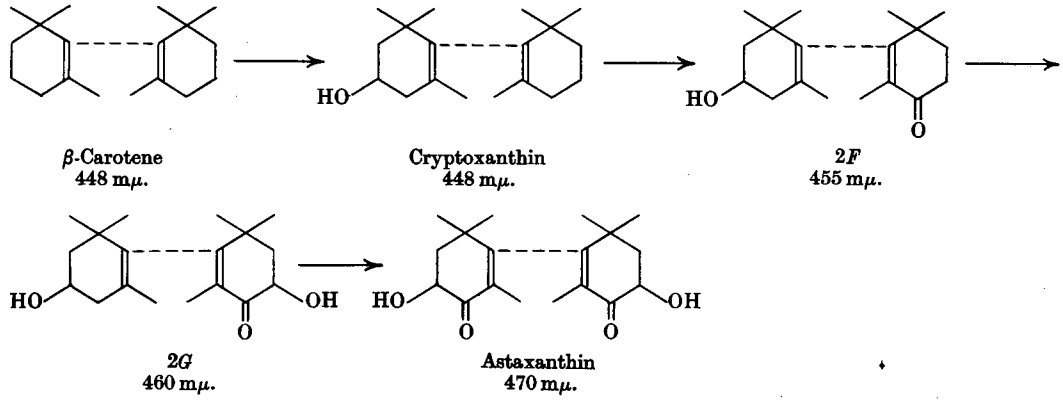

Much further work is required before this scheme can be fully established or otherwise, but it is important to point out that β-carotene has been shown to be almost certainly a precursor of astaxanthin in locust eggs (Goodwin & Srisukh, 1949).

This work on *O. ophidianus* emphasizes the importance of the study of the minor carotenoid components in living tissues.

SUMMARY

1. The presence of astaxanthin in the shell of *Ophidiaster ophidianus* is confirmed, and that of β-carotene and cryptoxanthin demonstrated. *neo*-β-Carotene B and γ-carotene are also probably present.

2. Three new pigments are reported: two are probably keto-carotenoids.

3. The quantitative distribution of carotenoids in the shell of *O. ophidianus* is recorded.

4. A possible biosynthetic route from β-carotene to astaxanthin is discussed.

I wish to express my deep gratitude to Dr T. W. Goodwin for reading the manuscript and for helpful advice and criticism.

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The Metabolism of Collagen and other Proteins of the Skin of Rabbits

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It has been shown with the aid of [α-^{14}C]glycine that, in the adult rat, collagen from a wide variety of sources is metabolically almost inert, if compared with proteins such as those of plasma or of liver (Neuberger, Perrone & Slack, 1951; Neuberger & Slack, 1953). Results indicating a relatively slow turnover of collagen in guinea pigs (Robertson, 1952) have also been reported. However, in the growing rat, labelled glycine is incorporated into collagen at a fairly fast rate (Neuberger *et al.* 1951) and the use of this amino acid thus seemed to afford a convenient tool to examine the mechanism by which this fibrous protein is formed.

It was reasonable to assume that insoluble collagen is derived from a more soluble precursor and the presence of soluble, collagen-like proteins in connective tissue supports such a hypothesis. It was first observed by Zachariades (1900) that the tendon of the rat tail, if suspended in very dilute formic acid or acetic acid, swelled markedly and

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then began to dissolve. This dissolution of collagen, particularly that of the tail tendon of the rat, was studied in great detail much later by other French workers, particularly Nageotte, Guyon and Fauré-Fremiet and also by Leplat. Nageotte (1927*a*, *b*) found that rat-tail tendon partly dissolved if placed in solution of about 8×10^{-3}M acetic acid; the dissolution was not complete, since after three extractions about 70% of the tendon remained undissolved (Nageotte & Guyon, 1934). Nageotte (1927*b*, 1928) at first believed that this phenomenon was peculiar to the tail tendon of the rat, but Leplat (1933*a*, *b*) showed that the presence of soluble collagen could be demonstrated in other tissues of the rat and also in other species; this observation was confirmed by Nageotte (1933). The solutions of collagen thus obtained, although extremely dilute, were very viscous (Nageotte, 1927*d*) and showed marked laevorotation (Fauré-Fremiet, 1933*a*); on heating to about 60° an apparently irreversible change occurred, akin to denaturation, and gelatin was produced (Nageotte & Guyon, 1933). Nageotte's results with rat-tail tendon were confirmed by Huzella (1932) and also by Fauré-Fremiet (1933*a*, *b*) who treated the tendon with trypsin and lime water, before exposing it to dilute acetic acid. It is of considerable physiological interest that the proportion of collagen which is soluble in dilute acid was found to decrease with age (Nageotte & Guyon, 1934). This acid-soluble collagen has been named collagen A by Nageotte (1930), while the term collagen B has been applied to material made soluble in acid by preliminary treatment with alkali.

On dialysing the collagen solution against distilled water a gel was formed (Nageotte, 1927*d*), if the pH was above 5·0 (Guyon, 1934): at a lower pH no visible precipitation occurred. Addition of sodium chloride in high concentration produced a massive clot, but at low ionic strength fibrils were formed, the size and shape of which appeared to vary with the salt concentration and probably also with pH (Nageotte, 1927*a*–*d*). Some of the fibrils had a crystalline appearance, resembling tyrosine crystals and were birefringent (Nageotte, 1927*a*, *b*; Fauré-Fremiet, 1933*a*, *b*). The reconstituted fibrils closely resembled native collagen fibres as observed in sections of connective tissue and their staining characteristics were those of collagen. Fauré-Fremiet (1933*a*) also observed that soluble collagen was precipitated by chondroitin sulphate. The X-ray diagrams of reconstituted collagen fibres were found to be closely similar to those of native mammalian collagen (Wyckoff & Corey, 1936; Champetier & Fauré-Fremiet, 1938) and the same applied to their electron micrographs (Schmitt, Hall & Jakus, 1942).

