A Study of the Biosynthesis of Collagenous and Non-collagenous Proteins and of the Proline/Hydroxyproline Ratios of Collagens Isolated from Embryonic Tissues

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1. After incubation of chick-embryo skin slices with [^{14}C]proline for 2 hr. the specific activities of [^{14}C]proline and [^{14}C]hydroxyproline in soluble and insoluble collagens and [^{14}C]proline in non-collagenous proteins were determined as well as the total amounts of both imino acids in these proteins. On the basis of these results it was demonstrated that soluble collagens having a high proline/hydroxyproline ratio are contaminated with non-collagenous proteins. 2. It was found that, in the presence of a mixture of amino acids in the incubation medium, the rate of synthesis of soluble collagen is significantly decreased. 3. The metabolic activity of collagenous proteins is related to their solubility, but that of non-collagenous proteins is not.

The study of the proline and hydroxyproline contents of collagens isolated mainly from embryonic and young tissues has been for many years a considerable problem in collagen chemistry.

There exists some experimental evidence that in newly formed collagen proteins the proline/hydroxyproline ratio is very high, several times surpassing the normal value for mature collagen, which is roughly 1.15. Tustanovsky, Mayagkaya & Volkova (1964), Saides, Tustanovsky, Mayagkaya & Orlovskaya (1964), Koblre & Chvapil (1961) and also others (Cadavid, Benduchis & Mancini, 1961; Chen & Postlethwait, 1961) have shown that, in spite of different purification procedures, the proline/hydroxyproline ratio for collagens extractable from pig-embryo skin remains high, about 5, and tends to decrease to 2.5 at parturition. The same change is apparent when the specific activity ratio of these imino acids or of lysine/hydroxylysine is studied (Robertson, Hiwett & Herman, 1959; Van Slyke & Sinex, 1958; Piez & Likins, 1957).

There is further experimental as well as theoretical evidence in favour of the existence of collagens with high proline and low hydroxyproline contents. When collagen biosynthesis is studied in tissue slices under the influence of hydroxylating blocking agents such as $\alpha\alpha'$ -bipyridyl, the specific activity of hydroxyproline of newly formed collagen approaches zero but the activity of proline is double that of the norm (Hurych & Chvapil, 1965). This type of collagen resembles the collagen in earthworm cuticle, the proportions of proline and hydroxyproline being 31.2 and 2.4% respectively.

Thus collagen molecules do exist with different proportions of proline and hydroxyproline. The structural stability of the linear molecule of collagen is dependent on the total imino acids present.

Robertson & Schwartz (1953) suggested that in scorbutic animals hydroxylation of proline is blocked and thus atypical collagen is formed. Collagens with a high proline/hydroxyproline ratio are mostly isolated from embryonic and scorbutic animals. Robertson & Schwartz (1953) and Gould, Manner, Goldman & Stolman (1960) thought that they had isolated such a collagen, but later both authors concluded that their collagens were contaminated.

It is commonly believed that the high ratio is caused by contamination with some non-collagenous proteins that are firmly bound only to young forms of collagen proteins. However, Lowther, Green & Chapman (1961), studying the amino acid composition of collagen of the microsomal fraction, found a normal ratio of the imino acids. Similar results have been presented by Hausmann & Neuman (1961) and by Popenoe & Van Slyke (1962), who stressed the importance of purifying the collagens.

In this Laboratory the study of atypical collagens has led us to the conclusion that with the usual protein purification procedures it is impossible to isolate from embryonic tissues soluble collagen with a normal proline/hydroxyproline ratio, 1.15. Even when collagenase was used for separating the

collagen part of the sample under investigation (V. Koblir & M. Chvapil, unpublished work) the ratio remained high. By the use of [^{14}C]proline the present results suggest that the high proline/hydroxyproline ratio is caused by contamination of collagen by other tissue proteins.

