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ENCLOSURE 5

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INCORPORATION OF TRITIUM INTO HAIR PROTEINS OF RAT*)

A simple and relatively rapid procedure for the extraction and fractionation of hair proteins, was elaborated and used for an analysis of rat hair proteins, tritiated in vivo. The most radioactive protein, containing over 6 per cent of the initial hair radioactivity, was isolated in a homogenous state. The protein had a molecular weight of about 190,000 daltons, and showed high proportions of glutamic acid, cysteine, aspartic acid, serine, and glycine and a low content of methionine and histidine.

More than 80 per cent of total tritium radioactivity incorporated into this protein was distributed among indispensable phenylalanine (30.3 per cent) and, isoleucine (17.2 per cent), valine (17.6 per cent), proline (10.5 per cent), and tyrosine (8.4 per cent). The highest values of specific radioactivity were recorded for phenylalanine, isoleucine, valine and methionine. The radioactivity recovered in the amino acids is due to the presence of firmly bound tritium.

1. Introduction

It has been known for more than twenty years, that stable or radioactive elements in a mammal are incorporated into hair. On the basis of this finding, hair is used in forensic medicine to detect poisonous elements in the body [1, 2]. It has also been suggested that hair might be used for determining body burdens of some radionuclides [3, 4, 5, 6, 7]. Therefore, the use of hair for monitoring accidental and chronic exposure to radionuclides seems to be of interest.

Hair proteins are synthetized in hair follicle cells during active growth anagen phase [8, 9]. Keratinisation, a specific form of differentiation of hair follicle cells, is associated with biosynthesis of keratins, specific fibrillar insoluble proteins of hair. During this process, metabolically active cells are transformed into inactive cells [10, 11].

Many data have been accumulated concerning tritium incorporation into the hair, however, no information is available on the distribution of tritium incorporated into hair proteins.

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The aim of the present study was to establish whether tritium administrated as tritiated water is preferentially incorporated into some protein (*s*) of hair, and what the distribution of tritium among amino acid residues is.

2. Materials and Methods

2.1. Chemicals

Dansyl chloride and diisopropylfluorophosphate were purchased from Sigma (St. Louis, Mo, USA); trypsin and human γ-globulin from Koch-Light (Colnbrock-Bucks, England); Sephadex G-100, G-200 and Blue Dextran from Pharmacia (Uppsala, Sweden), and bovine serum albumin form BDH (Poole, England). Tritiated water was obtained from the Isotope Production and Distribution Centre (Świerk, Poland). Porcine pancreatic elastases: I and II were prepared according to Ardelt method [12].

