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Structure and Composition of Soluble Feather Keratin

By A. M. WOODIN
Ophthalmological Research Unit (Medical Research Council), Institute of Ophthalmology, Judd Street, London, W.C. 1

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A large proportion of feather keratin can be extracted by urea and reducing agents, and by subsequent oxidation with performic acid converted into a form soluble in dilute salt solution. The extracted material has a narrow distribution of molecular weight and charge, and Woodin (1954) has suggested that it contains the feather keratin monomer, an asymmetric particle with molecular weight 10000. Rougvie (1954) independently found that solutions of feather keratin contain particles of these dimensions, and made the further observation that fibres reconstituted from feather keratin which had been oxidized with peracetic acid gave X-ray diffraction patterns with much of the detail of the original feather. It is therefore likely that the asymmetric particle of molecular weight 10 000 is responsible for the diffraction characteristics of feather keratin.

In this paper the results of an end-group analysis and some information on the amino acid composition of soluble feather keratin are reported.

METHODS

Preparation of keratin derivatives

White body feathers from hens were extracted with benzene, dried, extracted with 10% NaCl, washed with distilled water and dried over P_2O_5. Before solubilization the calmus was cut from each feather.

SH-Keratin. This was extracted in 10M urea, 0·1M-$NaHSO_3$, 0·05M phosphate (pH 5–6), for 18 hr. at 40°. The extract was filtered and dialysed against running tap water and then distilled water. The white gel was freeze-dried.

Cysteic acid-keratin. This was obtained by performic acid oxidation of SH-keratin. Formic acid was mixed with 0·115 vol. of 30% (w/v) H_2O_2, left at room temperature for 1 hr. and cooled to 0°. One-twentieth of its weight of SH-keratin was added and the mixture left overnight in the refrigerator, filtered and dialysed against tap water and then against distilled water. The resultant opalescent solution was freeze-dried. In the experiments to identify the *N*-terminal amino acids by reaction with 1-fluoro-2:4-dinitrobenzene (FDNB), cysteic acid-keratin was used after purification to this stage.

In all other experiments the cysteic acid-keratin was purified further. After drying, the solid was not completely soluble in water. When the suspension was adjusted to pH 9, more dissolved, but the pH fell during solution. When more alkali was added to keep the mixture at pH 9 all but a little dissolved. The solution was centrifuged at 20000 rev./min. and the supernatant passed down a column (15 mm. diameter) of Amberlite IR-120 in the H^+ form, and then down a column (15 mm. diameter) of Amberlite IRA-410 in the base form. Quantities of 20 ml. of each wet resin were used/g. of keratin. A solution was obtained which was very opalescent but free from visible particles. Examination in a flame photometer showed it to be free from metal cations. The solution was freeze-dried and gave a solid which readily dissolved in distilled water to give a very opalescent, acid solution.

Cysteic acid-keratin concentrations were determined from measurements of the N content. The N content of the dry protein was taken as 15·5% and the molecular weight as 10000.

Reaction with 1-fluoro-2:4-dinitrobenzene

A tenfold molar excess of FDNB was shaken with the protein, unless otherwise stated, in 50% (v/v) ethanol containing 1% $NaHCO_3$. After a known reaction time (3–42 hr.) the mixture was concentrated *in vacuo* and extracted with ether, acidified and extracted again. The aqueous phase was dialysed against changes of distilled water for several days in the dark. Some Amberlite IR-120 in the H^+ form was added to the final sample of distilled water. The dialysed 2:4-dinitrophenyl- (DNP)-keratin was freeze-dried.

Protein hydrolysis in redistilled 5·8 N-HCl or in 12 N-HCl was carried out in sealed tubes at 105°. Destruction of the DNP-amino acids was assessed by adding a standard solution of the DNP-amino acids to DNP-keratin.