More recently, Russian workers, especially Orekhovich and his co-workers, have studied the properties and distribution of soluble collagen in a great variety of species and tissues. Their method of isolation consisted of a preliminary extraction of the macerated tissue with a disodium hydrogen phosphate solution, which removed proteins of albumin and globulin type; this was followed by a further extraction with an acidic buffer such as citrate or oxalate (Tustanovskii, 1947; Orekhovich, Tustanovskii, Orekhovich & Plotnikova, 1948). Dialysis against a solution of low ionic strength and of a pH near neutrality gave needles of crystalline appearance of similar type, but of apparently more regular shape, than those described by Nageotte & Fauré-Fremiet. The material studied by Orekhovich *et al.* (1948) appears to be identical with, or closely similar to, that described by the French workers. Orekhovich called this soluble protein 'procollagen', implying that it is a precursor of insoluble collagen. The introduction of the term 'procollagen' has been deplored by Bear (1952), and it may be noted that the term 'précollagène' has been used by Laguesse (1903) to describe amorphous material present in connective tissues, lying between collagen fibres, and having some of the staining characteristics of collagen. The investigations of the Russian workers which have been summarized by Orekhovich (1952) show that the amino-acid composition of the soluble collagen is on the whole similar to that of insoluble collagen, although definite differences were observed. The ultraviolet absorption spectrum resembled that of gelatin. Bresler, Finogenov & Frenkel (1950) have studied the behaviour of 'procollagen' solutions in the ultracentrifuge and their results suggest that the molecules are rod shaped, and Orekhovich (1952) has reported on the electrophoretic behaviour of this protein. It has also been shown that soluble collagens occur in a great variety of tissues in all of the many vertebrates examined (Plotnikova, 1947). Earlier findings which suggested that the soluble material occurs in relatively large amounts only in connective tissue of young animals were extended and confirmed by Orekhovich (1950).

The presence of possibly another type of soluble collagen is suggested by the work of Highberger, Gross & Schmitt (1951), who found that an extract of skin made with slightly alkaline phosphate solutions contained a protein which in its appearance in the electron microscope resembled collagen.

The present work was carried out with young rabbits and was intended to answer the question whether soluble collagens could, as judged by their capacity to incorporate labelled glycine, be precursors of insoluble collagen. Some of our results have already been briefly reported (Harkness & Neuberger, 1952; Harkness, Marko, Muir & Neuberger, 1953), and observations similar to those contained in our first preliminary communication

have also been mentioned by Orekhovich (1952). In connexion with the isotope work a preliminary survey of proteins in rabbit skin was undertaken.

EXPERIMENTAL

Treatment of animals. Male and female albino rabbits of the Copenhagen or Himalayan strain were used throughout. Their weights at the time when the labelled glycine was given varied between 500 and 750 g. and they were about 4 weeks old. In some experiments litter mates were used. The animals were fed diet no. 18 (Bruce & Parkes, 1946) *ad lib.*

In the first series of experiments the [α-^{14}C]glycine of specific activity of 1·7 or 5·2 μc/mg. was given intraperitoneally in 0·5–1·0 ml. of 0·9% NaCl; the dose injected was 10 μc/100 g. body wt. In the second series of experiments the [α-^{14}C]glycine of specific activity 1 mc/19·3 mg. was given by mouth; rabbits were starved for the 24 hr. period preceding the feeding of the labelled glycine, but were allowed free access to water; total activity fed was 20 μc/100 g. body wt. The glycine was dissolved in a small amount of water and about 4 g. of pellets (diet no. 18) were impregnated with the glycine solution. The pellets after being dried were fed to the starved rabbits; they were generally eaten within 1 hr.

In the first series of experiments the rabbits were killed by breaking the neck. In later experiments, in which the radioactivity of the glycine in plasma proteins was examined, the animals were anaesthetized by injection of pentobarbitone followed by inhalation of chloroform, the thorax was opened and the heart exposed. About 20–30 ml. of blood were then removed by heart puncture, using a heparinized syringe.

Plasma proteins. The blood obtained by heart puncture was centrifuged to remove red and white cells. The plasma, to which was added non-radioactive glycine to a final concentration of 0·5%, was dialysed against frequent changes of distilled water.

Isolation of skin proteins

The general procedure is given below; any important deviations will be referred to under Results.

The hair was removed either with the aid of an electric clipper or by shaving and the skin and hypodermis were stripped off the body; the skin covering the paws, tail, ears and tip of nose was not used. The hypodermis, consisting of muscle, fat and connective tissue, was then scraped off. The remaining skin, consisting of dermis, epidermis and hair roots and stumps was then rolled up and frozen in solid CO_2, cut into small pieces with a scalpel and finely ground. Between 30 and 50 g. of skin were obtained from a rabbit. All further operations were carried out at 2° using 2-octanol as preservative. Extractions were done either on a mechanical shaker or with the aid of stirring; the solvents used were similar to those of Orekhovich *et al.* (1948). The main steps in the fractionation are shown in Fig. 1.

Proteins extracted by disodium hydrogen phosphate. The macerated skin was suspended in about 10 times its weight of a solution which had been saturated with Na_2HPO_4 at 2°. The extraction was continued for 8–20 hr., and the mixture was then centrifuged for 0·5 hr. at 2500 rev./min. The supernatant fluid was decanted and in most experiments filtered through a fine sintered-glass funnel (H3). The residue was re-extracted with the saturated phosphate solution up to six times until the last extract was water-clear. The extracts were centrifuged and filtered as described.

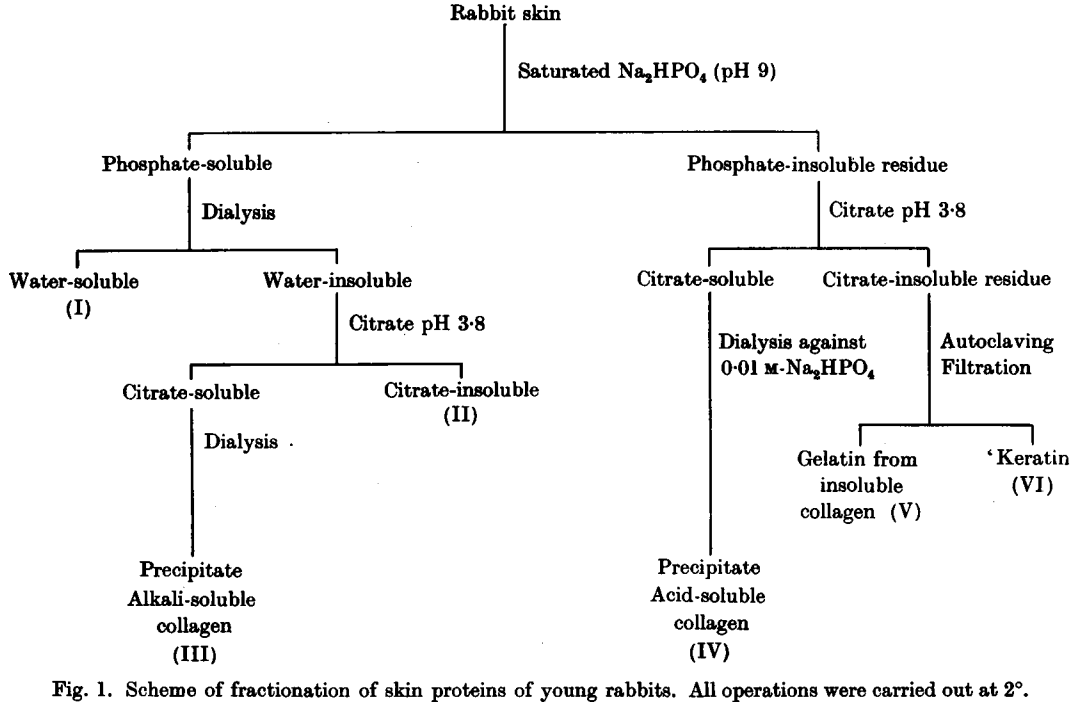

Fig. 1. Scheme of fractionation of skin proteins of young rabbits. All operations were carried out at 2°.

