DRUG DEVELOPMENT AND INDUSTRIAL PHARMACY, 20(3), 293-314 (1994)

THE INFLUENCE OF IONIZING RADIATIONS UPON DIFFERENT FORMS OF COLLAGEN

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ABSTRACT

Collagens received increasing doses of radiations β or γ The structural state of each of them is controlled by differential scanning calorimetry and by electrophoresis on polyacrylamide gel. Each of this method allows the study of only a part of the structure of collagen. The hemostatic activity is analysed by agregation test. The changes in the collagens in relation to irradiation are more important when studied by electrophoresis than by differential scanning calorimetry. Ionizing radiations change hemostatic activity of collagens.

INTRODUCTION

The aim of our work is to study the influence of ionizing rays upon a raw material, namely collagen, while evaluating the structural state of the molecule and its ability to maintain one of its activities.

Collagens are biopolymers whose uses are and will continue to be numerous in pharmaceutical, medical and cosmetic fields. As a biopolymer, collagen has two essential characteristics : biocompatibility and biodegradability. Moreover, these molecules are equiped with a certain number of properties which make them of interest to us. Amongst the most

important are its participation in the mechanical properties of the connective tissues, the activation of blood coagulation and its role in cellular growth.

Collagen is a macromolecule of the fibrous glycoprotein type. It is composed of 19 amino acids. The sequence of amino acids, which is formed by the linking of peptides, is a succession of type glycine X-Y, in which X and Y are often proline and hydroxyproline. The hydroxyproline is specific to collagen. The succession of different triplets is at the origin of the formation of a polypeptide chain, which is labelled as chain α. This chain is shaped like a helix with a left thread. Each chain is made up of a central helical pot, and two shapeless, amorphous, non-helical ends : telopeptides.

Three α chains wind round a central axis into the shape of a triple, right helix, which forms the structural unit of collagen : tropocollagen. The rigidity of this triple propeller is assured by hydrogen and Van Der Waals links which are created between the water molecules and the proline and hydroxyproline residues (1, 2, 3).

The different tropocollagen units merge, with a gap of a quarter of their lenght, into fibrils and then into fibres.

According to the degree of maturity of the collagen, different links within the molecule change, resulting in collagens with different levels of solubility (4).

It is in such a way that intramolecular links of the aldol type are built between the chains α of the same tropocollagen molecule, and define its subunits, β and γ. Moroever, intermolecular links are formed between different tropocollagen molecular chains. The latter become stable, constituting in reticulation of the collagen and leading to an insoluble fibrous collagen.

Different types of collagen are distinguishable in different tissues according to the distribution of the amino acids in the α chains, and the distribution of the α chains in the tropocollagen. Thus, as far as the dermus is concerned, it is the collagen types I and III which are predominant.

Being a forerunner in the chain for obtaining gelatine, collagen is a good environment for microbe culture.

For certain uses, a degree of microbiological purety or sterility may be demanded. (5, 6, 7, 8, 9).

MATERIALS AND METHODS

Obtaining the batches of raw material

Our study makes use of collagen types I and III, which are extracted from the dermus of calves, in two different forms of solubility :

- acid-soluble
- insoluble

From the treatment of calf dermus, we isolate :

- the supernatant which is purified by dialysis. It then undergoes precipitation and is reduced to a solution of acid soluble collagen.
- the residue of the extraction which constitues the insoluble collagen.

The raw materials we are using are a solution of 0,3 % acid-soluble collagen containing 1 % of sorbic acid, insoluble collagen fibres.

Lyophilisation and precipitation are the processes necessary in obtaining the collagen batches.

Using a solution of acid-soluble collagen

The fibres are obtained using precipitation which itself is obtained by adding acetone to the water dispersion (1000 ml of acetone to every 100 ml of dispersion). The precipitated fibres are spin on a buschner and dried on filter paper. The product obtained is made up of precipitated soluble fibres, which we have abbreviated as P.S.F.

The collagen water solution is subjected to lyophilisation and results in the formation of lyophilised soluble fibres, or L.S.F.

Using insoluble collagen fibres

A water dispersion of 0,6 % fibres is prepared and then undergoes lyophilisation. This process allows us to obtain lyophilised insoluble fibres, or L.I.F.

Whether the collagen is in an insoluble or soluble form, the conditions for lyophilisation are the same (10). However, there is an inherant constraint to this procedure, which is due to the protein structure of the collagen : a temperature of or below 30° C must be maintained.

Ionizing radiations

Our work aims to study the influence of ionizing radiation upon the four batches of collagen, since this treatment aims to obtain raw materials which satisfy either the sterility test or the microbial contamination limit test. Therefore, we subjected the different lots to 6 increasing doses of radiation (11, 12, 13).

3 g of each batch of collagen are put into an A type paper sachet which has been partially coated. A sample from each batch of collagen is prepared according to dose and to radiation.

The doses which are in fact used and which are controlled by dosimeter for γ rays are : 6.41, 10.01, 16.23, 20.14, 25.25, 31.28.

For the β rays the doses, which are controlled by a film of cellulose acetate with a precision of $\pm$ 15 %, are 5, 10, 15, 20, 25, 30 kgy.