DNP-amino acids were separated on buffered Celite columns or on paper chromatograms. The 'toluene' and 'phosphate' solvents of Levy (1954) were used, and irrigation with 'toluene' for 48 hr. and with 'phosphate' for 24 hr. separated all except two pairs, DNP-glutamic and DNP-aspartic acids and DNP-leucine and DNP-valine. The acid-soluble DNP-amino acids were separated on one-dimensional paper chromatograms with *tert.*-pentanol equilibrated with 0·05 M phthalate buffer (pH 6) as solvent (Blackburn & Lowther, 1951). After separation and elution the DNP-amino acids were assayed colorimetrically in 1% $NaHCO_3$ with the extinction coefficients of Rao & Sober (1954).

Direct spectrophotometry of DNP-keratin. Samples (1 ml.) of cysteic acid-keratin, 10^{-3} M, were treated with FDNB for varying times. The solutions were concentrated and extracted with ether. The aqueous phase was evaporated to dryness and the solid dissolved in 5 M guanidine-HCl in N-HCl. Blank solutions containing no keratin were put through the same procedure; after extraction they contained some material with ultraviolet absorption, presumably due to impurities in the FDNB. The absorption of the solutions in guanidine-HCl was measured from 270 to 400 mμ.

Reaction with phenyl iso*thiocyanate*

A volume of cysteic acid-keratin in distilled water (3 ml.), adjusted to pH 8, was stirred at 40° with 3 ml. of pyridine and 0·1 ml. of phenyl *iso*thiocyanate, in a small beaker containing a glass-electrode assembly. The mixture was kept at pH 8 for 2 hr. by addition of N-NaOH and then extracted 9 times with 20 ml. of benzene; 20 ml. of acetone was then added to the aqueous phase and the precipitate washed five times with ether. The last of the ether extracts was shaken with 4 ml. of N-HCl, and the ether layer evaporated to dryness, the solid dissolved in ethanol and its absorption curve recorded from 250 to 230 mμ. This formed the 'blank' in the assay of the phenylthiohydantoins of the *N*-terminal amino acids in keratin.

The solid phenyl *iso*thiocyanate derivative of keratin was heated with N-HCl at 100° for 1 hr. and the resultant solution of partly hydrolysed protein extracted with ether. The ether extracts were taken to dryness to give a solid which was dissolved in ethanol and the optical density recorded in the range 250–350 mμ.

The solution of partly hydrolysed protein, after extraction with ether, was extracted with ethyl acetate, the ethyl acetate phase evaporated to dryness to give a solid which was dissolved in ethanol, and the optical density of the solution recorded.

Finally, the solution of partly hydrolysed protein, after these extractions, was dried *in vacuo*, redissolved in water and the optical density recorded in the range 250–350 mμ.

The concentration of the phenylthiohydantoins of the amino acids in the extracts and the residual aqueous solution was assayed from a molar extinction coefficient at 267 mμ. of 16000 (Fraenkel-Conrat, Harris & Levy, 1955).

The phenyl thiocarbamate derivatives of keratin will be abbreviated PTC-keratin.

Reaction with carboxypeptidase

Carboxypeptidase (Armour Laboratories) was washed five times with water and kept suspended in water. A solution was obtained by adding 0·1 ml. of 1% $NaHCO_3$ to 1 ml. of suspension, and then NaOH was added to pH 9. When all the enzyme had dissolved HCl was added to give pH 8. Di*iso*propyl fluorophosphate (di*iso*propyl phosphorofluoridate) (0·1 M) in *iso*propanol was added and the solution incubated at 37° for 1 hr.

A 2×10^{-3} M solution of cysteic acid-keratin in distilled water, adjusted to pH 8, was incubated with an equal volume of the enzyme solution at 37°. Samples were withdrawn and acidified with HCl to pH 3. This precipitated some of the protein, which was centrifuged off. Samples of the supernatant were analysed for free NH_2 groups by the ninhydrin reaction, and further samples (1–2 ml.) were passed down columns containing 5 ml. of Zeo-Karb 225 in the H^+ form. These columns were then washed with water and eluted with 5 N-NH_3 soln. The eluate was dried and treated with FDNB for 4 hr., and the DNP-amino acids were extracted and separated on paper chromatograms. The optical density at 350 mμ. of the acid solution, after removal of the DNP-amino acids soluble in ether, indicated the absence of DNP-cysteic acid. Solutions of the substrate or enzyme alone showed no liberation of amino groups during the incubation.

