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Aspects of Calcification

THE AVAILABILITY OF ϵ -AMINO GROUPS IN COLLAGEN AGGREGATES

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Soluble collagen of connective tissue can be precipitated as six different types of fibril each representing a specific mode of aggregation of rod-like collagen molecules. Five of the reconstituted collagen aggregates are interconvertible, namely the 640 Å period, 220 Å period and structureless fibrils, the fibrous long-spacing fibril and the segment long-spacing aggregate, yet only the 640 Å fibril is found in Nature (for review see Schmitt, 1956). A nodular reconstituted collagen fibril has also been described (Kahn, Carrol & Witnauer, 1961).

Neuman & Neuman (1953) were the first to suggest that the mineralization of bone and dentine was initiated by collagen fibrils and their epitactic theory of calcification (Neuman & Neuman, 1958) has been further developed by Glimcher and his co-workers.

Glimcher, Hodge & Schmitt (1957) showed that the 640 Å fibril was the only collagen aggregate capable of nucleating hydroxyapatite (bone salt) crystals *in vitro* from metastable solutions (Strates, Neuman & Levinskas, 1957) of calcium phosphate.

By blocking the ϵ -amino groups of native collagen with FDNB*, Glimcher (1960) has established that these phosphate-binding groups are essential for calcification *in vitro*.

Solomons & Irving (1958) have demonstrated, however, that the ϵ -amino groups of soft-tissue collagen are less accessible to FDNB than those of bone or dentine. The unnatural collagen fibrils might, therefore, be prevented from calcifying *in*

vitro by a lower accessibility of their ϵ -amino groups rather than an absence of the unique stereo-structure of the 640 Å fibril. The present work was designed to investigate this possibility.

Collagen aggregates were dinitrophenylated by the procedure of Levy & Li (1955), a method discussed by Fraenkel-Conrat, Harris & Levy (1955). It was thought that, by causing the aggregates to react in purely aqueous media under simulated physiological conditions, the experimental results could be more closely related to those of Glimcher *et al.* (1957) than would be possible had the conventional Sanger (1945) dinitrophenylation procedure been employed.

EXPERIMENTAL

Materials

Acetic acid-soluble calf-skin collagen. Purified calf dermis (10 g.) was extracted with 0.5 N-acetic acid (600 ml.) for 24 hr. at 4° with occasional gentle stirring (Wood & Keech, 1960). The viscous collagen extract was strained through muslin and the residue of highly swollen insoluble collagen re-extracted (24 hr.) with sufficient 0.5 N-acetic acid to give a total volume of about 600 ml. After being strained through muslin, the combined extracts were clarified by filtration through coarse, medium and fine sintered glass.

A portion (500 ml.) of the acid filtrate was dialysed against three changes of 10 l. of 0.17 M-NaCl for 3 days at 4°. After being adjusted to pH 7.4 with 0.1 N-NaOH, the dialysed solution was incubated at 37° for 1 hr. The rigid collagen gel was spun down at 2500g, washed with water until free from Cl⁻ ions and freeze-dried. A portion (500 mg.) of the product was dissolved in 500 ml. of

* Abbreviation: FDNB, 1-fluoro-2,4-dinitrobenzene.

0.05% acetic acid at 4° (48 hr.), filtered, and dialysed once against 10 l. of 0.17M-NaCl at 4° for 24 hr. The dialysed solution was adjusted to pH 7.4 and incubated at 37° for 1 hr. The heat-precipitated collagen was centrifuged, washed with water and redissolved in cold 0.05% acetic acid (500 ml.). Repetition of the procedures outlined above with freeze-drying gave triple-reconstituted collagen.

A standard 0.1% collagen solution was prepared by dissolving 400 mg. of the purified collagen in 400 ml. of 0.05% acetic acid, the viscous solution being filtered through porosity 4 sintered glass and kept at 4°. The collagen content was checked by hydroxyproline and total nitrogen determinations.