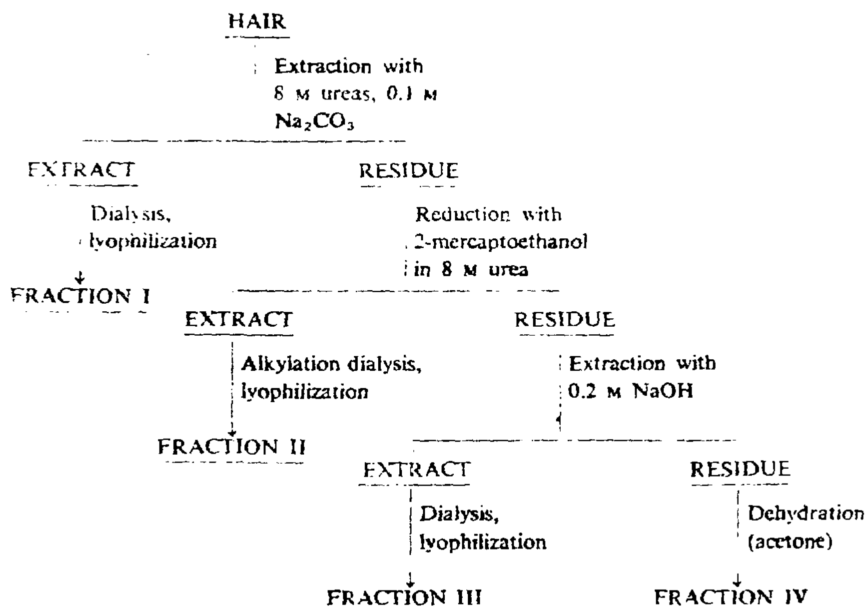
2.2. Preparation of Tritiated Hair

Female Wistar albino rats, 200 g of weight, were used. The animals were kept on a standard diet consisting of granulated food MURIGRAN, produced by BIOWET (Gorzów Wkp, Poland), supplemented with milk, carrots and water ad libitum. The synchronized anagen stage of the hair cycle was induced by plucking the back area of about 40 cm^2. Fifteen days later 20 mCi of tritiated water dissolved in saline containing 0.5 per cent of phenol was injected subcutaneously. The new anagen fleece was allowed to grow fully, and was plucked on the 22nd day after HTO injection. About 100 mg of tritiated hair was obtained from a single animal. The radioactivity of hair was about 0.0318 μCi/mg. The hair was washed with ethyl ether: ethanol (1:1), dried, pooled and used for further experiments.

2.3. Extraction and Preliminary Fractionation of Hair Proteins

Five-hundred mg portion of defatted hair was extracted (2 hr, 23°, constant stirring) with 80 ml of 8 M urea containing 0.1 M sodium carbonate. The extract was exhaustively dialysed against distilled water and freeze dried (Fraction I). The insoluble residue was treated (4 hr, 40°, constant stirring) with 80 ml of 8 M urea containing 0.1 sodium carbonate—HCl buffer, pH 8.7, and 0.5 M 2-mercaptoethanol. The solubilized proteins were alkylated in the following way: 7 g of iodoacetic acid were added to the extract and pH of the mixture was maintained at 8.0 with 5 M sodium hydroxide, until a cessation of proton release was observed. The mixture was then acidified to pH 4.0 with acetic acid, dialysed against distilled water and lyophilized (Fraction II). The residue was extracted (2 hr, 23°, constant stirring) with 0.2 M sodium hydroxide. The extract was dialysed against water and freeze dried (Fraction III). The insoluble residue was dried with acetone (Fraction IV). A summary of the procedure is given in scheme 1.

Diagram of preliminary fractionation of hair proteins



2.4. Protein Concentration

This was determined by the method of Lowry *et al.* [13].

2.5. Polyacrylamide Gel Electrophoresis

Protein samples were routinely analysed by disc electrophoresis at pH 8.6 [14]. Seven per cent running gels, occasionally containing 6 M urea or 0.5 per cent of sodium dodecyl sulphate were used. Gels were stained with Amido Black.

2.6. Determination of Tritium

Tritium radioactivity was measured by Schoeniger technique described by Kelly *et al.* [15] as modified by Bilkiewicz [16]. Measurement of tritium activity was performed in Spectrometer SL-30 (Intertechnique, France).

2.7. Molecular Weight Determination

An approximate molecular weight of the main protein of Fraction II was determined on Sephadex G-200 column. Diisopropoxy-trypsin, egg albumin, serum albumin and γ-globulin were used as standards, assuming the molecular weights of 24,000, 45,000, 69,000 and

16,0000 daltons, rspectively. Diisopropoxy-trypsin was prepared as follows: 10 mg of the enzyme was dissolved in 1.0 ml of a solution containing 0.9 ml of 0.16 M sodium phosphate buffer, pH 7.6 and 0.1 ml of 10 mM diisopropylfluorophosphate. The mixture was incubated for 10 min. and introduced into the column.

2.8. Amino Acid Analysis

Protein samples were hydrolysed in 6 M hydrochloric acid at 110° for 24 and 48 hr in sealed, evacuated tubes. Analyses were carried out using a Beckman model Unichrom automatic amino acid analyser. Halfcysteine was determined as a carboxymethyl derivative.

