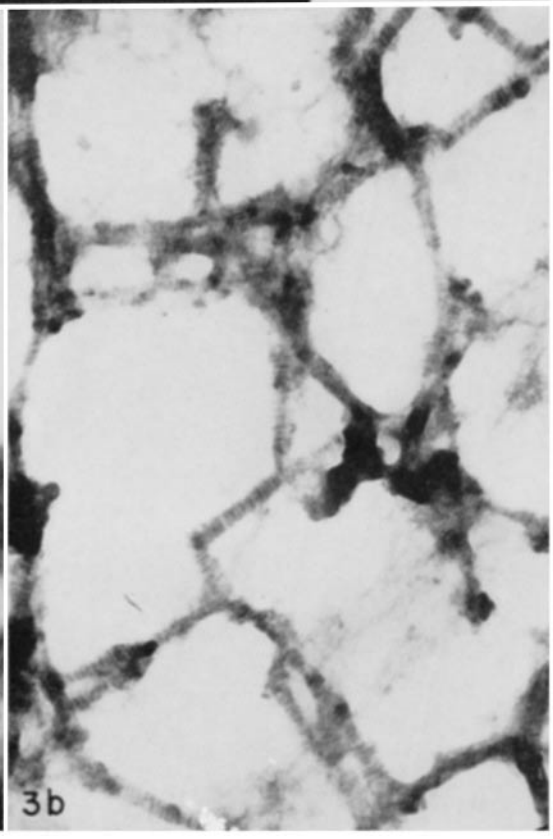
FIG. 2. Acetic acid solution of normal guinea pig collagen 5 minutes after addition of adenosine triphosphate; (*a*) normal collagen, showing heavy precipitate of SLS; (*b*) normal collagen pretreated with INAH showing lag in formation of very scanty SLS precipitate.

FIG. 3 a. SLS fibres formed from purified normal guinea pig collagen; stained with PTA. Magnification × 42,000.

FIG. 3 b. SLS fibres formed from same normal guinea pig collagen as Fig. 3 a after it had been pretreated *in vitro* with INAH; stained with PTA. These show the same periodicity as Fig. 3 a but are much thinner. × 42,000.







(Levene: Lathyrogenic compounds)