The combined phosphate extracts were dialysed for 36–48 hr. against distilled water. The precipitate which had formed was removed by centrifuging (1 hr. at 2500 rev./min.). The clear supernatant was concentrated to dryness and the residue designated 'water-soluble' fraction (Fig. 1, fraction I).

To the solids obtained from the dialysis against water were added 0·1 M sodium citrate/NaCl buffer, pH 3·8 (Sørensen, 1912). The suspension was shaken for 8–10 hr. and centrifugation and filtration through a sintered-glass filter or paper were done as described above. The extraction was repeated three or four times. The residue remaining after these extractions was designated the 'citrate-insoluble' fraction (Fig. 1, fraction II).

To the combined extracts which contained material, soluble in the citrate buffer but insoluble in water, was added non-radioactive glycine (final concentration 0·5%); the solution was dialysed first against running tap water and then against frequent changes of distilled water. After prolonged dialysis a fine fibrous precipitate appeared which was collected by centrifugation. It was named 'alkali-soluble' collagen (Fig. 1, fraction III), since it was originally soluble in phosphate buffer, pH 9. However, the solid material, obtained after the operations described, could not be redissolved in a neutral or alkaline buffer. The weight of this fraction varied between 10 and 25 mg. for one rabbit skin. The protein was dissolved in 5–10 ml. 0·1% (v/v) acetic acid, and the solution heated to 80–90° for 10 min. The solution, while hot, was filtered under slight pressure, through a fine sintered-glass funnel (H3). The filtrate was usually faintly opalescent.

Proteins soluble in citrate buffer, pH 3·8, *but insoluble in phosphate solution*, pH 9. To the residue remaining after thorough extraction with saturated Na_2HPO_4 solution was added citrate buffer, pH 3·8, in amounts corresponding to 10 times the weight of original skin. Extraction was carried out with stirring or shaking for about 18 hr.; when centrifuged and filtered, the extract gave a slightly viscous, but water-clear, solution. The solid was re-extracted in a similar manner 3 or 4 times. In some experiments, to be described later, the extracts were worked up separately, whilst in others they were combined. The solutions were dialysed against large volumes of 0·01 M-Na_2HPO_4. A heavy fibrous precipitate soon appeared; this solid was collected by centrifuging at 2500 rev./min. for 0·5 hr. In most isotope experiments the precipitate was redissolved in a small volume of citrate buffer and non-radioactive glycine was added to a final concentration of 0·5%. The solution was dialysed first against several changes of the citrate buffer and then against 0·01 M-Na_2HPO_4. The precipitate was collected as before, washed three times on the centrifuge with 100 ml. 10% (w/v) NaCl and finally with distilled water. Repeated washing with water converted the fibrous precipitate into a thixotropic gel. The precipitate of 'acid-soluble collagen' (Fig. 1, fraction IV) was generally left in contact with water for no longer than 1 hr. Conversion into gelatin was done by heating in water to 60° for 1 hr. or 80–90° for 15 min. The gelatin solution was filtered through a sintered-glass funnel under slight pressure. In some experiments the gelatin solution was poured into 10 vol. of acetone and the precipitate allowed to stand at 0° for 18 hr. It was then centrifuged, washed successively with ethanol:ether (3:1; v/v) and ether and dried over P_2O_5 *in vacuo*.

Insoluble collagen (Fig. 1, fraction V). The residue remaining after extraction with citrate was washed on the centrifuge three times with 10% (w/v) NaCl (500 ml. portions) and then three times with water (500 ml. portions). In earlier experiments the washings were carried out over periods of days or weeks at 0°, but this did not apparently affect the properties of the final product. The material which had swollen with water treatment was autoclaved at a pressure of 15 lb./sq.in. for 3 hr. in the presence of 500 ml. water. The hot mixture was then filtered by gravity and an insoluble residue (Fig. 1, fraction VI) was obtained, consisting presumably of keratin, elastin and proteins denatured during the various operations. The filtrate containing the gelatin was concentrated to about 50 ml. and any solid which appeared was removed by filtration. The gelatin was either precipitated with acetone as described above or concentrated to dryness and redissolved in about 50 ml. of water, filtered through a fine sintered-glass funnel (H3) and stored. The term 'insoluble collagen' used hereafter refers to this fraction.

Isolation of 2:4-dinitrophenylglycine (DNP-glycine) and radioactivity measurements

The various protein fractions were hydrolysed by heating with 6 N-HCl for 48 hr. in sealed tubes in an oven kept at 105°. The hydrolysates were concentrated to dryness *in vacuo* and the residues taken up in water; humin was removed by filtration before analyses were carried out. The preparation and isolation of DNP-glycine was generally done by the method of Perrone (1951) as described by Neuberger *et al.* (1951). In a few experiments in which the amount of material was small, the method of Campbell & Work (1952) was used for the isolation of DNP-glycine. Radioactivity measurements were made as described by Neuberger *et al.* (1951). Results are expressed as counts/min./sq.cm. (at 'infinite thickness').

Chemical analyses

Total nitrogen. In most experiments this was determined according to Chibnall, Rees & Williams (1943). For the fractionation experiments described in Table 1 the method of Ma & Zuazaga (1942) was used.

Tyrosine. This was determined according to Udenfriend & Cooper (1952). Readings were made on a Unicam Spectrophotometer at 450 mμ.

Hydroxyproline. The method of Neuman & Logan (1950) was used. Readings were made on a Unicam Spectrophotometer using a wavelength of 550 mμ.

Glycine. This amino acid was determined by the method of Kröl (1952). Readings were made on a Hilger Spekker Photometer using a violet spectrum filter (Ilford no. 601; 400–450 mμ.).

RESULTS

Characterization of the proteins of rabbit skin

The protein fractions obtained from the combined skins of two young rabbits by the methods outlined in Fig. 1 were analysed for total nitrogen and hydroxyproline, tyrosine and glycine contents (Table 1). It will be observed that only 75–80% of the total nitrogen, hydroxyproline and tyrosine of the skin are recovered in the various fractions listed in Table 1. This is due to the loss, which was not determined, of low-molecular nitrogenous substances by diffusion during dialysis, and adsorption

Table 1. *Distribution of nitrogen and of some amino acids in the various fractions of rabbit skin*

Roman numerals refer to fractions in Fig. 1 and text. Figures in brackets are calculated values of hydroxyproline-N, tyrosine-N and glycine-N, respectively, as percentage of total N of fraction.