When the ninhydrin reaction indicated that the digestion was complete, samples of the digest and of the enzyme and substrate were treated with FDNB for 3 hr. After extraction the extent of the reaction was assessed by direct spectrophotometry of the DNP-proteins in guanidine-HCl solution (pH 0·5).

Analytical methods

Titration curves. These were measured on 10 ml. samples of 5% cysteic acid-keratin in distilled water. Alkali was added from a microburette and a stream of nitrogen played on the surface of the solution. The pH changes were recorded on a Cambridge pH meter. At the end of the titration, samples of the alkaline solution were analysed for their N content and the protein concentration was calculated.

Absorption curves. These were measured on a Unicam spectrophotometer with 1 cm. cells. Optical densities were measured at 5 mμ. intervals.

Nitrogen. This was determined by Kjeldahl digestion with a selenium catalyst for 8 hr.

Amino groups. These were estimated by the ninhydrin reaction (Troll & Cannan, 1953).

Amide N. Cysteic acid-keratin was hydrolysed in 2 N-HCl for 4 hr. (further heating gave the same yield), brought to pH 5, and distilled with 2 M phosphate buffer, pH 10·8. The ammonia was collected in boric acid and titrated with HCl.

Urea. This was recrystallized three times from ethanol solution.

Guanidine hydrochloride. This was prepared from guanidine carbonate (British Drug Houses, Analar grade). Solutions (5M) showed some absorption in the ultraviolet, but with sufficient transmission to permit optical densities of solutes to be measured at 260 mμ.

Fluorodinitrobenzene and phenyl isothiocyanate. These were obtained from Light and Co., Colnbrook, Bucks.

Ion-exchange resins. Amberlite IR-120 and Zeo-Karb 225 in columns 15 mm. in diameter were washed with 2N-NaOH and then with distilled water, and converted into the H^+ form by treatment with 5 vol. of 3N-HCl and washed with distilled water. Amberlite IRA-410 was washed with distilled water and converted into the base form by treatment with 5 vol. of 5N-NH_3 soln. and washed free from NH_3 with distilled water which had been boiled in a stream of N_2 to lower its CO_2 content. The regenerated resins were stored wet.

RESULTS

Reaction with FDNB

(*a*) *Identification of* N*-terminal amino acids.* Samples of cysteic acid-keratin which had not been solubilized in alkali and treated with ion-exchange resins were used in these experiments.

Cysteic acid-keratin, coupled with FDNB for 24 hr. and subsequently hydrolysed in 5·8N-HCl for 8, 16 or 24 hr., or in 12N-HCl for 12 hr., liberated the DNP derivatives of aspartic acid, glutamic acid, serine, threonine, glycine, alanine and valine; ε-DNP-lysine was also present. The recovery of these DNP-amino acids during hydrolysis in 5·8N-HCl was determined, and the amounts of the *N*-terminal amino acids in cysteic acid-keratin were assessed. Table 1 records these results.

Cysteic acid-keratin coupled with FDNB in 2·5M guanidine-HCl solution or in aqueous solution at pH 11, or SH-keratin coupled under the usual conditions, has given results similar to these.

Table 1. N*-terminal amino acids of cysteic acid-keratin coupled with FDNB for* 24 *hr. in* 1% $NaHCO_3$ *in* 50% *ethanol*

Hydrolysis was for 24 hr. in 5·8N-HCl at 105°. Dry cysteic acid-keratin represents 80% of the weight of the air-dry DNP-keratin.