Collagen aggregates. The 640 Å, 220 Å, structureless, fibrous long-spacing and segment long-spacing aggregates of collagen were prepared from the 0.1% collagen solution according to the procedures of Gross, Highberger & Schmitt (for references see Schmitt, 1956). The fibrillar assignments of all aggregates were consistent with their electron micrographs. Pure serum α_1 -glycoprotein, required for the preparation of the fibrous long-spacing aggregate, was obtained by the subfractionation (Schmid, 1953) of a glycoprotein fraction isolated from human serum (human glycoprotein, fraction VI; Mann Research Laboratories Inc., New York, N.Y., U.S.A.).

Methods

Apparatus. Dinitrophenylations of collagen aggregates were carried out in a water-jacketed Pyrex titration cell, capacity 50 ml., equipped with a microburette, a combined glass electrode (Pye-Ingold Agroprobe) and a CO₂ guard tube. The reaction mixture was stirred by a magnetic stirrer mounted beneath the cell assembly. Since FDNB is only slightly soluble in water at room temperature, the reaction chamber was maintained at $37 \pm 0.1^\circ$ (cf. Fraenkel-Conrat *et al.* 1955).

General method for the dinitrophenylation of collagen aggregates in aqueous suspension at pH 7.4 and 37°. In the experiments of Glimcher *et al.* (1957), each aggregate was treated with a calcifying solution of the same total ionic strength as that used in its precipitation. Collagen aggregates were dinitrophenylated, therefore, (a) at a physiological ionic strength of 0.17, and (b) at the same ionic strengths as existed during aggregation. The native-type fibril has also been dinitrophenylated in water and in 5% NaCl (*I* 0.86).

(a) Dinitrophenylation in 1% NaCl (*I* 0.17). At least two dinitrophenylations of each collagen aggregate were carried out. Aggregates were prepared from 50 ml. portions of 0.1% collagen solution. Recoveries of collagen on aggregation were 80–100%. Hence the amount of collagen dinitrophenylated was 40–50 mg. After precipitation, the aggregate was sedimented at 2500g, resuspended in a filtered solution of the same ionic strength as used in its precipitation (i.e. water, 1%, 2% or 5% NaCl) and recentrifuged. The aggregate was then rinsed with 1% NaCl, transferred to the titration cell and resuspended in sufficient 1% NaCl to give a total volume of 20 ml. The suspension was warmed to 37° (water jacket) and the pH adjusted to 7.4 with 0.05N-NaOH in 0.12M-NaCl, added with stirring from the microburette, the tip of which dipped below the surface of the mixture. When the pH became steady at 7.4 (about 15–20 min.), 200 mg. of

FDNB was added. The reaction mixture was vigorously stirred (at 37°) and maintained at pH 7.4 by the continuous addition of standard alkali. Care was taken to exclude CO₂ (soda-lime tube). After a reaction period of 10 hr., the linear part of a plot of alkali uptake against time represented the hydrolysis of FDNB. Extrapolation of the linear portion gave the approximate alkali consumption due to reaction of the free ϵ -amino groups of the aggregate with FDNB, 2 OH⁻ ions being consumed for each ϵ -NH₂ group reacting (Fraenkel-Conrat *et al.* 1955). The reaction mixture was transferred, quantitatively, to a sintered-glass crucible and the aqueous phase removed by filtration. The DNP-aggregate was washed with three 50 ml. portions of ethanol to remove excess of FDNB and most of the 2,4-dinitrophenol. Sodium salts and some dinitrophenol were removed by extensive washing with water, the DNP-aggregate being freed from residual traces of this contaminant by continuous extraction with acetone (Soxhlet) for 24 hr. After a final rinse with ether, the product was dried to constant weight *in vacuo* over P₂O₅. On dinitrophenylation, the 640 Å aggregate (50 mg.) yielded about 52 mg. of DNP-aggregate.