Fraction	Nitrogen (mg.)	Hydroxyproline (mg.)	Tyrosine (mg.)	Glycine (mg.)
Total skin	1792	776 (4·6)	256 (1·1)	1432 (14·9)
Total phosphate soluble*	278	28 (1·1)	48 (1·1)	87 (5·8)
Water-soluble (I)	95	Approx. 1 (0·1)	28 (2·3)	21 (4·1)
Citrate-insoluble (II)	47	5 (1·1)	8 (1·3)	7 (2·7)
Alkali-soluble collagen (III)	21	16 (8·4)	0·2 (0·1)	29 (25·8)
Acid-soluble collagen (IV)	123	102 (8·8)	6 (0·37)	170 (25·7)
Water washings of citrate-treated skin residue	68	45 (7·1)	2·8 (0·32)	61 (16·8)
Skin residue†	964	442 (4·9)	154 (1·2)	1029 (19·9)
Gelatin from insoluble collagen (V)	598	421 (7·5)	35 (0·45)	858 (26·7)
'Keratin' (VI)‡	366	21 (0·6)	119 (2·2)	171 (8·7)

* Analyses on the material soluble in a saturated Na_2HPO_4 solution at 2° at a pH about 9.

† The residue remaining after the extraction of the skin with the saturated phosphate solution, followed by the extraction with the citrate buffer, washing with 10% NaCl and water.

‡ Obtained by difference: 'keratin' = skin residue − insoluble collagen.

of proteins on filter paper and membranes and by other mechanical causes. In addition, the various washing fluids contained small amounts of nitrogen and of the three amino acids which were determined in some cases. Making allowances for these losses the following conclusions can be drawn. The proteins of the skin of young rabbits can be divided into three broad groups: (*a*) Non-collagenous proteins soluble in phosphate buffer, pH 9 (fractions I and II). This group accounts for about 15% of the total nitrogen of the skin. (*b*) Proteins of collagen type. These account altogether for about 55% of the total nitrogen and can be divided into three fractions: material, extracted by disodium hydrogen phosphate (fraction III), representing about 4% of the total collagen and 2% of the skin nitrogen; material not extracted by disodium hydrogen phosphate, but extracted by citrate, pH 3·8, and subsequent washing with water (fraction IV), and accounting for about 20% of the total collagen and about 10% of the skin nitrogen; and lastly 'insoluble' collagen (fraction V), not extracted by any of these procedures and representing about 75% of the total collagen and 35–40% of the skin nitrogen. (*c*) Insoluble proteins other than collagen (fraction VI). These account for 20–25% of the total nitrogen of skin and have average contents of tyrosine of about 5%, of hydroxyproline of 1% and of glycine of 7·5% (assuming arbitrarily a nitrogen content of 16% for these proteins). The occurrence of hydroxyproline in this fraction is probably due to incomplete removal of collagen. The rest of this fraction consists probably of keratin-like material and of other, probably intracellular, proteins, either not extracted by the procedures used or denatured during the various operations.

Soluble, non-collagenous proteins

In order to obtain more information about this fraction, the following experiment was done.

Skin (45 g. wet wt.) obtained from one adult rabbit was extracted with Na_2HPO_4 as described. The milky extract (pH 8·35) was dialysed for 18 hr. against tap water and then for 2–3 days against three changes of distilled water; a precipitate formed, which was collected on the centrifuge. It could not be redissolved in the Na_2HPO_4 solution originally used for the extraction, nor in sodium phosphate buffer at pH 7·8 or 6·8.

The solution, after dialysis, was freeze-dried and yielded 1·375 g. of material, corresponding to about 3% of wet skin. The solid was extracted with sodium phosphate buffer, pH 7·8, $I = 0·2$. Only about 20% dissolved, and the residue had become insoluble in the Na_2HPO_4 solution used for extraction. The solution of the soluble protein material was concentrated by pressure dialysis against phosphate buffer, pH 7·8, until the concentration of protein was about 1%. Serum proteins of the rabbit were found to dissolve almost completely after freeze-drying. Electrophoresis in the Tiselius apparatus was done both with the soluble protein fraction described above and rabbit serum.

The patterns were similar (Fig. 2), both in the position of the various peaks and in their relative

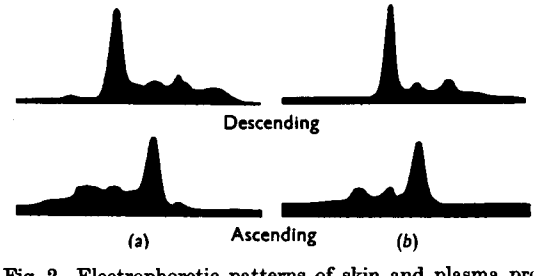

Fig. 2. Electrophoretic patterns of skin and plasma proteins of rabbit. (*a*) Pattern of skin protein fraction after 360 min.; buffer, potassium phosphate $I = 0·2$, pH 7·8; potential 2·9 v/cm.; protein concentration, approximately 1%. ($\Delta n_{25} = 0·00193$.) The skin protein is a soluble protein fraction of rabbit skin obtained on freeze-drying of a phosphate extract. (*b*) Pattern of plasma protein of a rabbit obtained under the same conditions as given above for the skin-protein fraction.

heights; but the skin sample also apparently contained a very small amount of material which migrated at pH 7·8 with a greater velocity than albumin.

Comparison of the three collagen fractions

Analyses for the hydroxyproline and tyrosine contents of the three collagen fractions are given in Table 2. The hydroxyproline values are of the same order as those found by other workers for mammalian collagens (Bowes & Kenten, 1948) and the tyrosine figures are all low. The variations between the values obtained for similar fractions in different experiments are somewhat larger than the experimental errors of the two methods, and it would appear that some of the differences shown in Table 2 between the three collagen fractions are significant.

Insoluble collagen has the lowest hydroxyproline and the highest tyrosine content; alkali-soluble collagen appears to have a slightly lower hydroxyproline content than acid-soluble collagen, but the tyrosine contents of the two fractions are similar and appreciably lower than that of insoluble collagen. From the data in Table 1 it can be calculated that the glycine content of all three samples is about the same, the glycine-nitrogen being 26–27 % of the total nitrogen.

The acid-soluble collagen fraction (IV) consisted mainly of needle-shaped 'crystals' similar to those described by Orekhovich *et al.* (1948) showing the typical banded structure of collagen in the electron microscope. There was usually also some amorphous material present. The solid alkali-soluble collagen (fraction III) consisted of a mass of fine fibres which showed a banded structure in the electron microscope. A two-dimensional paper chromatogram of an acid hydrolysate of this material showed a general picture similar to that obtained with a hydrolysate of the gelatin from insoluble collagen, or from acid-soluble collagen.