Due to the strong flow of the dose of electron accelerators, carboglace is used to avoid any overheating of the product.

Collagen content fo the batches

Hydroxyproline is the amino acid which characterises the collagen molecule (13,6 % of the weight of the collagen). Its dosage allows us to determine the collagen content. The conversion factor of hydroxyproline to collagen is 7.46. The dosage principle is based on the Stegeman ans Stadler method : after acid hydrolysis of the collagen, lasting 24 hours, the

oxidization product of the hydroxyproline, gained by chloramine T, gives along with the paradimethyl aminobenzaldehyde, a colourness which allows a dosage of up to 550 nm on the spectrophotometer.

Evaluation of the state of the molecule

We have chosen for examining the state of the molecule, and therefore the state of denaturation of the molecule (14) :

- differential scanning calorimetry
- analysis of the sub-units by electrophoresis

Differential scanning calorimetry

This method allows an appraisal of the helical structure of the collagen. When a collagen molecule is heated in an aqueous environment, it loses its helical structure. This denaturation process is accompanied by heat absorption which is a function in its native form which disappears.

The principle of this method is to record the variations in energy needed to maintain a sample at the same temperature as a reference cell. This variation is a function of the energy which is absorbed or given out by the sample during transition. Test were carried out on a Perkin-Elmer DSC.

2 to 2,5 mg of the product to be studied are weighed very accurately in the stainless-steel capsules. The product is moistened by approximately 70 mg of water.

The reference capsule is filled with the same weight in water. The crucibles are closed and put into the oven at 20° C.

Each control or irradiated sample is tested 2 or 3 times in the D.S.C. The enthalpy results are converted into Joules per gram of collagen and not per gram of product.

The denaturation of the collagen is expressed by a peak in heat absorption on the recording curve which shows the variation in power supplied to the two crucibles. The surface of

the peak of absorption is proportional to the enthalpy of the molecule denaturation (Δ H). This allows us to appraise the quantity of the helical structure of the collagen.

The analysis of a thermogram informs us about :

- the stability of the product, thanks to the temperature at the start of denaturation or even better to the temperature corresponding to the tangent of the peak. The higher the temperature, the more stable the product.

- the heterogenity of the product, thanks to the extent of denaturation zone (presence of soluble fibres in the insoluble fibres).

- the quantity of the helical structure, thanks to the calculation of denaturation enthalpy according to the surface under the peak.

Electrophoresis on polyacrylamide gel

This method allows an evaluation of the sub-units of collagen and an appreciation of its structural state.

The sample of collagen is subjected to a slow phase of denaturation by heating for 2 hours at 37° C in the presence of sodium dodecyl sulphate. The helical form collapses and the chains which are not connected by covalent links, separate.

The sub-units α, β and γ in the collagen become evident and migrate around differently, according to their molecular weight of 100 000, 200 000 and 300 000 daltons respectively.

A break in the covalent links between the tropocollagen molecules (dereticulation) brings about an increase in the number of α chains ; an increase in the γ chains would indicate reticulation.

The collagen dispersion is treated with a solution containing deposited then evaporated denaturing agent. After migration the gels are read on a photodensimeter, then dried.

Evaluation of hemostatic activity of collagens

Amongst the different properties of collagen, it was its hemostatic activity which was tested. On the collagen batches studied, we chose to apply the BORN method which studies the

activity of a certain number of agregation factors by using turbidimetry. It is a matter of recording the decrease in optical density of a plasma, which is rich in platelets, after adding the collagen.

RESULTS AND DISCUSSION

Collagen content of the batches

The percentages in collagen of the batches are :

Percentage in collagen in grams. per 100 g of product	P.S.F	L.S.F.	I.F.	L.I.F.
	83.24	78.63	76.92	77.74

Differential scanning calorimetry

At first, we compared the thermograms of fours batches before treatment by the ionizing radiations.

BEFORE TREATMENT

The quantitative results appear in table 1.

The aspect of the peaks of the soluble products is very much different to that of the insoluble products.

The denaturation peaks of the soluble products (P.S.F. and L.S.F.) are narrow with easy integration. Those of the insoluble products (I.F. and L.I.F.) are large with difficult integration, showing a greater heterogenity of the product (Figures 1 and 2).

The enthalpic value of the lyophilised soluble fibres (44° C) is markedly lower than that of the same non-lyophilised soluble fibres (54° C).

Lyophilisation of the soluble fibres seems to change their helical structure.

TABLE 1

Quantitative Results of Thermograms of Control Products

		Temperature		
Products	enthalpy ΔHJ/gr	T° of the start of the peak	T° of the end of the peak	T° of the tangent of the peak
P.S.F.	54.3	36.8	64.5	46.6
L.S.F.	44.7	35.4	66.6	42.2
I.F.	58.3	29.3	62.3	36.0
L.I.F.	58.9	30.4	60.5	35.3