(b) Dinitrophenylation at the ionic strengths existing during aggregation. Most techniques were identical with those described above. The individual ionic strengths of the reaction medium and standard alkali titrant (0.05N-NaOH) used in the dinitrophenylation of an aggregate were, as nearly as possible, the same as existed during aggregation, NaCl being added, as necessary, to adjust the total ionic strength. Salt-free 0.05N-NaOH was employed in dinitrophenylations of the long-spacing aggregates at near zero ionic strength.

Analytical methods. Portions (20 mg.) of the DNP-collagen aggregates were hydrolysed in sealed evacuated Pyrex tubes (calibrated at 3 ml.) with 2 ml. portions of 6N-HCl (constant-boiling HCl, redistilled from SnCl₄) for 16 hr. at 105° in the dark. Hydrolysis tubes were sealed after being cooled in liquid nitrogen and evacuated at 0.01 mm. for 5 min.

ϵ -Dinitrophenyl-lysine and -hydroxylysine contents of dinitrophenylated aggregates. After hydrolysis, peroxide-free ether (1 ml.) was added to the well-cooled contents of a hydrolysis tube. The mixture was shaken mechanically for 5 min. followed by a brief centrifugation at low speed. The ether extract was removed with a dropper pipette, fresh ether (1 ml.) was added to the hydrolysate and the extraction was repeated; in all, five extractions were performed. After removal of dissolved ether by air-blowing, the hydrolysate was made up to 3 ml. with water. A 1 ml. sample was diluted to 50 ml. with water and the total ϵ -DNP-lysine and ϵ -DNP-hydroxylysine concentration determined by recording the extinction at 360 m μ . Portions (50 μ l.) of the hydrolysate were applied to Whatman no. 20 paper and the descending chromatograms developed with butan-1-ol-acetic acid-water (4:1:5, by vol.) in the dark. As Solomons & Irving (1958) have observed, the spots corresponding to ϵ -DNP-lysine and ϵ -DNP-hydroxylysine are discrete when this solvent system is employed although the R_F values found in the present work were somewhat different (ϵ -DNP-lysine, R_F 0.63; ϵ -DNP-hydroxylysine, R_F 0.55). The spots were cut out, eluted with 1% (w/v) NaHCO₃ solution and their extinctions measured at 360 m μ , a paper blank correction being deducted from each reading.

Table 1. *Free ϵ -amino groups of collagen aggregates*

Experimental details are given in the text.

Aggregate (fibril type)	Ionic strength during aggregation	Dinitro- phenylation medium	Ionic strength during dinitro- phenylation	Mean no. of ϵ -NH ₂ groups* reacting with FDNB at pH 7.4 and 37° for 10 hr. (μ moles/g. of collagen)	Percentage availability ($\pm$ s.d.)	No. of dinitro- phenyl- ations
640 Å	0.17	1% NaCl	0.17	198	61.3 ($\pm$ 1.5)	3
220 Å	0.34	1% NaCl	0.17	189	58.6 ($\pm$ 1.7)	3
Structureless	0.86	1% NaCl	0.17	199	61.6 ($\pm$ 1.7)	3
Fibrous long-spacing	0.00	1% NaCl	0.17	178	55.0 ($\pm$ 1.6)	3
Segment long-spacing	0.00	1% NaCl	0.17	173	53.5 ($\pm$ 1.5)	3
640 Å	0.17	5% NaCl	0.86	223	69.1 ($\pm$ 1.4)	3
220 Å	0.34	2% NaCl	0.34	224	69.3	2
Structureless	0.86	5% NaCl	0.86	224	69.5	2
Fibrous long-spacing	0.00	Water	Nearly zero	139	43.1	2
Segment long-spacing	0.00	Water	Nearly zero	149	46.3	2
640 Å	0.17	Water	Nearly zero	128	39.8 ($\pm$ 1.7)	3

* Total number of ϵ -NH₂ groups/g. of acid-soluble calf-skin collagen = 323 μ moles (Bowes *et al.* 1955).