Table 2. *Hydroxyproline and tyrosine contents of the alkali-soluble, acid-soluble and insoluble collagen*

As percentage of total N of the hydrolysate.

Expt. no.	Hydroxyproline-N	Tyrosine-N
(*a*) Alkali-soluble collagen		
1	8·04	0·09
2	8·02	0·16
3	8·94	0·24
4	8·38	0·27
Av.	8·34	0·19
(*b*) Acid-soluble collagen		
1	8·79	0·37
2	8·77	0·21
3	9·55	0·34
4	8·79	0·29
Av.	8·98	0·30
(*c*) Insoluble collagen		
1	7·52	0·45
2	8·04*	0·47
3	7·02	0·62
4	7·74	0·49
Av.	7·58	0·51

* Gelatin solution refiltered to remove ppt. which had formed on standing at 0°.

For the interpretation of the isotope results it was important to assess the 'purity' of these fractions, i.e. to investigate whether these samples are free from non-collagenous proteins and also whether they represent chemically distinct groups. It has already been mentioned that the alkali-soluble collagen lost its solubility in the disodium hydrogen phosphate medium used for the original extraction, and the possibility existed that it was originally present in the form of fine fibres which passed through the filter used. Although this seemed unlikely, since filtration was done through a glass filter of low porosity, further evidence was sought. A phosphate extract of skin (25 ml.) was centrifuged at 10000 *g* for 1 hr. at 0°. A small amount of brown precipitate had formed and the supernatant was still faintly opalescent. Hydroxyproline estimation on measured samples indicated that the solution contained 0·518 mg. of this imino acid before centrifuging and 0·433 mg. after centrifuging. It would thus appear that the bulk of the alkali-soluble collagen was finely dispersed in the extraction fluid. The reason for the alteration of solubility of the alkali-soluble fraction during the isolation precedures is unknown, but may be due to a change akin to denaturation which occurred in spite of the low temperatures used. It is also possible that the material was kept in fine dispersion by other substances present in the original skin extract.

Acid-soluble collagen. If acid-soluble collagens were a group distinct from the insoluble collagens, it would be expected that, with repeated treatment of the skin residue with citrate, the amount of material removed by each successive extraction should decrease and ultimately approach zero. For the ten rabbits on which isotope data are given in Table 4, the total dry weight of acid-soluble collagen (fraction IV) was 17·6 ± 1·5 (S.E.M.) % of the total collagen (fractions IV and V), alkali-soluble collagen not being measured. Of the subfractions of the acid-soluble collagen, the mean weight of the first was 7·7, the second 4·6, the third 3·3 and the fourth 2·0 % of the total collagen. Extrapolation of the mean figures to an infinite number of extractions, suggests that the average proportion of acid-soluble collagen as percentage of total collagen would be about 20 %. However, it was observed that further extraction of the citrate-treated residue with water removed a further, relatively large amount of hydroxyproline-containing material (Table 1).

Although the amounts of collagen removed by successive extractions decreased progressively, there is not conclusive evidence that the change from acid-soluble to insoluble collagen is discontinuous.

The effectiveness of various solutions in dissolving acid-soluble collagen is shown in Table 3. The data were obtained by shaking portions of the damp fibres (fraction IV) with the various solutions at room temperature, and removing undissolved material on the centrifuge. There is no evidence that equilibration had taken place, but the relatively short period of extraction was used in order to reduce any irreversible changes which take place slowly at room temperature. The best pH for extraction appears to be near 3·0. With rising pH, extractability decreases markedly and becomes zero above pH 6·0. The slight effect of 0·1 N sodium hydroxide may be due to degradation. The slight effect of this solvent suggests that no significant amounts of acid-soluble collagen would be lost in the preliminary extraction used in the method of Lowry, Gilligan & Katersky (1941) for estimation of collagen. The effect of increasing concentration of calcium chloride in dissolving this collagen fraction is of interest. It has been noted already by Nageotte (1927*c*) that calcium chloride, unlike sodium or potassium chlorides, does not precipitate collagen which had been dissolved by acetic acid.

Table 3. *Extractability of acid-soluble collagen by different solvents*

The buffer covering the range of pH to 8·0 was that described by McIlvaine (1921), whilst that for the range pH 8·0–12·0 was that of Britton & Welford (1937). The molarities used were those given by the authors quoted. Extractability is given as mg. N/100 ml. solvent.

Solvent	Extractability
Citrate/phosphate, pH 2·5	30·2
Citrate/phosphate, pH 3·0	36·8
Citrate/phosphate, pH 3·5	24·6
Citrate/phosphate, pH 4·0	22·2
Citrate/phosphate, pH 4·5	6·2
Citrate/phosphate, pH 5·0	9·6
Citrate/phosphate, pH 6·0	3·1
Citrate/phosphate, pH 7·0	0·0
Citrate/phosphate, pH 8·0	0·0
Phosphate, pH 8·0	0·0
Phosphate, pH 10·0	0·0
Phosphate, pH 11·0	0·0
Phosphate, pH 12·0	0·0
0·3 M-Na_2HPO_4	0·0
0·1 M-NaOH	7·7
0·02 M-$Ca(OH)_2$	1·7
0·1 N-HCl	23·7
$1·6 \times 10^{-2}$ N acetic acid	48·7
$6·5 \times 10^{-4}$ N acetic acid	6·3
0·16 M-NaCl	1·4
0·8 M-NaCl	1·0
1·6 M-NaCl	1·0
0·13 M-KCl	1·1
0·65 M-KCl	2·3
1·3 M-KCl	0·7
0·09 M-$CaCl_2$	4·7
0·45 M-$CaCl_2$	12·6
0·9 M-$CaCl_2$	17·9

Insoluble collagen. Gelatin samples obtained by different methods and from different sources contain small, but varying, amounts of a substance which behaves in the different colorimetric tests like tyrosine. It has also been mentioned (see Table 2) that insoluble collagen, isolated as gelatin, contains more tyrosine than either of the two soluble collagen fractions. It thus appeared likely that the relatively high tyrosine content of the gelatin from insoluble collagen might be due to contamination by a non-collagenous protein. An attempt was therefore made to fractionate this gelatin. To such a gelatin solution at 0° was added trichloroacetic acid (TCA) to a final concentration of 5% (w/v); a precipitate was formed. The tyrosine content of the original gelatin was about 1%, whilst that of the material isolated from the TCA-supernatant was 0·5%. In another experiment in which gelatin, isolated from rabbits fed 24 hr. previously with [α-^{14}C]glycine, was used, the radioactivity of the DNP-glycine obtained from the TCA-supernatant was significantly less than the DNP-glycine of the untreated gelatin. Thus two samples from unfractionated gelatin gave values of 135 ± 1 and 131 ± 1 counts/min./sq.cm.; the corresponding samples from the TCA-supernatants were 97 ± 1 and 86 ± 1. Similar fractionation of gelatin from rabbits killed at later times after the administration of the isotope produced no significant change in the radioactivity of the isolated DNP-glycine.