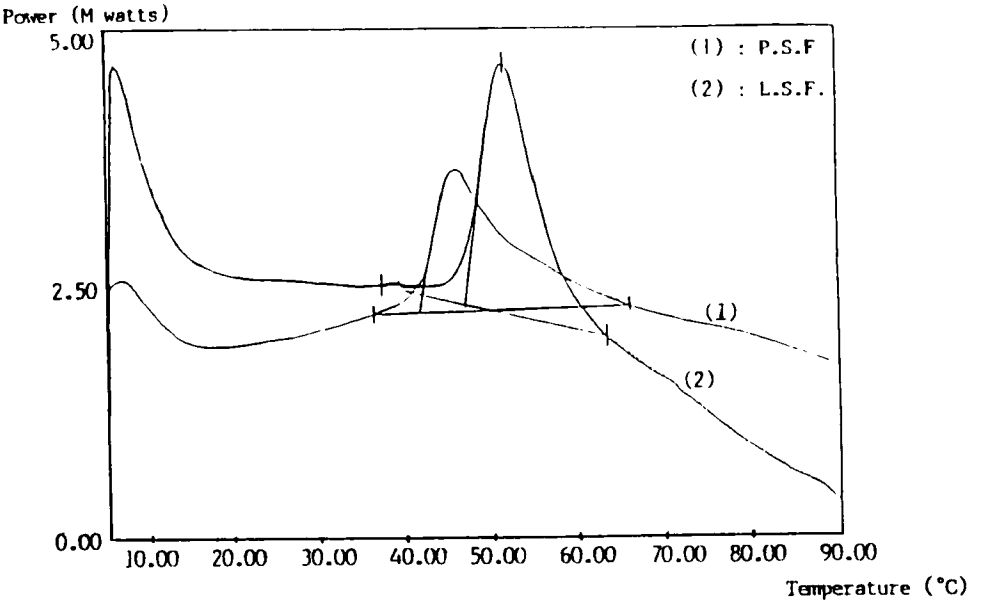

FIGURE 1 : Thermograms of non irradiated soluble collagens (PS.F. and L.S.F.)

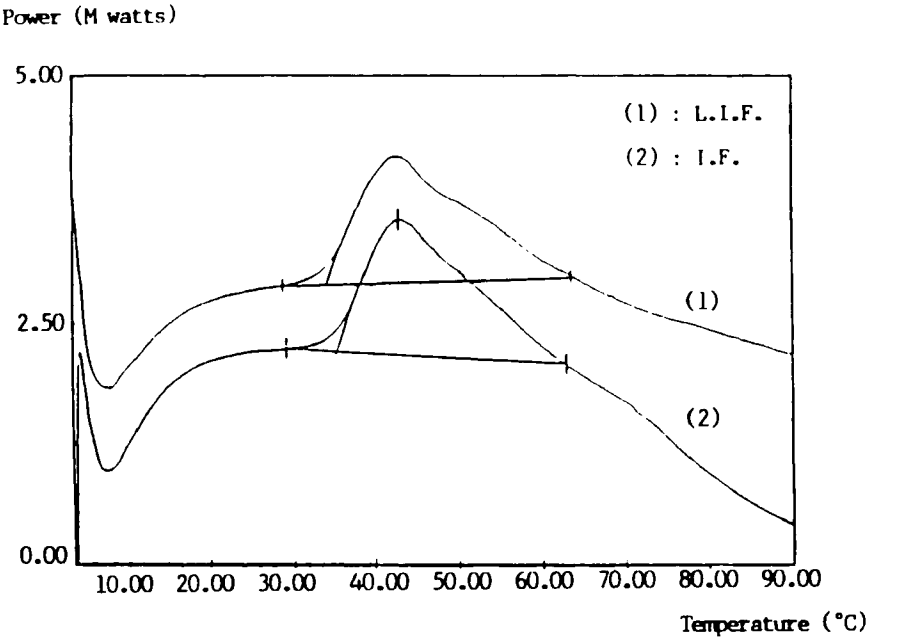

FIGURE 2 : Thermograms of insoluble collagens (I.F. and L.I.F.) non irradiated

TABLE 2

Mean Values of Thermograms of Precipitated Fibres Treated by Two Types of Radiations and Different Doses

Radiation doses	Enthalpy J/gr	T° of the start of the peak	T° of the end of the peak	T° of the tangent of the peak
Control	54,35	36,80	64,53	46,64
5 γ	51,05	35,47	64,06	44,76
10 γ	53,34	38,99	65,53	42,25
15 γ	54,99	36,49	64,06	42,17
20 γ	53,52	35,98	63,56	41,69
25 γ	52,63	34,72	63,05	41,34
30 γ	57,70	34,34	66,72	40,01
5 B	53,65	35,48	63,56	44,27
10 B	52,67	39,52	65,07	43,92
15 B	50,58	37,47	64,51	41,99
20 B	52,94	36,44	63,45	41,43
25 B	54,23	36,20	68,31	42,90
30 B	54,37	34,52	60,71	39,35

AFTER TREATMENT

The average values of the thermograms for the two types of collagen, according to the type of radiation, appear in tables 2, 3, 4, and 5.

According to these results, the enthalpy for most of the products (L.S.F. γ, L.S.F. β, L.I.F. β, I.F. γ, I.F.β) undergoes a slight fall, in relation to the irradiation dose received. But this evolution does not show itself markedly (Figure 3).

The enthalpy values of the products (P.S.F. γ, P.S.F.β, F.I.L. γ) do not seem to change under the doses of radation applied. It appears that the denaturation peaks become rounded off with the increase in the radiation dosage.