2.9. N-terminal Amino Acid Analysis

Determination of N-terminal amino acid residue was performed using the dansyl chloride method described by Fuller *at al.* [17].

2.10. Enzymatic and Acid Hydrolysis

Ten mg protein samples were digested for 15 hrs at 30° with a mixture of pancreatic elastases: I and II (125 μg each) in 15 ml of 0.08 M ammonium chloride buffer, pH 8.5. The lysates were then freeze dried and hydrolysed in 6 M HCl at 110° for 45 min. Hydrochloric acid was removed *in vacuo*.

2.11. Paper Chromatography

Two dimensional chromatography on Whatman No 2 sheets was employed [18]. N-butanol: acetic acid: water (12:3:5), and phenol:ammonia : water (160:40:1) were used as solvents. Amino acid spots were developed with a ninhydrin reagent.

3. Results

3.1. Extraction and Fractionation of Hair Proteins

The method described in the previous section, was used for the extraction and fractionation of hair proteins of the animals treated with HTO and of the control ones. The protein recovery in the four protein fractions isolated is given in Table 1. Tritium radioactivity was determined in the consecutive extracts (Fig. 1) before and after dialysis, and in freeze dried preparations of fractions I, II and III, as well as in the insoluble residue (IV). A considerable part of the activity in the extracts appeared dialysable and volatile and only small amount of tritium could be recovered in the final preparations. The results are col-

Table 1. Recovery of hair proteins during the extraction and preliminary fractionation

Material	Protein	
	(mg)	(%)
Starting hair	500*)	100
Fraction I	57	11.4
II	210	41.6
III	50	10.0
IV	170	34.0
Total recovery	487	97.0

*) Dry weight

Table 2. Recovery of tritium radioactivity during the course of extraction and preliminary fractionation of hair proteins

Material	Tritium radioactivity			
	Before dialysis %	After dialysis %	After lyophilization %	Specific radioactivity (μCi × 10⁻³/ /mg)
Starting hair*)	100.0	—	—	—
Fraction I	11.8	3.7	0.7	0.1
II	68.0	30.8	12.6	1.0
III	5.5	2.7	1.0	0.3
IV	11.5	—	—	0.7
Total recovery	96.8	37.2	14.3	

*) 0.0318 μCi/mg

lected in Table 2. The highest proportion of the total hair radiosensitivity and the highest specific activity was found in fraction II. It contained 66 per cent of hair proteins solubilized under the provided conditions. Therefore, further fractionation was limited to this fraction only. The elution pattern of this fraction from Sephadex G-100 gel column (Figure 1) shows seven peaks. The elements were pooled as indicated in the figure, dialysed against destilled water and freeze dried. Table 3 presents the recovery of protein and radioactivity in the pooled fractions. The highest total activity and the specific activity were detected in fraction (a).

Only negligible labelling of pooled protein fractions (*a* to *e*) was obtained when fraction II of untreated animals was incubated for 15 min at room temp. with 1 μCi of HTO (Table 3) 48 per cent of total radioactivity applied for *in vitro* labelling emerged from the column after the last protein fraction, presumably as unchanged HTO.