Recovery of ϵ -dinitrophenyl-lysine on hydrolysis. This was estimated by heating collagen aggregates (20 mg. portions) with 2 ml. portions of standard 2 mM- ϵ -DNP-lysine solution in 6N-HCl under conditions identical with those described above. After extraction with ether (see above) the control hydrolysates were diluted with water and their extinctions determined at 360 m μ . Paper chromatography indicated that ϵ -DNP-lysine was the sole DNP component of control hydrolysates after ether extraction.

Total lysine and hydroxylysine content of collagen aggregates and dinitrophenylated aggregates. Free lysine and hydroxylysine in hydrolysates were assayed by the method of Porter (1950). This method depends on the fact that Cu²⁺ ion will protect the α - but not the ϵ -amino groups of free dibasic amino acids from reacting with FDNB. The total number of ϵ -amino groups present in a collagen aggregate was calculated from the extinction of its hydrolysate at 360 m μ after the latter had been neutralized with excess of NaHCO₃, treated at the boil with CuCO₃, treated (24 hr. at 25°) with FDNB, reacidified with dilute HCl and exhaustively extracted with ether.

Other analytical procedures. Hydroxyproline was determined by a modified Stegemann procedure due to Woessner (1961). Hexosamine was estimated as glucosamine by the Boas (1953) modification of the Elson-Morgan method. Total nitrogen determinations were carried out by the micro-Kjeldahl method, digests being analysed by the Conway (1950) microdiffusion technique.

Calculations. The following data were adopted. Soluble calf-skin collagen has an ϵ -amino group content of 323 μ -moles/g. (Bowes, Elliot & Moss, 1955), a hydroxyproline content of 14.4% (Neuman, 1949) and a total nitrogen content of 17.7% (Bowes *et al.* 1955). The extinction coefficients and hydrolytic recoveries of ϵ -DNP-lysine and ϵ -DNP-hydroxylysine are given in the Results section.

RESULTS

The proportions of ϵ -amino groups available for dinitrophenylation under the described conditions are shown in Table 1.

Availability of ϵ -amino groups in aggregates dinitrophenylated in 1% sodium chloride. All collagen aggregates treated at a constant ionic strength of 0.17 had similar availabilities. The proportions of ϵ -amino groups reacting with FDNB in the 640 Å, 220 Å and structureless aggregates were identical to within the experimental error although the proportions were significantly lower in both long-spacing aggregates. All dinitrophenylations were incomplete at this ionic strength (*I* 0.17) after a reaction period of 10 hr.

Effect of ionic strength on the availability of ϵ -amino groups to fluorodinitrobenzene. Table 1 shows that aggregates were completely dinitrophenylated at higher ionic strengths (*I* 0.86 and 0.34) if one accepts values of 69 and 63% (Bowes & Moss, 1953; Bowes *et al.* 1955) for the respective availabilities of calf-skin procollagen and ox-hide collagen. Ox achilles tendon and rat-tail tendon have availabilities of 67 and 68% respectively (Solomons & Irving, 1958). At near zero ionic strength only about 40% of the ϵ -amino groups present in the 640 Å and the two long-spacing aggregates had reacted with FDNB during the same interval of time.

Molar ratio of lysyl to hydroxylysyl ϵ -amino groups reacting with fluorodinitrobenzene. When the 640 Å and structureless aggregates were dinitrophenylated in 5% sodium chloride and the 220 Å aggregate in 2% sodium chloride respectively, the molar ratio of ϵ -DNP-lysine to ϵ -DNP-hydroxylysine was 3.4–3.8:1. Lower ratios (1.9–2.4:1) were observed for collagen aggregates (640 Å, both long-spacing aggregates) dinitrophenylated at almost zero ionic strength.