TABLE 3

Mean Values of Thermograms of Lyophilised Soluble Fibres Treated by Two Types of Radiations and Different Doses

Radiation doses	Enthalpy J/gr	T° of the start of the peak	T° of the end of the peak	T° of the tangent of the peak
Control	44,79	35,48	66,60	42,24
5 γ	40,46	36,10	67,14	45,49
10 γ	35,93	35,48	64,91	43,61
15 γ	38,84	36,46	67,55	45,58
20 γ	41,06	36,49	68,62	45,75
25 γ	40,08	36,96	69,63	45,63
30 γ	33,16	36,96	68,56	45,94
5 ß	47,85	37,47	66,54	43,00
10 ß	48,89	35,98	66,60	41,71
15 ß	37,21	35,92	66,98	41,49
20 ß	41,38	36,49	68,11	44,71
25 ß	40,32	36,46	67,04	45,30
30 ß	36,14	38,00	68,62	44,24

The aspect of the enthalpic peaks differs according to the nature of the product. One may note also a change in appearance of the peaks between the control products and the irradiated products. The enthalpy of the majority of the products undergoes a slight drop in relation to the irradiation dose received, but this evolution is not marked.

Irradiation has little influence on the collagen's helicoid structure.

However, the enthalpic values of the lyophilised soluble fibres are markedly lower than those of the non lyophilised soluble fibres. Is seems therefore that lyophilisation modifies the helicoid structure of the soluble fibres ; this action could take place upon the hydrogen and electrostatic links which maintain the triple propeller shape.

TABLE 4

Mean Values of Thermograms of Insoluble Fibres Treated by Two Types of Radiations and Different Doses

Radiation doses	Enthalpy J/gr	T° of the start of the peak	T° of the end of the peak	T° of the tangent of the peak
Control	58.30	29.28	62.32	36.00
5 γ	60.95	30.40	61.50	35.00
10 γ	58.59	30.42	61.53	35.00
15 γ	56.53	30.39	59.96	33.93
20 γ	54.60	30.39	61.48	34.60
25 γ	49.78	30.08	58.32	34.29
30 γ	44.76	30.77	58.14	34.32
5 β	53.07	29.91	60.53	35.37
10 β	55.83	31.41	61.49	34.97
15 β	54.06	30.42	60.53	35.29
20 β	59.56	30.39	59.97	34.26
25 β	43.91	30.96	55.92	33.73
30 β	47.64	31.78	56.73	34.52

Electrophoresis on polyacrylamide gel

BEFORE TREATMENT WITH RADIATION

* Soluble fibres

The electrophoresis profiles of the non-irradiated controls of the precipitated and lyophilised soluble fibres are similar to that of the collagen solution used in their preparation.

Lyophilisation and precipitation by aceton of the soluble fibres do not seem to influence the subunits of this soluble collagen (Figure 4).

TABLE 5

Mean Values of Thermograms of Lyophilised Insoluble Fibres Treated by Two Types of Radiations and Different Doses

Radiation doses	Enthalpy J/gr	T° of the start of the peak	T° of the end of the peak	T° of the tangent of the peak
Control	58.92	30.42	60.57	35.34
5 γ	54.13	31.09	61.54	35.05
10 γ	57.80	31.39	60.76	35.06
15 γ	52.74	30.42	60.53	34.88
20 γ	56.11	29.72	63.49	33.85
25 γ	47.32	30.92	59.26	33.86
30 γ	55.31	30.08	63.00	33.76
5 β	51.43	30.42	62.55	35.45
10 β	57.58	30.40	60.48	34.54
15 β	51.95	30.40	60.47	34.35
20 β	52.78	30.54	61.73	33.58
25 β	48.57	30.78	56.30	33.69
30 β	49.75	31.78	57.76	34.32

* Insoluble fibres

The profiles of the lyophilised and non-lyophilised insoluble fibres are identical. Lyophilisation does not modify the subunits of this collagen (Figure 5).

AFTER TREATMENT

The electrophoresis of the products are changed greatly according to the irradiation dose.

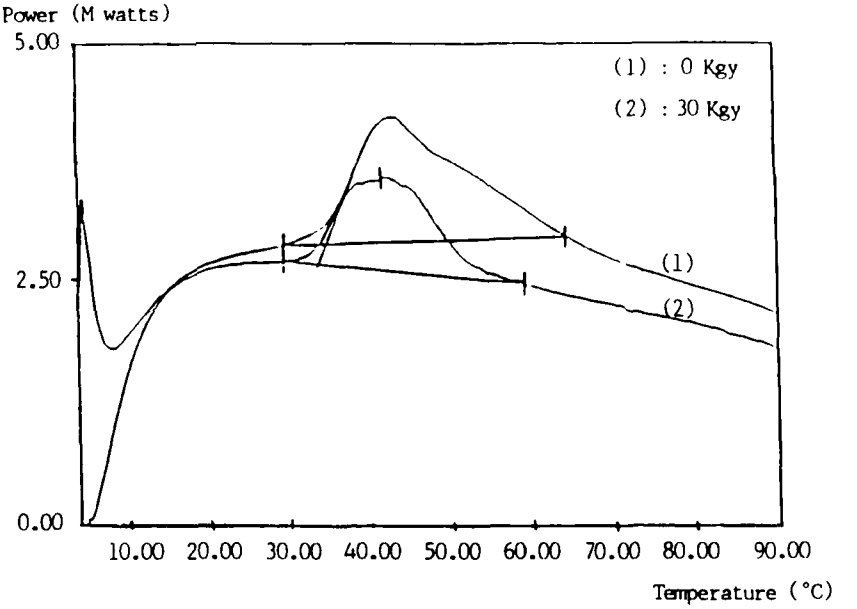

FIGURE 3 : Thermograms of lyophilised insoluble fibres (L.I.F.) non irradiated and irradiated with a dose of 30 kgy