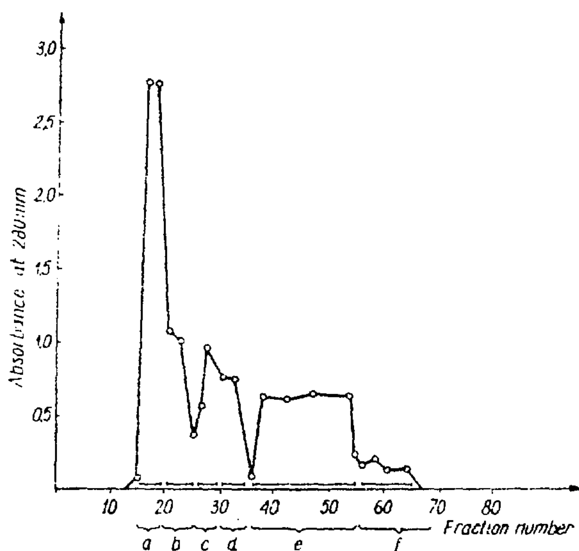


Fig. 1. Gel filtration of hair protein Fraction II on Sephadex G-100 column, The column (3 × 57 cm) was equilibrated and developed (at room temp.) with 0.05 M sodium carbonate buffer of pH 8.7, containing urea 3 M. The sample of Fraction II (80 mg) was passed in a downward flow of 30 ml/hr. Four ml fractions were collected. The effluents, were pooled into 6 fractions labelled *a* to *f*

Table 3. Recovery of proteins and tritium radioactivity in the pooled element fractions obtained during the Sephadex G-100 chromatography of fraction II, labelled *in vivo* and *in vitro*

Material	Protein %	*In vivo* Radioactivity %	*In vivo* Radioactivity μCi × 10⁻³/mg	*in vitro* Radioactivity %
Starting Fraction II*)	100.0	100.0	1.4	
Fraction II pool *a*	27.6	48.6	2.3	0.26
b	12.9	10.7	0.7	0.04
c	7.8	7.7	1.2	0.14
d	8.8	11.2	1.8	0.024
e	37.9	14.8	0.5	1.2
f	4.4	2.3	0.2	14.0
				48.0
Total recovered		99.4	95.3	64

*) Total activity 0.1137 uCi

The protein present in fraction (*a*) appeared homogenous in disc electrophoresis without and in the presence of 0.5 per cent of sodium dodecyl sulphate or 6 M urea (Fig. 2). Molecular sieve chromatography on Sephadex G-200 column indicated that the apparent

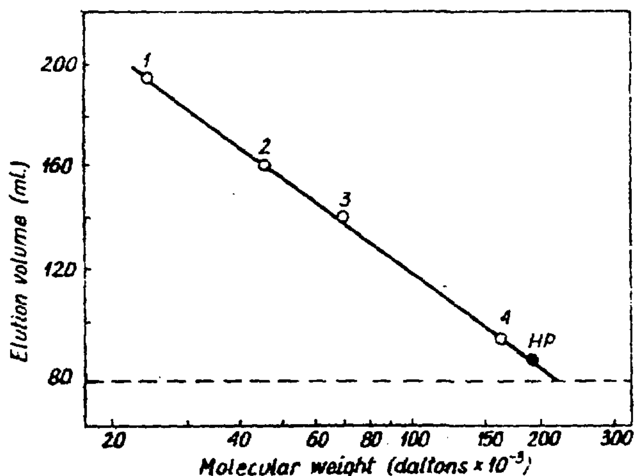


Fig. 2. Determination of the apparent molecular weight of the tritiated rat hair protein (fraction *a*). The Sephadex G-200 column (2×76 cm) was equilibrated and eluted at 22°C, with 0.2 M sodium phosphate buffer of pH 7.6, containing 0.03 per cent of sodium azide. A downward flow rate of 16 ml/hr was employed. Standard proteins (10 mg each, 1 ml): 1, diisopropoxy-trypsin; 2, egg albumin; 3, serum albumin; 4, γ-globulin; HP—hair protein. Broken line represents the void volume (Blue Dextran)

molecular weight of this protein was about 190,000 daltons (Fig. 3). Its amino acid composition is given in Table 4. The protein showed a high content of glutamic acid, cysteine, aspartic acid and serine and low content of methionine and histidine. The only N-terminal amino acid was valine.