Recovery of ϵ -dinitrophenyl-lysine and -hydroxylysine on hydrolysis. Very high recoveries of ϵ -DNP-lysine were obtained in the present work. Apart

from the fibrous long-spacing aggregate, recoveries of ϵ -DNP-lysine were identical ($96.9 \pm 0.1\%$) when a 2 mm solution in 6N-hydrochloric acid was used to hydrolyse the various collagen aggregates under standard conditions. In the presence of the fibrous long-spacing aggregate the recovery was only $88.4 \pm 0.2\%$, and the latter value has been used in calculations involving this fibril.

Preliminary experiments in which various amounts of starch were hydrolysed with standard ϵ -DNP-lysine have shown that the destruction of the DNP-amino acid was proportional to the amount of carbohydrate added. About 33 % of the original ϵ -DNP-lysine was lost from solution when the latter (1 ml.) was heated under standard conditions with 5 mg. of starch. Quantitative recoveries of ϵ -DNP-lysine were observed when a 2 mm solution in 6N-hydrochloric acid was heated under standard conditions in the absence of collagen or carbohydrate or both.

Since an authentic sample of ϵ -DNP-hydroxylysine was not available, its recovery has been assumed to be identical with that of ϵ -DNP-lysine under the same conditions (Solomons & Irving, 1958).

Total lysine and hydroxylysine content of collagen aggregates and dinitrophenylated aggregates. Values for the total ϵ -amino group content of collagen aggregates ($322\text{--}327\text{ }\mu\text{moles/g.}$) obtained by using the copper complex method (Porter, 1950; cf. Solomons & Irving, 1958) were in excellent agreement with that ($323\text{ }\mu\text{moles/g.}$) obtained by Bowes *et al.* (1955) for citrate-soluble calf-skin collagen. About 95 % of the total ϵ -amino groups present in a DNP-aggregate could be accounted for after corrections had been applied for the destruction of ϵ -DNP-lysine and hydroxylysine during hydrolysis (see above).

Extinction coefficients of ϵ -dinitrophenyl-lysine and -hydroxylysine. Although ϵ -DNP-lysine has a molar extinction of 17700 in 1 % sodium bicarbonate at $360\text{ m}\mu$ (Fletcher, Lowther & Reith, 1954), its extinction in strongly acidic solution has not been recorded. This has been measured (in 1N-hydrochloric acid) and found to be virtually identical with the extinction in 1 % sodium bicarbonate. No data being available, the molar extinction of ϵ -DNP-hydroxylysine has been assumed to be the same as that of ϵ -DNP-lysine. The assumption seems valid since the ultraviolet-absorption spectrum of ϵ -DNP-allohydroxylysine, as measured by Zahn & Zürn (1958), indicates that the extinction should be about 17000 at $360\text{ m}\mu$.

Hexosamine content of collagen aggregates. Apart from the fibrous long-spacing aggregate, the hexosamine content of all aggregates was about 0.3 %, a value corresponding to the hexosamine content of the purified collagen used in their preparation.

Most samples of the fibrous long-spacing aggregate contained about 1.5 % of hexosamine. Since serum α_1 -glycoprotein contains 11.5 % of hexosamine (Schmid, 1953), the hexosamine present in this aggregate was equivalent to a glycoprotein content of about 10 %. The latter was strongly bound, apparently, since extensive washing of the fibrils with water did not appreciably affect their hexosamine content.

DISCUSSION

A significant finding of the present investigation is that the ϵ -amino group availabilities of all collagen aggregates are of the same order when the aggregates are dinitrophenylated under identical conditions in 1 % sodium chloride. Moreover, no significant differences exist between the availabilities of the 640 Å, 220 Å and structureless aggregates on dinitrophenylation in this medium. Though all dinitrophenylations in 1 % sodium chloride were incomplete, the ϵ -amino group availabilities of the 640 Å, 220 Å and structureless aggregates were again identical at higher ionic strengths. Under these conditions the free ϵ -amino groups of the 640 Å fibril were completely dinitrophenylated (69.1 %; cf. Bowes *et al.* 1955) after 10 hr.