* Soluble fibres

Whether lyophilised or precipitated, the soluble fibres give identical electrophoretic profiles. As from the dose of 5 Kgy, there is a great decrease, especially in the β subunits, followed by the α subunits (Figure 6).

* Insoluble fibres

Lyophilised or not, they have the same electrophoretic behavior (Figure 7).

Although their electrophoretic profile is greatly modified, they seem to be more resistant than the soluble fibres.

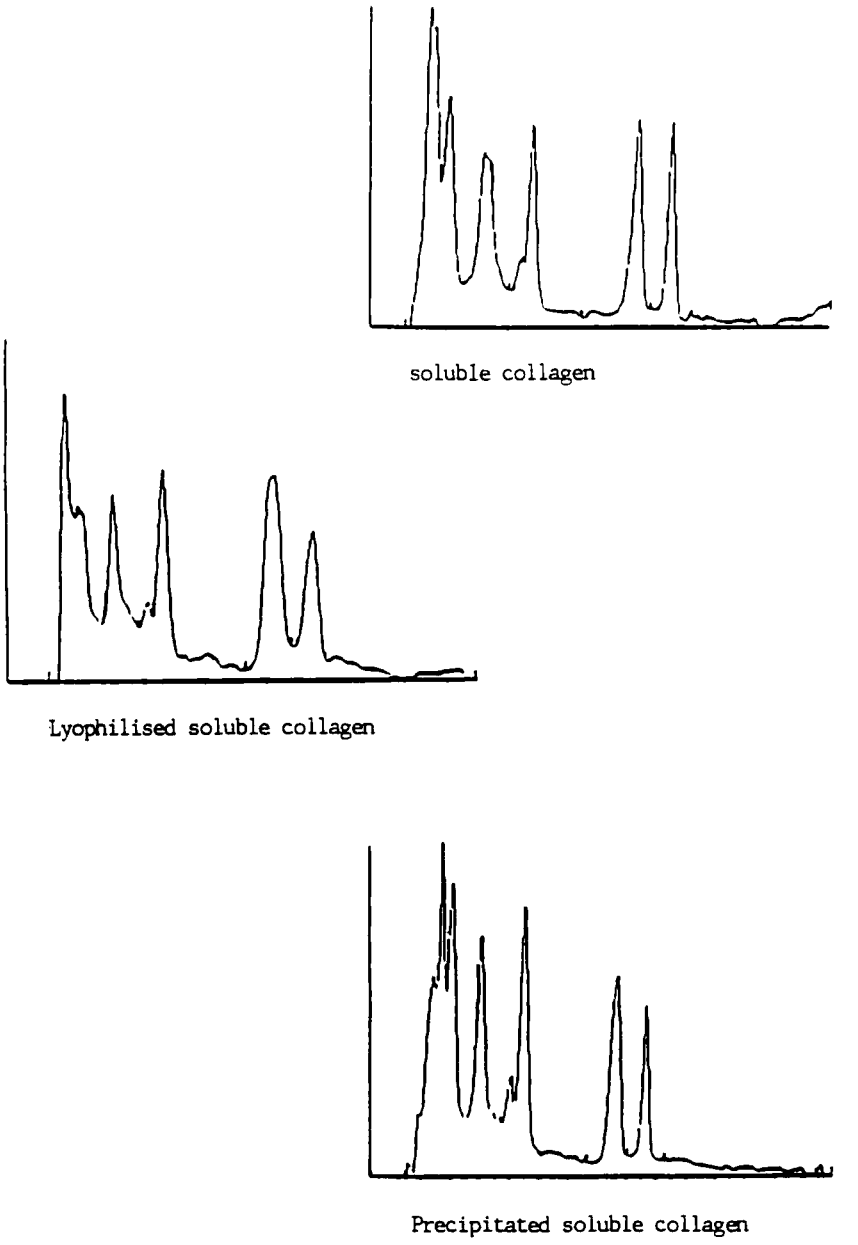

FIGURE 4 : Electrophoresis profile of soluble collagens, non irradiated

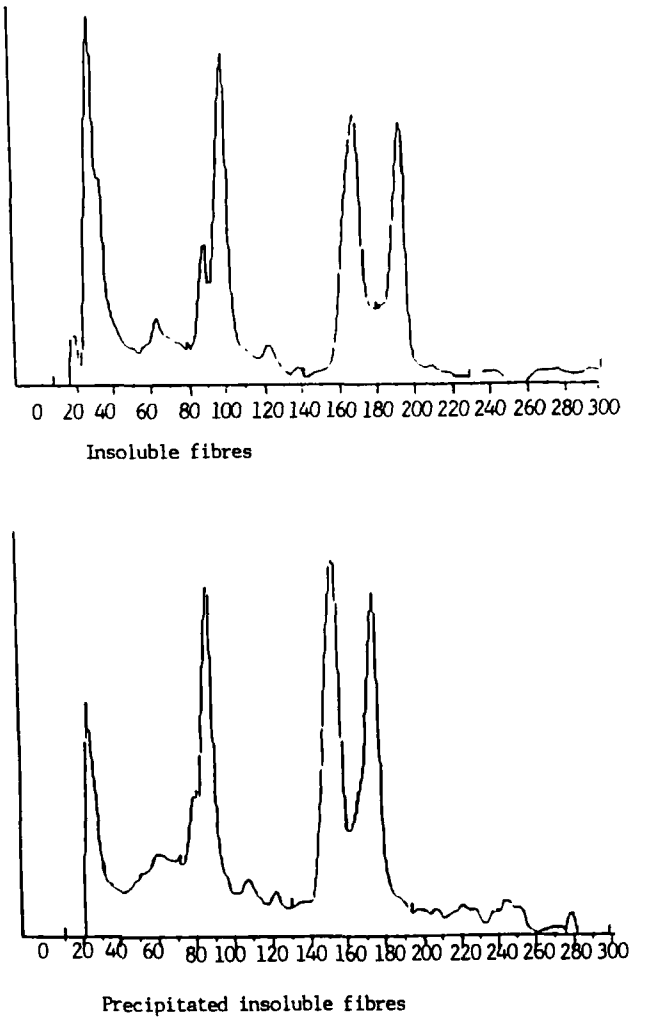

FIGURE 5 : Electrophoresis profile of insoluble collagens. non irradiated

The irradiated products show marked changes in the electrophoretic migration. It seems therefore that irradiation acts upon the collagen subunits. It is to be noted that the insoluble fibres appear more resistant to irradiation than the soluble fibres.

Hemostatic activity of collagen

Our non-irradiated collagen controls (insoluble and soluble fibres) cause platelet agregation. It is to be noted that the insoluble fibre controls seem more agregate (84 per cent