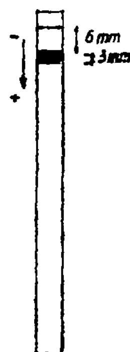


Fig. 3. Disc electrophoresis of the protein of fraction (*a*) eluted from the Sephadex G-100 column. The protein was separated using 7 per cent Polyacrylamide gel, buffer Tris-glycine, pH 8.7, 4 mA per tube 230 V, 1.5 hr

To determine the extent of tritium incorporation into each amino acid residue the protein was degraded by a mixture of porcine pancreatic elastases followed by a short acid hydrolysis. The obtained hydrolysate was subjected to two-dimensional paper chromatography. Ninhidrin spots of individual amino acids were cut out and tested for tritium by using combustion and liquid scintillation technique. It was found the distribution of tritium among amino acids was uneven (Table 5). The relative content of tritium ranged from 0.82–0.25 (Phenylalanine, isoleucine, valine) to 0.20–0.10 (Methionine, proline, tyrosine, lysine, leucine) and to 0 (all other amino acids).

Table 4. Amino acid composition of the most radioactive soluble proteins isolated from rat hair (Fraction)

Amino acid residue	μmoles/100 μmoles of amino acids recovered[a]	No. of residues per 190,000 daltons	Nearest integer per 190,000 daltons
Lysine	2.60	45.71	46
Histidine	1.10	19.34	19
Arginine	5.90	103.73	104
Aspartic acid[b]	9.10	159.99	160
Threonine	4.88	85.79	86
Serine	9.00	158.23	158
Glutamic acid[b]	15.60	274.26	274
Proline	6.22	109.35	109
Glycine[c]	8.15	143.28	143
Alanine	6.80	119.55	120
Cysteine[c]	10.50	184.60	185
Valine[d]	4.05	71.20	71
Methionine	0.85	14.94	15
Isoleucine[d]	2.60	45.71	46
Leucine	7.65	134.49	134
Tyrosine	2.78	48.87	49
Phenylalanine	2.10	36.92	37

Amino acid composition was determined automatically; the protein obtained from untreated animals was used.
a) 24 hr hydrolysis; b) free amide; c) as carboxymethyl derivative; d) 48 hr hydrolysis.

When hydrolysate of the same protein obtained from untreated animals was labelled *in vitro* (10 mg, 0.1 mCi, room temperature) the incorporation of tritium into amino acids was negligible and uniform.

4. Discussion

A procedure for extraction and fractionation of hair proteins, suitable for tritiated material has been developed. Three soluble protein fractions could be obtained by this procedure from tritiated rat hair. The fraction containing the highest total and specific radioactivity (Fraction II) could be rendered soluble after reduction of S-S-bridges with 2-mercaptoethanol. Therefore, the proteins of this fraction could be regarded as keratcins [19]. From this fraction the most radioactive high molecular protein was isolated in a pure form. Amino acid composition of this protein was similar to that of kerateins, although the protein could not be identified with any well characterized protein of hair. The presence of only one N-terminal amino acid residue indicates one polypeptide chain or a number of similar subunits. Over 50 per cent of the radioactivity of this protein was found in indispensable amino acids. Half of this value was connected with phenylalanine. Forty per cent

Table 5. Distribution of tritium radioactivity among amino acid residues of the most radioactive rat hair protein (Fraction(a))

Amino acid residue	Tritium radioactivity (% of radioactivity recovered)	Relative radioactivity per one acid amino residue
Lysine	5.55	0.12
Histidine	trace	—
Arginine	0.00	—
Aspartic acid	trace	—
Threonine	0.00	—
Serine	0.00	—
Glutamic acid	trace	—
Proline	10.48	0.10
Glycine	0.00	—
Alanine	0.00	—
Cysteine	1.10	0.01
Valine	17.61	0.25
Methionine	3.14	0.21
Isoleucine	17.19	0.37
Leucine	4.82	0.04
Tyrosine	8.43	0.17
Phenylalanine	30.30	0.82

Tritium radioactivity was determined in the active protein, degraded by enzymatic digestion followed by acid hydrolysis. The amino acids were separated by two dimensional paper chromatography.

of the radioactivity was fixed to dispensable amino acids with a considerable contribution of another aromatic amino acid residue: tyrosine.