Since only the 640 Å fibril could nucleate hydroxyapatite *in vitro* (Glimcher *et al.* 1957), the inability of the 220 Å and structureless fibrils to calcify under the same conditions can hardly be attributed to a lower accessibility of their ϵ -amino groups. Further, as the preparation of all three reconstituted collagen fibrils only requires the presence of salt, there can be no possibility of strongly adsorbed contaminants affecting either the calcification or dinitrophenylation experiments. Conclusions based on ϵ -amino group-availability studies are, however, subject to the reservations (Glimcher, 1960) on the analogy between the reaction of ϵ -amino groups with FDNB and the binding of phosphate ion by the same groups.

When dinitrophenylated in 1 % sodium chloride, the proportions of reactive ϵ -amino groups in the two long-spacing aggregates were significantly less (55 %) than those (61 %) of the other three aggregates. The former value is, however, not thought to be sufficiently low to prevent calcification *in vitro*. The ϵ -amino group-availability values of all aggregates would probably have been several per cent higher had dinitrophenylation in 1 % sodium chloride been more prolonged. Glimcher (1960) could not accurately determine the critical number of free ϵ -amino groups below which partially dinitrophenylated native collagen lost the ability to calcify *in vitro*. Nevertheless, he states that the critical value is well below the 64–70 % value found

by Solomons & Irving (1958) for the free ϵ -amino group availability of the soft-tissue collagens.

The lower percentage of reactive ϵ -amino groups (53.5 %) observed on dinitrophenylation of the segmented aggregate in 1 % sodium chloride may be due to diffusion barriers set up by the close parallel packing of collagen molecules in the aggregate. In dentine, Solomons & Irving (1956) and Solomons, Irving & Neuman (1960) showed that the number of ϵ -amino groups available for dinitrophenylation was inversely proportional to the total mineral content of the tissue. Diffusion barriers created by the close proximity of hydroxyapatite crystals to the collagen fibril were considered (Glimcher, 1960) to be responsible for the latter result rather than a chemical interaction of ϵ -amino groups and hydroxyapatite.

Steric effects may also have decreased the percentage of reactive ϵ -amino groups (55 %) in the fibrous long-spacing aggregate on dinitrophenylation in 1 % sodium chloride. This fibril has, however, been found to contain appreciable amounts (approx. 10 %) of serum α_1 -glycoprotein. Some ϵ -amino groups might, therefore, have been made unavailable by the formation of complexes with glycoprotein. Though it is possible that tightly bound glycoprotein might have inhibited the calcification (Glimcher *et al.* 1957) of the fibrous long-spacing fibril this seems rather unlikely in view of the highly specific stereochemical conditions required for the epitactic nucleation of hydroxyapatite (Glimcher *et al.* 1957; Glimcher, 1960).

The very low availability of ϵ -amino groups (approx. 44 %) in the 640 Å, fibrous long-spacing and segment long-spacing aggregates when dinitrophenylated in water is not easily explained, since the swelling of collagen aggregates at low ionic strength might be expected to increase the accessibility of these groups. At higher ionic strengths, however, the ϵ -amino groups of collagen might be made more accessible by a reorientation of lysyl and hydroxylysyl side chains away from the polypeptide backbone of the molecule due to compensation of the negative charges on glutamyl and aspartyl residues by Na^+ ions (cf. the interchain lysine-glutamic acid salt linkage discussed by Gustavson, 1956).

There appears to be no adequate explanation for the lower molar ratio of lysyl to hydroxylysyl ϵ -amino groups available to FDNB at low ionic strength.

From the calcification studies of Glimcher *et al.* (1957) and Glimcher (1960) and the findings of the present work it would appear that the unique stereostructure of the natural (640 Å) collagen fibril is of even greater importance than the number of its free ϵ -amino groups in effecting the epitactic calcification of collagen *in vitro*.