Amino acid derivative	Recovery after 24 hr. in 5·8N-HCl at 105° (%)	Moles of DNP-amino acid/100 moles of keratin*
α-DNP-aspartic acid	46	4·50
α-DNP-glutamic acid	85	0·63
α-DNP-serine	90	0·60
α-DNP-threonine	87	1·50
α-DNP-glycine	35	1·10
α-DNP-alanine	70	1·12
α-DNP-valine	88	1·08
ε-DNP-lysine	80	12·5

* Molecular weight of keratin is taken as 10000.

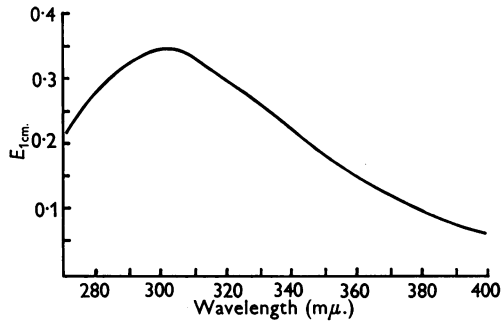

Fig. 1. Absorption spectrum of the DNP groups of DNP-keratin in 5M guanidine-HCl (pH 0·5). Protein concentration corresponds to 0·034 μmole of cysteic acid-keratin/ml.

(*b*) *Direct spectrophotometry of DNP-cysteic acid-keratin.* DNP-cysteic acid-keratin is soluble in 5M guanidine-HCl (pH 0·5). This permitted the measurement of the optical density of the DNP-protein before hydrolysis. In particular it has been shown that the absorption characteristics of the DNP derivatives of proline peptides are similar to those of DNP-proline (Schroeder, Honnen & Green, 1953; Phillips, 1955). Accordingly, the optical density of DNP-keratin at 390 mμ. and at 360 mμ. affords a measure of *N*-terminal proline and of other *N*-terminal amino acids respectively.

The absorption spectra of keratin and of its DNP-derivative in 5M guanidine-HCl (pH 0·5) were measured. The difference between the two is taken as the contribution of the DNP groups to the absorption. Fig. 1 describes the absorption of the DNP groups of a sample of cysteic acid-keratin coupled with FDNB during 4 hr. Coupling with FDNB for longer periods increases the optical density only slightly; after 42 hr. the optical density was 10% greater than at 4 hr. The optical density at 360 mμ., if it is due to combined DNP-amino acids with an assumed molar extinction coefficient of 17 500, corresponds to 0·38 equivalent of DNP groups/mole of protein. The absorption peak at 290–300 mμ. may be due to *O*-DNP-tyrosine and this material may be responsible for some of the absorption at 360 mμ. Rao & Sober (1954) give the molar extinction coefficient of *O*-DNP-tyrosine at 360 mμ. as 290. As there is no evidence of an absorption peak at 390 mμ. *N*-terminal proline is not present to any great extent.

Reaction with phenyl isothiocyanate

Fig. 2 describes the absorption spectrum of the material extracted by ether from PTC-keratin heated in N-HCl for 1 hr. The peak at 267 mμ. is characteristic of a phenylthiohydantoin of an amino acid. The small peak at 310 mμ. is similar to those found for materials liberated from PTC-serine and

PTC-threonine on acid hydrolysis (Levy, 1955). The optical density at 267 mμ., if due to a phenylthiohydantoin of an amino acid with a molar extinction coefficient 16 000 (Fraenkel-Conrat *et al.* 1955), corresponds to 0·1 equivalent of *N*-terminal amino acid/mole of protein.

No further phenylthiohydantoins are extracted from the partly hydrolysed PTC-protein by ethyl acetate, and the absorption of the neutral solution of the partly hydrolysed PTC-protein after extraction with ether and ethyl acetate was identical with that of a solution containing an equivalent concentration of cysteic acid-keratin.