METHODS

Skin tissue slices (2g.) from 14–17-day-old chick embryos (the average weight is mentioned in each experiment) were incubated in 10ml. of Krebs–Ringer bicarbonate buffer (Krebs & Henseleit, 1932) enriched with ATP (sodium salt) (2.0mm). L-[U- ^{14}C]Proline was repurified by paper chromatography and its purity was checked by radioautography. In some experiments the medium contained a mixture of amino acids having the same composition as collagen hydrolysate but without proline and hydroxyproline. The total concentration of amino acids in the medium was 10mm. The molar proportions of individual amino acids were as follows: glycine, 34; alanine, 10; lysine, serine, valine, phenylalanine, leucine, isoleucine, methionine and threonine, each 6.2; glutamic acid, 10; aspartic acid and arginine, each 9; tyrosine and tryptophan, each 0.7. Incubation with labelled proline was carried out at 37° in an O_2 atmosphere for 2hr. with moderate shaking. The concentration of [^{14}C]proline in the medium as well as its specific activity are given in each experiment. When the incubation was finished, cooled samples were spun and the incubation medium was discarded.

The sediment was homogenized in a Potter–Elvehjem homogenizer with 4ml. of 0.2M-NaCl, and the volume of the tissue homogenate was adjusted to 6ml., which corresponds approximately to the ratio 3:1 (v/w). Soluble collagens were extracted twice at 4° with 0.2M-NaCl at pH 7.4 for 16hr. The solution of 0.2M-NaCl contained EDTA (2mm). In pooled experiments 50% (w/v) trichloroacetic acid was

added so that the final concentration amounted to 5% (w/v). The total volume was approx. 12ml. The extraction and purification of soluble collagens into trichloroacetic acid was performed three times: twice at 90° for 20min., and the last by rinsing the sediment with cold 5% trichloroacetic acid. Free amino acids as well as trichloroacetic acid were removed by dialysis against tap water. Insoluble collagen was extracted from the residue according to the method of Fitch, Harkness & Harkness (1955) and the final extract was again dialysed.

The residue after trichloroacetic acid extraction (non-collagenous proteins) was rinsed three times with 2ml. of 5% trichloroacetic acid. The sediment was washed with 5ml. of ethanol, 5ml. of ethanol-ether (1:4, v/v) and finally with 5ml. of ether.

After hydrolysis in 6N-HCl at 105° for 16hr. the amounts of proline (Troll & Lindsey, 1955) and hydroxyproline (Stegemann, 1958) in each individual protein were determined.

Isolation and determination of the specific activity of [^{14}C]proline and hydroxy[^{14}C]proline were carried out according to the method of Peterkofsky & Prockop (1962) in a liquid-scintillation counter (Tracerlab model LSC10B). The efficiency of the apparatus was 67–82%.

RESULTS

The results show a striking difference in the metabolic turnover of collagenous and non-collagenous proteins in relation to their solubility (Table 1). The specific activity of collagens extracted with 0.2M-sodium chloride was about 10 times that of non-collagenous proteins extracted under the same experimental conditions. The specific activity of [^{14}C]proline in extractable collagens was only one-third of that of hydroxy[^{14}C]proline.

Table 1. *Incorporation of hydroxy[^{14}C]proline and [^{14}C]proline into collagenous and non-collagenous proteins*

The average weight of chick embryo was 9.9g. The specific activity of [^{14}C]proline was 37.5 $\mu\text{C}/\mu\text{mole}$. The final activity in the medium was 0.552 $\mu\text{C}/\text{ml}$. The incubation medium was without any amino acid mixture.

Total imino acids ($\mu\text{g./flask}$)		Proline/hydroxy- proline ratio	$10^{-4} \times \text{Sp. activity (counts/min./}\mu\text{mole)}$		
Hydroxyproline	Proline		Collagenous		Non-collagenous Proline
			Hydroxyproline	Proline	
			(a) Insoluble proteins		
1780	2180	1.22	2.00	2.14	8.60
1640	1860	1.14	1.77	2.23	7.84
1780	2100	1.18	1.97	2.31	8.30
1820	2320	1.27	1.90	2.12	8.54
			(b) Soluble proteins		
28.2	102	3.62	123.0	42.4	11.23
28.2	114	3.86	120.0	40.7	11.76
29.7	102	3.43	140.9	54.9	12.50
26.5	102	3.86	143.0	50.7	12.90
26.5	91	3.44	143.7	54.3	12.10

The situation is different with the insoluble proteins. In comparison with soluble collagens the specific activities of [^{14}C]proline and hydroxy[^{14}C]proline were 50-fold less and both imino acids were incorporated at the same rate. Metabolic turnover of insoluble non-collagenous proteins was higher than that of insoluble collagen proteins. There was, however, no great difference in the specific activities of [^{14}C]proline in extractable and insoluble non-collagenous proteins, the latter being about 20% less.