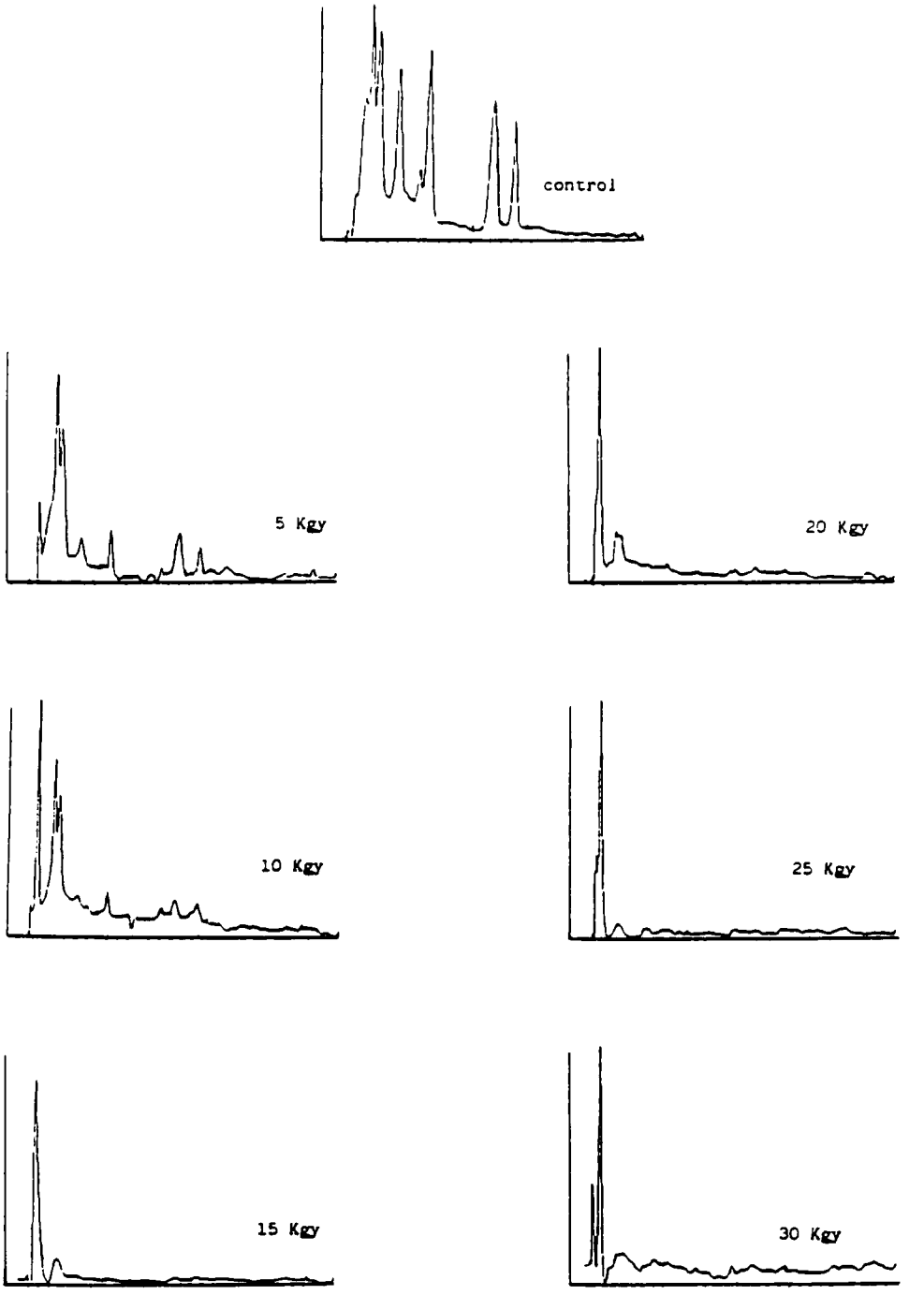

FIGURE 6 : Eclectrophoresis profile of precipitated soluble fibres as a function of the doses of γ radiations