Isotope experiments

In the first series of experiments, labelled glycine was injected intraperitoneally. With this route of administration surprisingly large variations were observed between the values obtained for similar protein fractions in different rabbits given identical doses of labelled glycine and killed after the same time intervals. Thus, two rabbits of similar body weight (nos. 3 and 7, Table 4) were given the same doses of [α-^{14}C]glycine 7 days before killing; it will be seen that radioactivities of all the fractions obtained from rabbit no. 3 are about 15 to 20 times higher than the corresponding values for rabbit no. 7. However, the ratios of radioactivities for different protein fractions obtained from the same rabbit showed a fairly regular behaviour; they were similar to those of other rabbits which had received identical treatment and depended almost entirely on the time interval between injection of labelled glycine and the killing of the animal. It has been our experience that similar relationships were found to apply to plasma and muscle proteins in rabbits which had been intraperitoneally injected with

Table 4. *Radioactivities of DNP-glycine samples prepared from skin proteins of rabbits which had received* [α-^{14}C]*glycine by intraperitoneal administration*

Fractions were not treated with unlabelled glycine.

Rabbit no. and sex	Interval between injection and killing (days)	Radioactivities of DNP-glycine (counts/min./sq.cm. at 'infinite thickness')						
		Total phosphate-soluble	Alkali-soluble collagen	Acid-soluble collagen				Insoluble collagen
				Extract 1	Extract 2	Extract 3	Extract 4	
A1 (M.)	1	28	—	4·8	2·6	2·8†		1·3
A5 (F.)	1	15·4	—	0	0	0 †		0
B1 (F.)	1	53*	169	9·5	3·6	2·4†		3·5
B2 (M.)	1	860*	1100	107	64	43 †		65
A2 (M.)	3	29·3	—	—	4·1	3·7†		2·4
A6 (F.)	3	490	—	370	284	263 †		134
A3 (M.)	7	194	—	270	330	306	303	186
A7 (F.)	7	12·3	—	21·2	20·5	15·3	13·0	8·8
A4 (M.)	21	34·4	—	36·1	55·6	62·0	55·1	65·6
A8 (F.)	21	8·7	—	12·5	13·0	14·5†		15·6

— Not isolated or estimated.
* These figures refer to the water-soluble fractions (I, Fig. 1) only. † Extracts 3 and 4 combined.

glycine; on intravenous or oral administration, radioactivities, even in rabbits, were much more reproducible. Rats which had received isotopic glycine by interperitoneal injection behaved in a fairly predictable manner (Arnstein & Neuberger, 1951). The reasons for these great variations of radioactivities after intraperitoneal injection in rabbits are obscure. It may be that in some cases the needle penetrated the caecum.

In most of the experiments of the first series glycine was isolated from the total phosphate-soluble protein fraction which included alkali-soluble collagen. On the other hand, the four fractions of acid-soluble collagen obtained from successive extractions with citrate were worked up separately. The phosphate-soluble proteins showed very high radioactivities relative to that of collagen 24 hr. after injection; after 3 days values for this fraction were still very much higher than the corresponding figures for insoluble collagen and appreciably higher than those found for the acid-soluble collagen fractions. The phosphate-soluble proteins had activities below those of any of the acid-soluble collagen fractions 7 days after injection and were similar to the values found for insoluble collagen, whilst after 21 days the radioactivities found for the phosphate-soluble fraction were lower even than those of the least active collagen.

The radioactivities of successive fractions of acid-soluble collagen relative to each other and relative to those of insoluble collagen appeared to show a fairly regular behaviour. With short intervals—1 and 3 days—radioactivities decreased sharply from the first to the fourth acid-soluble collagen fraction and then to insoluble collagen. With the 7-day samples insoluble collagen had still the lowest activity, but the differences between the various acid-soluble collagen fractions were small, whilst after 21 days insoluble collagen appeared to have the highest activity. These results are similar to those reported briefly by Orekhovich (1952) who determined the radioactivity of the whole protein.

In the second group of experiments (Table 5) glycine was fed by mouth over a very short period, the three fractions of the phosphate extract were worked up separately, but the acid-soluble collagen samples were combined. Plasma proteins were also prepared and from them glycine was isolated and its radioactivity assayed. In these experiments, as in the earlier series, insoluble collagen was less active than acid-soluble collagen 3 and 7 days after injection, but surprisingly this relationship was reversed in almost all the experiments in which the animals had been killed 8 or 24 hr., after injection of the labelled glycine. It has already been mentioned (p. 564) that the radioactivity of these insoluble collagen samples was reduced by treatment with TCA. The behaviour of alkali-soluble collagen was found to differ greatly from all other collagen fractions. At 8 hr. its activity was relatively low, but after 24 hr. the radioactivities of this fraction were of the same order as those found for plasma proteins, or citrate-insoluble or water-soluble skin proteins (Fig. 3). It may be noted that the 24 hr. values showed marked variations, but in some cases the radioactivities found for the alkali-soluble collagen fraction at 24 hr. exceeded significantly those found for any other protein in the same experiment.

The values obtained for the citrate-soluble and water-soluble non-collagenous fractions of the phosphate extracts of skin and those for plasma proteins were closely similar throughout. It follows that all these fractions must contain proteins of similar average turnover rates.

Table 5. *Radioactivities of DNP-glycine samples prepared from the skin and plasma proteins of female rabbits which had been fed* [α-^{14}C]*glycine by mouth*

Rabbit no.	Time between feeding of labelled glycine and killing (days)	Wt. of rabbits (g.)	Radioactivities of DNP-glycine (counts/min./sq.cm. at 'infinite thickness')					
			Plasma proteins	Water-sol. proteins of skin	Citrate-insol. proteins of skin	Alkali-sol. collagen	Acid-sol. collagen	Insoluble collagen
5	0·3	554	844	893	713	67·6	3·6	56·6
6	0·3	612	904	1051	1019	131·5	7·0	84·0
7	1·0	536	677	834	848	104·2	55·8	74·6
8	1·0	560	512	825	677	390	33·4	71·5
9	1·0	585	739	588	938	1362	150·8	83·3
10	1·0	490	782	542	562	682	41·6	37·3
3	3·0	605	—	—	157	61·0	86·3	41·8
4	3·0	640	475	380	351	73·5	172	74
1	7·0	530	135·1	—	108	20·8	106	68·4
2	7·0	550	151·5	—	97	19·8	142·9	128

— Sample lost or not isolated.