The content of both imino acids presented as the proline/hydroxyproline ratio in insoluble collagen approached the value for pure native collagen. Although the high proline/hydroxyproline ratio of extractable collagens might indicate that anomalous collagens exist with a high proline content, nevertheless Table 2 shows that, as the proline/hydroxyproline ratio decreased, the specific activity of proline approached that of hydroxyproline, sug-

gesting that collagen fractions were contaminated with non-collagenous proteins of low specific activity.

From the inverse relationship between the specific activity of proline and the proline/hydroxyproline ratio, it was possible to calculate how much contamination of collagen by non-collagenous proteins of low metabolic activity would be required to decrease the specific activity of proline in isolated soluble collagens.

The calculation is based on the following assumptions. (1) The rates of incorporation of both proline and hydroxyproline into collagen are the same (see Table 1: results for insoluble collagen), and thus values of the specific activity found for hydroxyproline can also be used for collagen proline. (2) The synthesis of the tropocollagen molecule is completed at the ribosomes and thus there is no reason for additional hydroxylation of proline in collagen molecules (Lowther *et al.* 1961). (3) The dilution

Table 2. Comparison of the proline/hydroxyproline ratio with their specific activities in soluble collagen

In the incubation medium a mixture of amino acids was added. The final concentration of total amino acids in the medium was 10 mM.

Expt. no.	Proline/hydroxyproline ratio	$10^{-4} \times$ Sp. activity of hydroxyproline (counts/min./ μmole)	$10^{-4} \times$ Sp. activity of proline (counts/min./ μmole)	Proline/hydroxyproline sp. activity ratio	Notes		
					Sp. activity of proline used ($\mu\text{C}/\mu\text{mole}$)	Average wt. of chick embryos (g.)	Final activity in incubation medium ($\mu\text{C}/\text{ml.}$)
1	4.95	13.0	6.0	0.46	17.4	17.5	0.548
2	3.85	31.0	17.6	0.57	36.4	10.5	0.556
3	3.72	36.0	21.3	0.59			
4	2.05	25.6	29.2	1.14	42.8	12.4	0.633

Table 3. Comparison of the calculated total activity of proline in soluble collagens with values found in experiments

The values used are those given in Table 1.

Total proline in fraction (μmole)	Collagen proline* (μmole)	Non-collagen proline (μmole)	$10^{-4} \times$ Sp. activity of proline per sample (counts/min./ μmole)		Difference§ (%)
			Calc.†	Found‡	
0.89	0.27	0.62	40.1	37.6	+6.7
0.99	0.27	0.72	40.9	40.3	+1.5
0.89	0.28	0.60	47.5	48.7	-2.5
0.89	0.25	0.63	44.5	45.0	-1.1
0.79	0.25	0.54	43.0	43.0	0

* $\mu\text{g.}$ of hydroxyproline $\times 1.15$.

† Counts/min./ μmole of hydroxyproline $\times \mu\text{mole}$ of collagen proline + counts/min./ μmole of non-collagen proline $\times \mu\text{mole}$ of non-collagen proline.

‡ Counts/min./ μmole of collagen proline $\times \mu\text{moles}$ of total proline.

§ Calc. activity - found activity
calc. activity $\times 100$.

of labelled hydroxyproline with unlabelled free hydroxyproline can be excluded since collagen hydroxyproline is formed from labelled proline. (4) Of the total proline present in soluble collagens, only part is actually in true collagen. The remainder is non-collagenous proline. The value for collagen proline was calculated from the amount of hydroxyproline $\times 1.15$, which is the commonly accepted value for the ratio of these imino acids.

Table 3 shows the results of a comparison of theoretically calculated activities with those experimentally found. The difference between both procedures is negligible, varying between $+6.7\%$ and -2.5% .

The incorporation studies were carried out either in a medium containing a mixture of amino acids resembling collagen hydrolysate in their composition (see Table 2) or without any amino acid mixture (Table 1). The other additions to the medium were the same in both series of experiments. When skin slices were incubated with amino acids, the specific activity of hydroxy[^{14}C]proline of soluble collagen was fivefold less than that of hydroxy[^{14}C]proline of soluble collagens synthesized in the medium without amino acids.