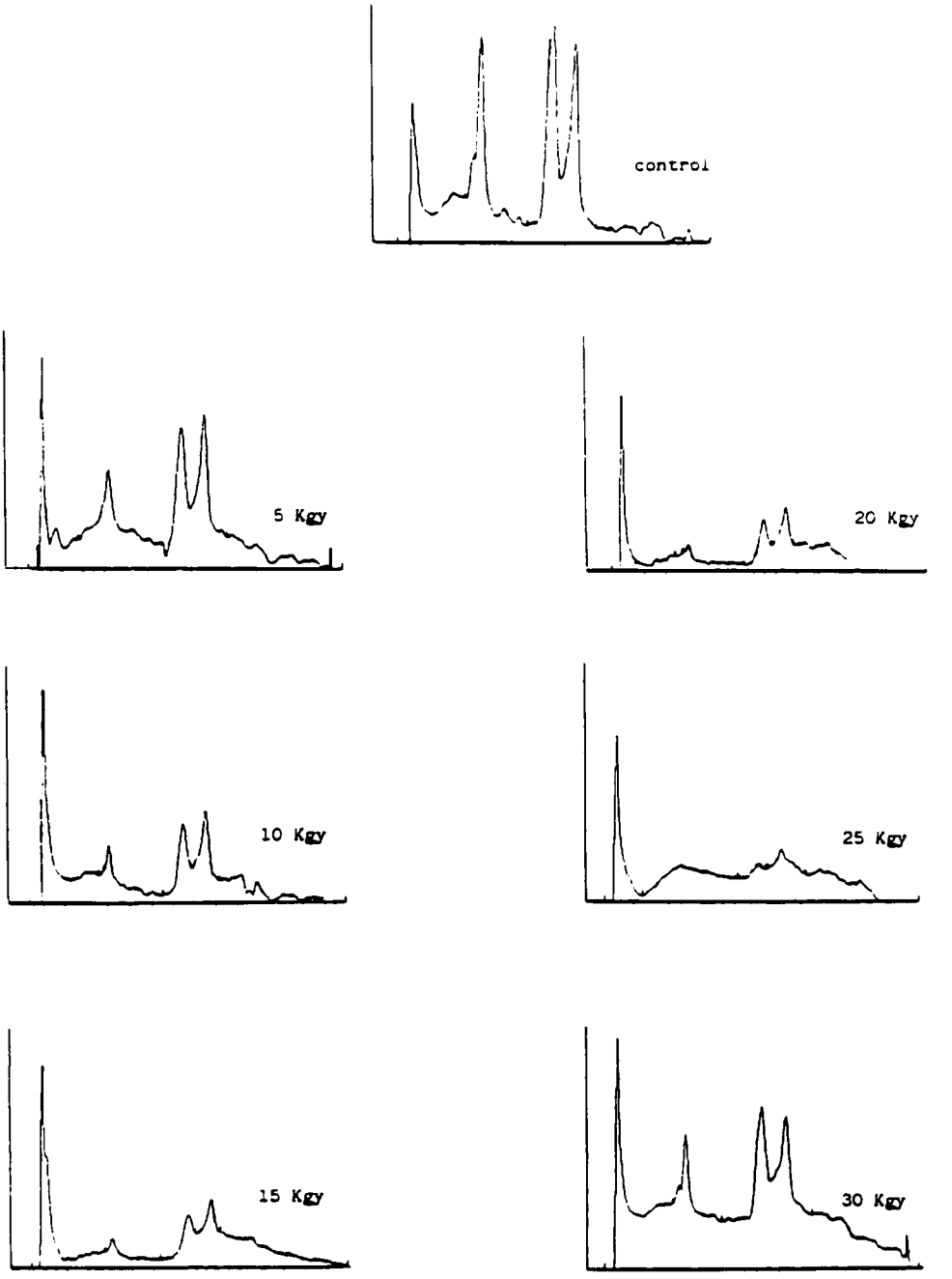

FIGURE 7 : Electrophoresis profile of lyophilised insoluble fibres as a function of doses of γ radiations