Reaction with carboxypeptidase

On incubation with carboxypeptidase in the presence of di*iso*propyl fluorophosphate amino acids are liberated from cysteic acid-keratin, and the ninhydrin reaction shows the liberation to proceed to a maximum of 0·9–1·0 equivalent/mole of substrate. With a final keratin concentration of 1 μmole/ml. this result has been obtained with di*iso*propyl fluorophosphate concentrations of 0·005 M–0·0003 M and enzyme concentrations of 0·01–0·05 μmole/ml. With 0·0004 μmole of enzyme/ml. the reaction was slow and 0·25 μmole of amino acids was liberated after 6 hr.

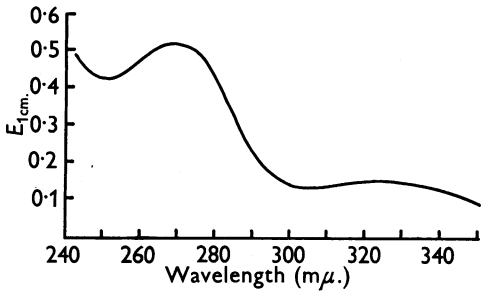

Fig. 2. Absorption curve of the phenylthiohydantoins derived from PTC-cysteic acid-keratin. The optical densities are for the extract from 3 μmoles of protein in 10 ml. of ethanol.

Table 2. *Liberation of amino acids from cysteic acid-keratin incubated with carboxypeptidase*

Substrate concn., 1 μmole/ml.; enzyme concn., 0·02 μmole/ml. Incubation was in the presence of 0·005 M di*iso*propyl fluorophosphate.

Amino acid	Final concn. in digest (equiv./mole of keratin*)	Percentage of final amount liberated after incubation for 10 min.	60 min.
Threonine	0·088	40	67
Glycine	0·095	25	67
Asparagine or glutamine	0·08	63	90
Alanine	0·135	46	64
Serine	0·175	20	52
Phenylalanine	0·083	60	88
Valine and leucine	0·250	68	92

* Molecular weight of keratin is taken as 10000.

Table 3. *Amino acid composition of cysteic acid-keratin hydrolysed in* 5·8 N-HCl *for* 24 *hr.*

Amino acid	Equiv./mole of keratin*
Glycine	9·8
Alanine	4·0
Serine	13·7
Threonine	5·0
Proline	13·0
Valine and leucine	19·0
Aspartic and glutamic acids	10·2
Arginine	3·9
Cysteic acid	6·1
Phenylalanine	3·2
Lysine	0·1–0·2

* Molecular weight of keratin is taken as 10000.

The liberated amino acids, separated from protein by ion-exchange treatment and subsequently coupled with FDNB, gave the DNP derivatives of glycine, alanine, phenylalanine, valine, serine, leucine, threonine and asparagine or glutamine. Table 2 gives their relative concentrations and some information on the rate at which they are liberated.

The protein present in the digest, after removal of amino acids by treatment with Zeo-Karb 225, treated with FDNB for 3 hr. and examined by direct spectrophotometry was found to contain fewer than 0·3 equiv. of DNP groups/mole of protein. That is, the liberation of the amino acids by carboxypeptidase does not simultaneously change the reactivity of the amino groups to FDNB, and it is unlikely that the action of the enzyme preparation is due to hydrolysis other than that of peptide bonds at the *C*-terminal end of a polypeptide chain.

Amino acid analysis of cysteic acid-keratin

The interpretation of the reactivity of cysteic acid-keratin to some of the reagents used here required quantitative information on the composition of the protein. This has been provided by separation and assay of the DNP derivatives of the hydrolysate.

Hydrolysis in 5·8 N-HCl or 12 N-HCl has given the same result, summarized in Table 3. DNP-valine and DNP-leucine and also DNP-glutamic acid and DNP-aspartic acid were respectively imperfectly resolved on the chromatograms and they are reported in pairs.

The lysine content is such that it must be absent from most of the monomers of feather keratin. The cysteic acid content corresponds well with the number of potential –SH groups reported by Woodin (1954) to be present.

Amide N determinations indicate the presence of 1 mole of asparagine or glutamine/mole of protein.

