Table 2. *Resistance of enzyme to acid and alkali*

Treatment*	Activity remaining (% of initial activity)
0.1N-acetic acid	96
0.2N-acetic acid	67
0.4N-acetic acid	0
0.01N-H ₂ SO ₄	41
0.1N-H ₂ SO ₄	0
0.01N-NaOH	97
0.02N-NaOH	33
0.04N-NaOH	0

* 1% enzyme solution kept for 1 hr. at room temperature with acid or alkali, then neutralized. Destruction expressed as percentage of activity of untreated control.

the substrate concentration is reduced the rate of hydrolysis decreases; with 0.001 M-acetylcholine the velocity of the reaction is only one-quarter of the maximum rate (Table 3).

Table 3. *Enzymic hydrolysis at various concentrations of acetylcholine*

Molarity of acetylcholine	μ l. CO ₂ evolved in 10 min.
0.056	162
0.042	155
0.028	158
0.016	142
0.008	131
0.004	107
0.002	69
0.001	42

Specificity. The purified enzyme does not act exclusively on acetylcholine but to a lesser degree hydrolyzes non-choline esters as well. A common esterase, present in horse serum and mainly responsible for the hydrolysis of tributyrin and methyl butyrate, is removed in the early stages of purification. However, as the purification proceeds, the

rate of hydrolysis of tributyrin, as compared with that of acetylcholine, remains constant. As the purest preparations so far obtained still retain the power to hydrolyze tributyrin at high velocity (30% of that for acetylcholine) it would seem justified to assume that the same enzyme catalyzes both reactions (Table 4).

Table 4. *Hydrolysis of tributyrin by enzyme preparations at various stages of purification*

Enzyme preparation		Hydrolytic activity towards tributyrin* (% of activity towards acetylcholine)
Q_{ACh}	Degree of purification	
50	—	139
1,600	32-fold	31
10,000	200-fold	30
300,000	6000-fold	30

* Substrate concentration 0.03M. Because of the low solubility of the substrate it was placed in the main compartment of the Warburg vessel, while the side-bulb contained the enzyme.

From the facts discussed above it is evident that the enzyme should be classified as a pseudo-cholinesterase (Mendel & Rudney, 1943), since it displays its maximum activity at high substrate concentrations and does not hydrolyze exclusively esters of choline.

SUMMARY

A method for the purification of pseudo-cholinesterase from horse serum is described and some of the properties of the purified preparation are discussed.

The author wishes to thank Dr B. Mendel, at whose suggestion this work was undertaken, for his continued interest in the progress of this investigation.

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The Analysis of Hair Keratin

2. THE DICARBOXYLIC AND BASIC AMINO-ACIDS OF HUMAN HAIR

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Data concerning the composition of many keratins is still very incomplete, so a study of human-hair hydrolysates was undertaken. A more thorough examination of such hydrolysates would give in-

formation of theoretical importance to those interested in protein constitution and might possibly be of practical value in revealing new sources for some of the amino-acids. The fractionations described

herein were done during 1938–40, but not until now has it been possible to prepare the data for publication.

The study has been confined, as far as possible, to the use of characteristic crystalline derivatives suitable for quantitative isolations, since we were also interested in obtaining quantities of amino-acids for use in other studies. The importance of the order of separation of the amino-acids and its influence upon the success of the isolations appears not to be realized by some workers. The practical difficulties involved, due to mutual solubility phenomena and co-precipitation, sometimes seem to have been overlooked by those who attempt quantitative separations of the amino-acids. Procedures which prove suitable for one type of hydrolysate (say of zein) may prove quite inadequate for the study of another (say of gelatin or of human hair) because of the marked differences in the ratios of the amino-acids present. It is unwise, therefore, to attempt to apply isolation procedures (the details of which may have been very carefully worked out for one particular type of hydrolysate, but for one only) to a new mixture of unknown nature, without careful preliminary checking of the applicability of the method to the new situation. In spite of enthusiastic reports concerning specificity made for certain precipitants of amino-acids, very few (if any) of these reagents possess the highly desirable characteristics claimed for them, although in certain mixtures the interference caused by other amino-acids may be negligible. When interference is encountered, a suitable order of separation may minimize the difficulties.