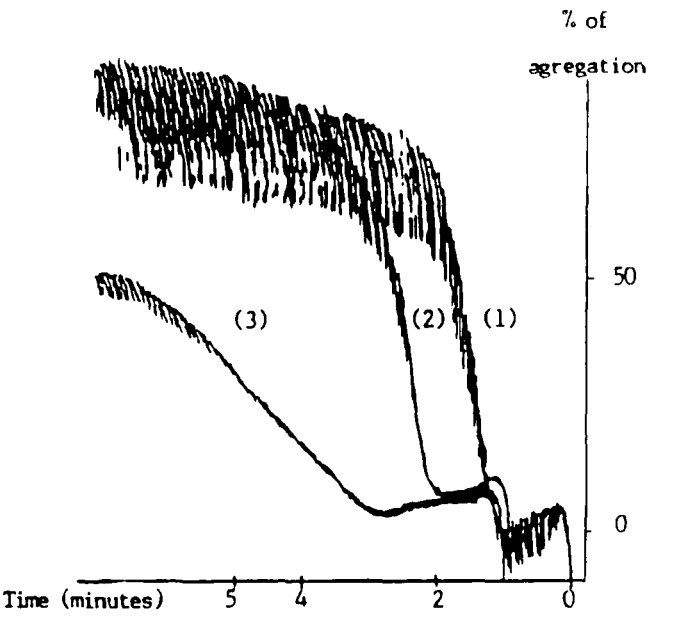

FIGURE 8 : Influence of the γ radiation on the ability of agregation of insoluble fibres 1 : I.F. 0 γ , 2 : I.F. 5 γ, 3 : I.F. 30 γ

agregation per 25µg of collagen) than the precipitated soluble fibre controls (65 per cent per 25µg of collagen).

We tested the precipitated soluble fibres (P.S.F.) and the insoluble fibres (I.F.) under γ ray doses of 0.5 and 30 kgy.

The results are presented in figures 8 and 9.

These agregations however are markedly lower than that obtained by the reactive collagen used as a reference (85 per cent per 0,5µg of collagen).

The ability for agregation of the patelets by the soluble and insoluble fibres falls with the increase in irradiation doses on these products.

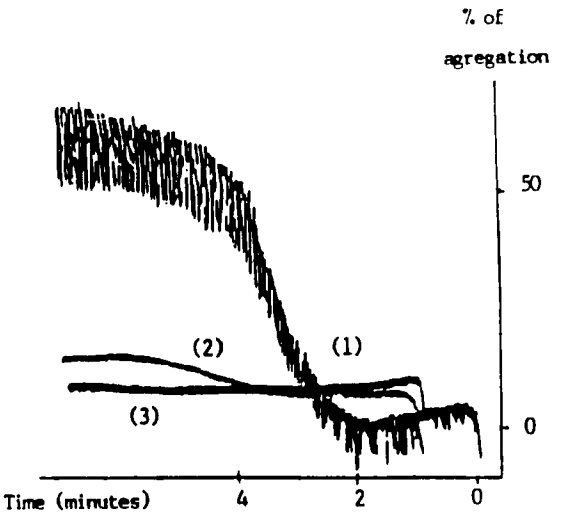

FIGURE 9 : Influence of the radiation on the ability of agregation of the precipitated soluble fibres 1 : P.S.F. 0 $\mathcal{V}$, 2 : P.S.F. 5 $\mathcal{V}$ 3 : P.S.F. 30 $\mathcal{V}$

The decline in this ability for agregation is more clearly marked in the soluble fibres than in the insoluble fibres, since the soluble fibres no longer agregate the platelets at 30 kgy.

Irradiation changes the agregation power of the collagen fibres. The precipitated soluble fibres seem to be more affected than the insoluble fibres.

CONCLUSION

The simultaneous study of non-irradiated controls of the different collagen forms, underlined the markedly lower enthalpic values of denaturation for the lyophilised soluble fibres compare to the other collagen forms. It therefore seems that lyophilisation of the soluble fibres, under our conditions changes their helicoid structure. This action might take place upon the hydrogen and electrostatic links which maintain the triple helix.

As for the structural state of the raw material after irradiation, it seems that all the collagen forms undergo changes according to the dose of β or $\mathcal{V}$ rays received.

These changes in the collagens in relation to their irradiation become very evident with electrophoresis, whereas one notes little difference with differential scanning calorimetry.

The explanation could be a follows :

irradiation may act upon the collagen by changing the subunits, and that is to say probably by changing the covalent links connecting these subunits (henceforth modified electrophoresis), but on the other hand, it would have little effect on the hydrogen and electrostatic links binding the triple helix (henceforth little change in differential calorimetry).

The comparison of the ability for platelet agregation between the non-irradiated soluble and insoluble fibres allows us to establish that the insoluble collagen is more agregate than the soluble collagen. This ability for agregation of the soluble and insoluble fibres falls with the increase in irradiation dosage.

This study allows us to underline the fact that ionizing rays do not act solely on the bacterial structure, but also act on the collagen structure and changes its hemostatic activity.

It also allows us to establish that ionizing radiations are not the only operation able to change the collagen molecule ; it seems that lyophilisation is also capable of changing the soluble collagen.

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