Reaction with the hydroxyl ion

A 5% solution of cysteic acid-keratin in distilled water had a pH 1·8–2·0 and was very opalescent. On addition of alkali the opalescence remained until between pH 5 and 6 a clear, yellowish solution was obtained. Fig. 3 records the power of cysteic acid-keratin to combine with base under various conditions. There is little combination with base between pH 6·5 and 8·0, a finding consistent with the absence of free α-amino groups and histidine. In the absence of salt, base is neutralized between pH 8·0 and 10, which is unexpected, as the lysine content is so low. Addition of KCl decreases the amount of base bound in this region, and simultaneously increases the amount bound below pH 6. Urea and formaldehyde have the same effect but to a greater extent.

As we have chemical evidence that free amino groups are largely absent, the effects of salt, urea and formaldehyde have to be explained in terms of their action on the acidic groups. It has been noted above that cysteic acid-keratin recovered from solution in performic acid is not readily soluble in water, and that solution in dilute alkali is accompanied by liberation of acid. This is consistent with the view that the uncharged acid groups are stabilized in the solid state, or that protons cannot readily diffuse away from the protein surface.

The large increase in combination with base above pH 10 is ascribed to the arginine residues. The base neutralized in reaching pH 10 is due to the acid groups.

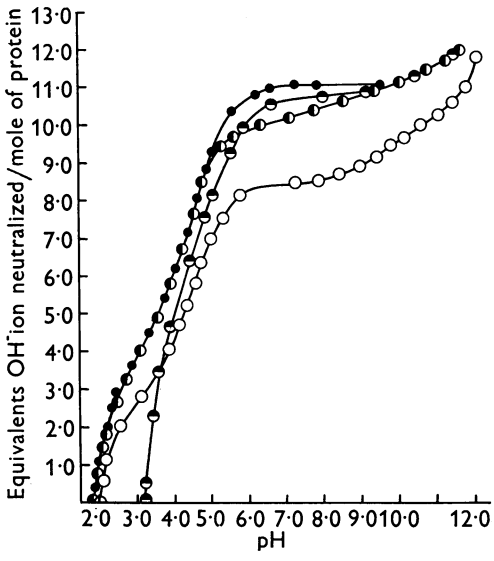

Fig. 3. Titration curves of cysteic acid-keratin. ○, In distilled water; ◐, in 0·5 M-KCl; ●, in 2 M formaldehyde, 0·5 M-KCl; ◒, in 5 M urea, 0·5 M-KCl.

Chemical analysis has shown the presence of six cysteic acid residues, ten aspartic or glutamic acid residues and one amide group in each protein molecule. There are four arginine residues, so that the net combination with base to be expected is 11 equivalents/mole of protein. This is the amount found by titration, and the agreement is evidence for the absence of free α-carboxyl groups.

DISCUSSION

The yield of DNP-amino acids from hydrolysed DNP-keratin corresponds to 1 equivalent of *N*-terminal amino acids in 10 moles of protein. It is unlikely that this low yield is due to destruction during hydrolysis, for spectrophotometry of DNP-keratin before hydrolysis gives a similar result. The reaction with phenyl *iso*thiocyanate also reveals a deficiency of *N*-terminal amino acids. It is, therefore, fairly certain that only 0·1 equivalent of *N*-terminal amino acids in each mole of keratin are available to FDNB and phenyl *iso*thiocyanate. There is no evidence for the masking of amino groups other than by peptide-bond formation, so the absence of free α-amino groups is evidence for a cyclic structure in the molecule. The many amino acids liberated by carboxypeptidase amount, together, to 1 equivalent/mole of protein, and if these come from the keratin molecule it is necessary to assume that feather keratin consists of a collection of polypeptides each with only one amino acid capable of being hydrolysed by carboxypeptidase. This is rather improbable, and the liberation of amino acids by carboxypeptidase is likely to be due to stepwise hydrolysis of an impurity. The reaction of cysteic acid-keratin with the OH^- ion is also consistent with the absence of a molar proportion of α-carboxyl groups. A branched chain is therefore unlikely in this case.