The very high recoveries (88.4–96.9 %) of ϵ -DNP-lysine obtained in control hydrolyses require comment. Considerably lower recoveries of ϵ -DNP-lysine have been reported by others (e.g. 80 % by Solomons & Irving, 1958) for hydrolyses in the presence of collagen and under similar conditions of acid strength, temperature and duration. In previous hydrolyses, however, appreciable amounts of oxygen (air) were present. The present improved recovery of ϵ -DNP-lysine is attributed to the virtual absence of free oxygen, peroxides and light during hydrolysis. Fraenkel-Conrat *et al.* (1955) obtained better recoveries of DNP-amino acids when they carried out hydrolyses in sealed evacuated tubes rather than by open reflux.

The increased destruction of ϵ -DNP-lysine on hydrolysis of the fibrous long-spacing aggregate was doubtless due to the higher carbohydrate content of this collagen-glycoprotein complex, as the preliminary experiments suggest. Fraenkel-Conrat & Porter (1952) ascribed the extensive destruction of ϵ -DNP-lysine and DNP-alanine on hydrolysis of DNP-ovomucoid to the protein-bound carbohydrate moiety. Since both the α - and the ϵ -amino groups of casein (Lea & Hannan, 1950), α -N-acetyl-L-lysine and polylysine (Hannan & Lea, 1952) react with glucose, the α -amino group of ϵ -DNP-lysine should also be capable of reaction with glucose or similar sugars. Gottschalk & Partridge (1950) found that free lysine reacted with glucose to yield 5-hydroxymethylfurfural derivatives, the latter rapidly forming humin in hot acid solution.

However, as considerable losses of DNP-alanine (no free amino group) occurred on hydrolysis in the presence of carbohydrate (Fraenkel-Conrat & Porter, 1952), other interactions, chemical or physical, must take place between DNP-amino acids and carbohydrate. Adsorption of DNP-amino acids by humin or reduction of their nitro groups by carbohydrate degradation products, or both, are possibilities to be considered.

SUMMARY

1. The free ϵ -amino groups of five types of reconstituted collagen aggregate have been determined by reaction with 1-fluoro-2,4-dinitrobenzene in aqueous media of various ionic strengths at pH 7.4 and 37°.

2. No significant differences existed between the ϵ -amino group availabilities (approx. 61 %) of the 640 Å, 220 Å and structureless fibrillar aggregates on dinitrophenylation in 1 % sodium chloride, although the availabilities of the fibrous long-spacing and segment long-spacing aggregates were appreciably lower (55 %) in this medium.

3. The ϵ -amino group availabilities of the 640 Å, 220 Å and structureless fibrils were also identical (69.5 %) on dinitrophenylation at higher ionic strengths.

4. When dinitrophenylated in water, the proportions of reactive ϵ -amino groups in the 640 Å and the two long-spacing aggregates were considerably less (approx. 44 %) than those of the same aggregates in 1 % sodium chloride.

5. As the ionic strength of the aqueous dinitrophenylation medium was decreased, the molar ratio of lysyl to hydroxylysyl ϵ -amino groups available to fluorodinitrobenzene in the 640 Å and the two long-spacing aggregates also decreased.

6. Most samples of the fibrous long-spacing aggregate contained about 10 % of the glycoprotein used in their preparation.

7. The significance of these findings is discussed in relation to the calcification of collagen fibrils *in vitro*.

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Changes in Liver Nucleotide Concentrations in Experimental Liver Injury

1. CARBON TETRACHLORIDE POISONING

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A single dose of carbon tetrachloride administered to rats causes centrilobular necrosis and fatty degeneration of the liver (for early references see Van Oettingen, 1955). After a suitable enteral dose, large increases in liver fat can be detected at

6 hr. and hepatic necrosis is easily recognizable at 12 hr., reaching a maximum 1 day after administration.

Much biochemical work has been done in attempts to elucidate the pathogenesis of the