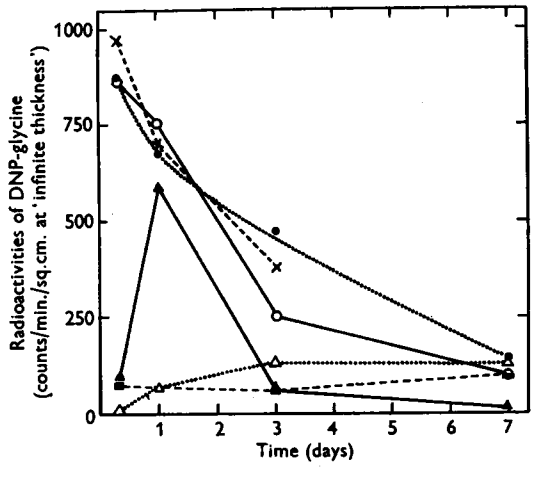

Fig. 3. Time/activity curves of proteins from the skin and plasma of rabbit. The averaged values of the radioactivities of the DNP-glycine samples given in Table 5 have been used to plot the above curves: ▲, alkali-soluble collagen; △, acid-soluble collagen; ×, water-soluble protein; ●, plasma proteins; ○, citrate-insoluble protein; ■, insoluble collagen.

DISCUSSION

The metabolism of proteins of the skin other than collagens

The present experiments show clearly that rabbit skin contains proteins of greatly differing renewal rates. A similar conclusion had already been reached by Ussing (1941), who investigated the incorporation into proteins of deuterium supplied as deuterium oxide. It is reasonable to assume that these varying turnover rates are somehow related to differences in the organized structures with which these proteins are associated. The skin preparations used consisted mainly of dermis, but contained also epidermis and may have been contaminated with small amounts of hypodermis. Both the dermis and epidermis contain a great variety of cells, but a large part of the total mass of the skin is not cellular. This 'extracellular' phase, which has been estimated to occupy in the rabbit about 55 % of the total skin volume (Manery & Hastings, 1939), contains collagen as the quantitatively most important solid. In addition, it may be assumed that there will be present in this phase, as in other parts of the extracellular space, plasma proteins which have escaped through the capillary lining. The protein content of tissue fluids has never been directly measured, but good reasons have been put forward to suggest that the plasma protein concentration in the extracellular fluids of the limbs and the skin may be about 0·5–1 %, i.e. similar to that of the peripheral lymph (Landis, 1934, 1946; Drinker, 1946). The protein fraction isolated in the present experiments after freeze-drying was found to resemble plasma proteins electrophoretically (Fig. 2). It represented about 0·4–0·5 % of the total wet weight of the skin, or about 0·8 % of the estimated extracellular mass. We may therefore assume that this fraction is indeed composed of plasma proteins and is derived mainly from the extracellular phase of the dermis. The fluid or gel between the collagen fibrils is generally considered to have a low protein content and this is supported by the fact that connective tissue such as tendon which has only a small number of cells contains very little non-collagen protein. It can be deduced, therefore, that those fractions of the phosphate extract of skin, which are not composed of alkali-soluble collagen or plasma proteins, i.e. the citrate-insoluble fraction and part of the water-soluble fraction, are derived mainly from the cells of the skin, probably largely from those of the dermis. The high radioactivities found for these fractions

shortly after feeding of the labelled glycine and the rates of decrease of radioactivity with time indicate that the cell proteins of the skin have turnover rates of the same order as those of the mixed proteins of the liver, kidney, etc. The very low overall renewal rates of skin proteins observed are thus caused mainly by the high collagen content of the skin. These low average figures will be particularly misleading, if glycine, which is present in such large amounts in collagen, is used as a label.

The metabolism of skin collagens

Acid-soluble and insoluble collagen. The results presented in Table 4, and similar observations reported by Orekhovich (1952), indicate that the first extract of acid-soluble collagen has a relatively high radioactivity soon after administration of labelled glycine; this activity decreases in successive fractions of this acid-soluble material and is lowest in insoluble collagen. Later, radioactivity becomes equal in the different fractions, until ultimately the order of activities is reversed and is highest in insoluble collagen. These findings have led Orekhovich (1952) to conclude that acid-soluble collagen is a precursor of insoluble or 'true' collagen and to suggest that the name 'procollagen' is indeed justified. It will be observed that, compared with the non-collagen proteins of skin, the radioactivities of acid-soluble collagen are very low, at least at periods up to 3 days after labelled glycine has been given. The activity/time curves of the various fractions of acid-soluble and insoluble collagen are of such a shape (Fig. 3) that no calculations of turnover values can be made, and it is doubtful whether the isotope data can be explained on the assumption that all insoluble collagen passes through the stage of acid-soluble collagen. Rabbits of the age used increase their body weight and probably also their total collagen content by approx. 2% per day. Since acid-soluble collagen was found to be about 20% of the total collagen, approx. 10% of the acid-soluble collagen would have to be synthesized per day and a similar amount would have to be converted into insoluble collagen. The activity/time curve to be expected on such a basis should thus resemble those of plasma or liver proteins; but this is obviously not the case.

It is felt that a more satisfactory explanation is the following: the two fractions, i.e. acid-soluble and acid-insoluble collagen are not distinct groups of proteins and both are obtained from fibres deposited outside cells. It is true that gelatin obtained from insoluble collagen has been found in the present experiments to have a higher tyrosine and lower hydroxyproline content than acid-soluble collagen and similar results have been reported by Bowes, Elliott & Moss (1953). But these workers have reported, on the basis of a comprehensive analysis, that there is a very close resemblance between these two protein fractions with respect to all other amino acids. Bowes *et al.* (1953) suggested that the insoluble collagen, identical in composition with acid-soluble collagen, is associated with some other substance, relatively rich in tyrosine and poor in hydroxyproline. It is also possible, and in our opinion more likely, that this tyrosine-rich material is not chemically linked with collagen in living tissue, but represents a contamination difficult to remove. The differences between these two collagen fractions would then not be of a chemical nature, but be due to physical factors affecting extractability by acidic solvents. The recent experiments of Jackson (1953) also indicate that there is no sharp division between acid-soluble and insoluble collagen. It is known (Gross, 1950) that in the young animal collagen fibrils are relatively thin and increase in size with age. The collagen of small, isolated fibres may be more readily extracted than the massive network found in the more adult animal. There may also be increasing aggregation of collagen molecules with age or increasing association with another substance (Bowes *et al.* 1953) affecting extractability with a citrate solution. The first extraction with weakly acidic solvents will thus remove mainly the collagen recently laid down, i.e. the material derived from small fibres, or from the outer layers of larger fibres. As extractions are repeated, more of the 'older' collagen will be made soluble, until an apparent limit is reached, due to denaturation or other unknown factors. Such an explanation fits satisfactorily the isotope results reported by Orekhovich (1952) and by ourselves. Indeed it is difficult to reconcile the simple precursor hypothesis with the finding that the maximum activity 'migrates' gradually with time from the first to the last extract of acid-soluble collagen and ultimately to insoluble collagen.