A cyclic structure for soluble feather keratin can give an axial ratio consistent with the results of dynamic methods of determining the shape of keratin in solution. The 3·1 Å meridional arc of the feather-keratin diffraction pattern corresponds to the projected length of the amino acid residues along the axis. A cyclic polypeptide of 90 residues will have a maximum length of 140 Å, and if the diameter of the double chain is 14–20 Å the axial ratio will be 10–7. Axial ratios calculated from the viscosity, assuming a prolate ellipsoidal shape and a hydration of 0–30%, will be in this range.

The slight reaction with FDNB and the other reagents can be due to impurities which, if they have the same molecular weight as the bulk of the particles in soluble feather keratin, will represent 10% of the weight. The heterogeneity of soluble feather keratin makes a more detailed amino acid analysis than has been attempted of doubtful

value. The analysis has indicated that histidine and lysine are absent from cysteic acid-keratin. The proportions of the other amino acids can be affected by the contaminants. The findings of Schroeder & Kay (1955) that the parts of feather differ slightly in composition can, of course, reflect a variation in the composition of the monomer, but are more likely to be due to different amounts of contaminants in the different parts.

SUMMARY

1. The reaction of soluble feather keratin with 1-fluoro-2:4-dinitrobenzene and with phenyl *iso*-thiocyanate indicates that the protein contains only one-tenth equivalents of *N*-terminal amino acids/mole of protein.

2. The reaction of soluble feather keratin with carboxypeptidase and with the OH^- ion can be interpreted as evidence for the absence of a molar proportion of α-carboxyl groups.

3. Some of the amino acids in soluble feather keratin have been estimated quantitatively.

4. It is concluded that the feather keratin monomer is an unbranched cyclic peptide.

I wish to thank Mr G. Cook for his technical assistance.

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The Iodine-containing Proteins of Normal and Abnormal Human Thyroid Tissue

By P. G. STANLEY*
Department of Clinical Research (Medical Research Council), University College Hospital Medical School, London, W.C. 1

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Derrien, Michel & Roche (1948) studied the nature of the iodine-containing proteins present in the thyroid glands of hogs, cattle and horses by a method involving the fractional precipitation of the proteins from saline extracts and the determination of the iodine and nitrogen contents of the precipitates. They used either a 3·5 M mixture of equimolar mono- and di-potassium phosphate (pH 6·5) or a saturated solution of ammonium sulphate (pH 6·5) as a precipitating agent. The presence of at least five different protein components which contained iodine could be demonstrated in the crude extracts, and a method of preparing a purified thyroglobulin was developed. The preparation of thyroglobulin, however, was not a homogeneous protein, but one containing three components (Derrien *et al.* 1948; Derrien, Michel, Pedersen & Roche, 1949).

Only two investigations of the iodine-containing proteins of abnormal tissue from human thyroid glands have so far been published. Roche, Michel, Deltour & Michel (1952) concluded on the basis of solubility and other tests that the iodine-containing protein of the thyroid glands of hyperthyroid patients did not differ significantly from that of a normal control. Trunnell & Wade (1955) compared semi-quantitatively, by paper chromatography and autoradiography, the ^{131}I-contents of the iodo-amino acid and iodide fractions of alkali-hydrolysed normal and adenomatous thyroid tissue from each of two patients who had been given sodium iodide containing ^{131}I. Their results indicated differences in the distribution of ^{131}I between the various fractions from the two types of tissue. In one case no labelled thyroxine or triiodothyronine appeared on the chromatograms of the hydrolysed thyroid adenoma tissue.

The present investigation is divided into three parts. The first consists of an examination of the distribution of ^{131}I between the various fractions obtained in the course of the preparation of purified thyroglobulin by the method of Derrien *et al.* (1948).

* Present address: Department of Chemistry, Indiana University, Bloomington, Indiana.