The present study, therefore, resolved itself into two phases: (1) determining the most suitable reagents for and (2) the best order of separation of the amino-acids in the hair hydrolysate. Preliminary removal of cystine, for example, was found to be essential in order to attain any semblance of accuracy in the determination of the dicarboxylic acids, and it simplified the separation of the basic amino-acids. Development of a method for the complete removal of cystine without the introduction of undesirable inorganic matter (Lucas & Beveridge, 1940) greatly facilitated subsequent procedures (see Flow Sheet 1). Suitable concentration of the filtrate (freed from copper with H_2S) resulted in the crystallization of a large proportion of the tyrosine. The removal of the bulk of this amino-acid aided the purification of the dicarboxylic acids. Attempts to separate the basic amino-acids from the cystine-free hair hydrolysate (now low in tyrosine) by the silver-baryta procedure (Vickery & Leavenworth, 1928b) before the removal of the dicarboxylic acids involved heavy co-precipitation of the latter, thus complicating the determination of all the amino-acids concerned. After a number of variations in

the order of separation had been investigated, the scheme here reported was found to give maximal results. Arginine was removed as flavianate, the dicarboxylic acids were precipitated with baryta and ethanol, histidine was taken out with silver and baryta, and lysine was separated as the phosphotungstate (see Flow Sheet 2).

Arginine accounts for about 10 % of human-hair keratin. The total crude flavianate obtained by direct isolation was equivalent to 10.7 % arginine. In spite of a fairly satisfactory decomposition temperature (260–261° for the main crops; other crops 255–257 and 242–244°) the combined flavianates were treated to remove impurities. The purified monoflavianate finally obtained corresponded to only 8.86 % arginine, but several impure flavianate fractions, from which no more pure arginine flavianate was isolated, contained further small amounts of the amino-acid—less than 1 % of the keratin as indicated by colorimetric assay.

The quantities of histidine (0.63 %) and lysine (2.40 %) isolated in the present study are in good agreement with the findings of others (Vickery & Leavenworth, 1929; Block, Bolling, Brand & Schein, 1939) and require no further comment.

The dicarboxylic acids were precipitated by Dakin's (1920) modification of Foreman's method (1914) after removal of cystine, tyrosine and arginine, and, of course, after the removal of the excess flavianic acid with baryta. It was found that under these conditions more than one reprecipitation of the barium salts was unnecessary, since very little improvement in the fraction was effected. After exact removal of barium, glutamic acid was separated as hydrochloride in the conventional manner and aspartic acid as the copper salt.

The amount of glutamic acid isolated in this study (10.6 %) is significantly greater than that (8 %) reported by Buchtala (1913); no other estimation of this amino-acid in human hair was discovered in the literature. The figure given here for aspartic acid (3.5 %) is apparently the first report on the occurrence of this amino-acid in human hair.

After the above procedures for the removal of glutamic and aspartic acids from the dicarboxylic acid fraction had been repeated, an amorphous substance (mixture?) was isolated from the mother liquors in a yield of 1 %. Many of the properties of this fraction agreed with those of ' β -hydroxyglutamic acid', a substance whose existence in proteins has been the subject of prolonged controversy. It was soluble in glacial acetic acid and the N content of both the free compound and its silver salt was correct for hydroxyglutamic acid. The apparent absence of glutamic acid, as shown by failure of this fraction to form an insoluble zinc salt, made these data seem more significant. The material after

oxidation with chloramine-T gave a *p*-nitrophenyl-osazone (m.p. 297–299°). This is the accepted melting-point of malic semi-aldehyde *p*-nitrophenyl-osazone, which is the derivative usually used for identification of this oxidation product of β -hydroxyglutamic acid. However, the N content (24.5%) of the osazone obtained is too high for that of the expected product (21.75%) and is more in agreement with the *p*-nitrophenylosazone of glyoxal (25.6%). If serine had been present it would have given rise to the *p*-nitrophenylosazone of glyoxal following the above treatment. All of our fraction was used up before the papers of Nicolet & Shinn (1939) or of Van Slyke, Hiller, MacFadyen, Hastings & Klemperer (1940) appeared. Periodate oxidation would have given valuable information as to the nature of this product from human hair.