When an animal is injected with tritiated water almost each compound of the body can be labelled with tritium. Most of the tritium atoms replaced with easily exchangeable hydrogen are labile. However, some of tritium atoms owing to an exchange with slowly exchangeable hydrogen and due to metabolic processes, become firmly bound as a stable label. The content of stable labelled compounds increases with time after an exposure to tritium. The sufficient labelling of hair protein in this experiment was obtained after 22 days of hair growth.

The results showed that the hair proteins were labelled to a different extent and that in a single, isolated protein the radioactivity of each amino acid was also different. Since under the condition of applied isolation procedure the labile tritium was removed (21) and the established radioactivity can be regarded (22) as derived from firmly bound tritium atoms, our results indicate the specificity of the *in vivo* incorporation of tritium into components of biological interest (proteins). However, the mechanism od this incorporation, kinetics and stability of the label in the living organism, remain unknown. The fact, that some of amino acids not contained tritium lead us to assume that the synthesized protein was labelled mostly during its biosynthesis, not afterwards. This assumption was confirmed

by negligible labelling of an isolated protein exposed to tritiated water *in vitro*. Experiments *in vitro* showed also that amino acids in hydrolysates exposed to HTO were labelled uniformly, though to a very small extent. In vivo, the nonuniform labelling of amino acids utilized for keratin biosynthesis indicates possible selective metabolism of tritium. This indication could be of interest for studies of tritium metabolism and of its biological effects. It is tempting to speculate that tritium label of essential amino acids was introduced mostly by exchange, while to labelling of non essential amino acids, exchange and/or incorporation of tritium in metabolic processes may contribute.

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INKORPORACJA TRYTU DO BIAŁEK WŁOSA SZCZURA

Streszczenie

Opracowano prosty i względnie szybki sposób ekstrakcji i frakcjonowania białek włosa. Opracowaną metodę zastosowano do rozdziału białek z włosów szczurów, skażanych wodą trytową. Najbardziej aktywne białko, zawierające ponad 6% wyjściowej aktywności włosów, zostało wydzielone w postaci jednorodnej. Posiada ono masę cząsteczkową około 190000 daltonów i odznacza się wysoką zawartością reszt kwasu glutaminowego, cysteiny, kwasu asparaginowego, seryny i glicyny, jak również niską zawartością reszt metioniny i histydyny.

Ponad 80% aktywności całkowitej trytu włączonej do badanego białka zawarte jest w dwóch aminokwasach egzogennych: fenyloalaninie (30,3%) i izoleucynie (17,2%) oraz trzech aminokwasach endogennych: walinie, prolinie, tyrozynie (17,6%; 10,5%; 8,4% aktywności odpowiednio). Najwyższą aktywność właściwą wykazano w resztach fenyloalaniny, izoleucyny, waliny i metioniny.

Aktywność aminokwasów wynika z obecności trytu związanego trwale.

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ДЕПОЗИЦИЯ ТРИТИЯ В БЕЛКАХ ВОЛОС КРЫС

Резюме

Разработано простой и сравнительно быстрый метод выделения и разделения белков волос. Этот метод применено для разделения белков волос крыс, занечищенных окисью трития.

Наиболее активный белок, содержающий больше чем 6% первоначальней активности волос, был выделен в гомогенном виде. Имеет он молекулярный вес около 190 000 дальтонов и отличается высокой емкостью остатков глютаминовой кислоты, цистеина, аспарагиновой кислоты, серина и глицина, но низкой емкостью по отношению к остаткам метионина и хистидина.

Больше чем 80% общей активности окиси трития введеной в исследованный белок находится в двух эгзогенных аминокислотах: феньлоаланине (30,3) и изолейцине (17,2%) и трех эндогенных аминокислотах: валине, пролине и тирозине (17,6%, 10,5% и 8,4% активности соответственно). Самую большую специфическую активность обнаружено в остатках феньлаланина, изолейцина, валина и метионина.

Обнаружено также активность аминокислот вследствие присутствия сильно связанного трития.