An association or contamination of insoluble collagen with a tyrosine-rich material would also explain the surprisingly high apparent radioactivity found for this collagen in some of the 8 and 24 hr. experiments of the second series (Table 5). This interpretation is supported by the observation that on fractionation of the gelatin, high tyrosine content and high radioactivity of the isolated glycine are apparently associated, at least with earlier collagen samples. It is clear that the gelatin from insoluble collagen is heterogeneous and is probably contaminated with a non-collagen protein of relatively high turnover rate.

Alkali-soluble collagen. This fraction was found to differ completely from the other two collagen fractions and indeed from all other proteins investigated. The radioactivity was high after 24 hr., but had decreased sharply at 3 days (Fig. 3). The

variations between the results obtained in altogether six 24 hr. experiments (Tables 4 and 5) were large, but this may be explained as follows: owing to differences between animals it is unlikely that the maximum activity in this fraction occurred in all experiments at the same time after injection. It is indeed doubtful whether in any of these experiments a value was obtained which was close to the maximum, particularly since both the ascending and descending parts of the activity/time curve appear to be very steep. On this basis, the high radioactivities at 24 hr. which exceeded, in three out of six experiments, those of any protein fraction measured, and the general character of the curve (Fig. 3) suggest that alkali-soluble collagen is formed and removed rapidly with a half-life of 2 days or less. Such a value is compatible with the assumption that this material is indeed a precursor of all other collagens of the skin. The initial solubility in disodium hydrogen phosphate solution suggests that the state of this alkali-soluble material in the tissue differs from that of the other collagen fractions. Its initial solubility may be due to a lower particle weight or to association with another substance. During isolation this association may be broken or polymerization may occur, leading to a change in solubility. We have no information about the morphological origin of this fraction. It may be suggested, tentatively, however, that alkali-soluble collagen is not derived from microscopically visible fibres, but may have been present either in the space between the fibres or even in or near the fibroblasts which are generally assumed to be responsible for the formation of collagen.

SUMMARY

1. The proteins present in the skin of growing rabbits have been separated into the following main fractions: (*a*) material soluble in phosphate buffer pH 9; (*b*) proteins insoluble in phosphate, but soluble in citrate pH 3·8; (*c*) collagen not extracted by these procedures, but converted into gelatin by autoclaving; (*d*) an insoluble residue.

2. Evidence was obtained that the phosphate-soluble material was a mixture of cellular proteins, plasma proteins derived from the extracellular space and a small amount of a collagenous protein ('alkali-soluble collagen'). The citrate-soluble fraction consisted of collagen-like material which is called acid-soluble collagen ('procollagen' of Orekhovich).

3. The amounts of hydroxyproline, glycine and tyrosine in the three collagenous fractions were similar, although there were some significant differences. Evidence was obtained that gelatin from insoluble collagen is either heterogeneous with respect to amino-acid composition or contaminated with protein of non-collagen type.

4. Experiments using a single dose of [α-^{14}C]-glycine and isolating the glycine as the 2:4-dinitrophenyl derivative showed that the proteins of the phosphate-soluble fraction, other than alkali-soluble collagen, had turnover rates similar to those of plasma and liver proteins. The highest radioactivity of the alkali-soluble collagen was observed about 24 hr. after administration of the labelled glycine and exceeded in some experiments the maximum values found for other proteins of the phosphate fraction or plasma proteins. Thereafter it dropped rapidly to values below those of acid-soluble or insoluble collagen.

5. The radioactivity of the glycine of the acid-soluble collagen by comparison with that of alkali-soluble collagen increased and decreased very slowly and the activity/time curve of insoluble collagen was almost flat.

6. It is concluded that alkali-soluble collagen is almost certainly a true precursor of the other collagen fractions. The metabolic role of acid-soluble collagen is more uncertain and its activity/time curve suggests that it is not a necessary intermediate in the formation of all the insoluble collagen of the skin.

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Content of Vitamin A_2 in some Nile Fishes

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Heilbron, Gillam & Morton (1931) observed that in the antimony trichloride colour test, an absorption band at 693 mμ. accompanied the 617 mμ. band in many fish-liver oils. For several years, little progress was made in characterizing the new chromogen. It accompanied vitamin A in the richest concentrates and no separation could be effected either by chromatography or molecular distillation. Edisbury, Morton & Simpkins (1937) found relatively large quantities of the 693 mμ. chromogen in the eyes of gold-fish and the livers of trout. They therefore suggested that the 693 mμ. chromogen should be designated vitamin A_2, because its distribution suggested that it could replace vitamin A, particularly in fresh-water fish.

Morton, Salah & Stubbs (1946) prepared vitamin A_2 free from vitamin A_1, and later Morton, Salah & Stubbs (1947) suggested that it was dehydrovitamin A_1, with the extra double bond in the β-ionone ring.

From the retinas of marine fishes, Wald (1935) obtained rhodopsin from which, after bleaching, retinene$_1$ could be extracted by light petroleum. An analogous pigment, porphyropsin, was obtained from the eyes of fresh-water fishes (Wald, 1936); from this retinene$_2$ could be obtained. Morton & Goodwin (1944) proved that retinene$_1$ is vitamin-A_1 aldehyde and retinene$_2$ was later shown to be vitamin-A_2 aldehyde (Morton *et al.* 1947).

Wald (1937) has made the interesting observation that, in general, the eye tissues of fresh-water fishes contain vitamin A_2, while those of marine fishes contain vitamin A_1. Fishes which migrate from the one environment to the other contain both vitamins. It is with these facts in mind that Nile fishes have been examined. The amounts of both vitamin A_1 and A_2 in such fishes have not hitherto been determined. The results may help in accounting for distribution of vitamins A_1 and A_2 in nature.

EXPERIMENTAL

Materials. The fishes reached the laboratory within a few hours of being caught and were immediately dissected. The livers were removed and wet weights recorded.

Extraction of oils. The oils were prepared by grinding the livers with silver sand and anhydrous Na_2SO_4 and extracting with light petroleum 3 or 4 times. The solvent was completely distilled off in an atmosphere of CO_2 and the residue was weighed. The light petroleum used was purified by refluxing for 30 min. with $KMnO_4$ and dilute H_2SO_4; washed with water, then NaOH and water, and then dried over anhydrous $CaCl_2$. It was then fractionated and the fraction boiling between 40 and 60° was used.

Alumina. Alumina for chromatography (British Drug Houses Ltd.) was weakened by the addition of 10% of its weight of water and used immediately. The following is the procedure which was found suitable for weakening the active alumina. Alumina (10 g.) is made into a paste with