DISCUSSION

The results of this study enable one to discuss three problems of collagen biochemistry: (1) the existence of collagen with atypical imino acid (proline/hydroxyproline composition); (2) the relationship of the metabolic turnover of collagenous and non-collagenous proteins with their solubility; (3) the effect of some factors, in our case of a mixture of amino acids, on collagen biosynthesis.

The good agreement between the calculated activities and those found experimentally favours the hypothesis that soluble collagens isolated from embryonic tissues and having a high proline/hydroxyproline ratio are associated with some non-collagenous proteins.

It is remarkable that, despite repeated attempts to isolate and purify the soluble collagens from embryonic and young tissues, the collagen did not have a typical proline/hydroxyproline ratio of pure mammalian collagen (Kobler & Chvapil, 1961). The results do not exclude the possibility that the atypical collagens formed under artificial or pathological situations exist (Hurych & Chvapil, 1965).

Collagen is often regarded as being metabolically inert. The present work shows, however, that in embryonic tissues extractable collagens have a higher metabolic activity than the analogous globular proteins, although the term 'solubility' has only relative importance with reference to molecular structural stability. The results of the present study show the interrelation between extrac-

tability and molecular activity of collagenous proteins only, but not of globular proteins whose activity remains the same in proteins differing in solubility.

Amino acids are important in controlling protein biosynthesis in tissue slices. However, in contrast with our original supposition that protein biosynthesis was stimulated by amino acids, we found the reverse to be the case. A similar striking inhibition of protein biosynthesis in the presence of amino acids in the incubating medium was recently found by J. Folbergrová (personal communication), who studied the incorporation of methionine into brain proteins. Hitt, Dawson & McGavack (1963) and Flanagan & Nichols (1964) have reported on the inhibition of the incorporation of labelled amino acid into proteins in the presence of a mixture of amino acids. It is difficult to explain, however, the inhibitory effect of amino acids, i.e. whether some feedback control mechanism of energy competition is involved in this process.

REFERENCES

- Cadavid, N. G., Benduchis, B. & Mancini, R. E. (1961). *Nature, Lond.*, **191**, 1297.
- Chen, R. W. & Postlethwait, R. W. (1961). *Proc. 5th int. Congr. Biochem., Moscow*, p. 57.
- Fitch, S. M., Harkness, M. L. & Harkness, R. D. (1955). *Nature, Lond.*, **176**, 163.
- Flanagan, B. & Nichols, G. J. (1964). *J. biol. Chem.* **239**, 1261.
- Gould, B. S., Manner, G., Goldman, H. M. & Stolman, J. (1960). *Ann. N.Y. Acad. Sci.* **85**, 385.
- Hausmann, E. & Neuman, W. F. (1961). *J. biol. Chem.* **236**, 149.
- Hurych, J. & Chvapil, M. (1965). *Biochim. biophys. Acta*, **97**, 361.
- Kao, K. Y. T., Hitt, W. E., Dawson, R. L. & McGavack, T. H. (1963). *Proc. Soc. exp. Biol., N.Y.*, **113**, 762.
- Kobler, V. & Chvapil, M. (1961). *Experientia*, **17**, 519.
- Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyl. Z.* **210**, 33.
- Lowther, D. A., Green, N. M. & Chapman, J. A. (1961). *J. biophys. biochem. Cytol.* **10**, 373.
- Peterkofsky, B. & Prockop, D. J. (1962). *Analyt. Biochem.* **4**, 400.
- Piez, K. A. & Likins, R. C. (1957). *J. biol. Chem.* **229**, 101.
- Popenoe, E. A. & Van Slyke, D. D. (1962). *J. biol. Chem.* **237**, 3491.
- Robertson, W. van B., Hiwett, J. & Herman, C. (1959). *J. biol. Chem.* **234**, 105.
- Robertson, W. van B. & Schwartz, B. (1953). *J. biol. Chem.* **201**, 689.
- Saides, A. L., Tustanovsky, A. A., Mayagkaya, G. L. & Orlovskaya, G. V. (1964). *Biofizika*, **9**, 441.
- Stegemann, H. (1958). *Hoppe-Seyl. Z.* **187**, 41.
- Troll, W. & Lindsey, J. (1955). *J. biol. Chem.* **215**, 655.
- Tustanovsky, A. A., Mayagkaya, G. L. & Volkova, Z. J. (1964). *Gerontologia*, **9**, 28.
- Van Slyke, D. D. & Sinex, F. M. (1958). *J. biol. Chem.* **232**, 797.