Nicolet & Shinn (1942) have cast some doubt on the occurrence of β -hydroxyglutamic acid in proteins. Although the methods used by Dakin (1918) and Gulland & Morris (1934) appeared to characterize the substances isolated by them and to constitute very strong evidence for the presence of this amino-acid in certain protein hydrolysates, Dakin himself (1941) has recently admitted that it seems impossible for his product to be a β -hydroxy- α -amino acid. The fact that the products isolated by Gulland & Morris (1934) and others give the colour reactions described by Dakin (1918) and Harington & Randall (1931), coupled with the analytical data on the free acid, silver salt and particularly on the derived *p*-nitrophenylosazone, adds to the confusion in this field.

EXPERIMENTAL

General. Certain general precautions have been followed throughout the work. All evaporations were done *in vacuo*, unless otherwise stated. All inorganic precipitates (CuS, BaSO₄, AgCl, etc.) were washed repeatedly until the ninhydrin test on the final filtrate was negative or very faint. Usually at least four washings were necessary. These were done by rubbing the precipitate with water (or 0.01N-HCl) to a smooth paste, which was then diluted to a volume 15–40 times the bulk of the precipitate; the mixture was thoroughly stirred and usually digested at 80–90° for at least 15 min. Filtrations were done on Büchner funnels, with suction; a thin layer of Celite Analytical Filter-Aid on the paper proved useful on occasion to improve clarification and hasten the filtration when removing inorganic precipitates. Such precipitates were analyzed for adsorbed N in order to keep as complete a record as possible of the N partition during the fractionation of the hydrolysate. All yields of amino-acids isolated are corrected for the amounts removed for analytical control.

After considerable experience with four different hydrolysates, portions of which were fractionated by the usual standard methods with variations in the order of separation, the scheme presented here was finally evolved and applied to two hydrolysates referred to as H5 and H6. Details of the preliminary studies will not be presented.

Hydrolysate H5 was derived from 328 g. of carefully cleaned hair (moisture, 8.06%—constant weight over P₂O₅ *in vacuo*, at room temp.; ash, 0.50%) equivalent to 300 g. keratin containing N, 16.2%; S, 5.00%. Hydrolysate H6 was made from 111 g. of hair (moisture, 9.48%; ash, 0.25%) equivalent to 100.3 g. of keratin with N, 15.8%; S, 5.05%. N was determined by weighing about 150 mg. air-dried hair into a small Kjeldahl flask. About 10 mg. Cu wire and 10 ml. conc. H₂SO₄ were added and a preliminary digestion was carried out for about 2 hr. The resulting solution was made to 50.0 ml. in a volumetric flask and 2.00 ml. portions digested with 1 ml. conc. H₂SO₄ in the presence of CuSO₄·SeO₃ catalyst (2:1). When the solution was almost clear (1–2 hr.), 2 drops of 30% H₂O₂ (perhydrol, C.P.) were added, with more later, if necessary. Heating was then continued for 0.5–1 hr. after clearing. Duplicate determinations on diluted samples from two preparations of hair gave quadruplicate values in good agreement. Sulphur was determined by the method of Trotman & Bell (1926) with the use of Robison's reagent (1922) in place of the Benedict-Denis mixture.

In the case of H5 the hydrolysate was made to a volume of 1200 ml.; 1.00 ml. was removed (and diluted to 50.0 ml.) for N and cystine determinations; all of the remainder was used for the analysis. Hydrolysate H6 was made to 500 ml.; 450 ml. were used for the fractionations described.

Hydrolysis of hair and removal of cystine from hydrolysate. Human hair (from a men's barber shop) was thoroughly cleaned, hydrolyzed with 8–9N-HCl for 11 hr., freed from excess HCl and melanin and treated with Cu₂O to remove cystine, as already described in detail (Lucas & Beveridge, (1940). The main filtrate (F1), containing the remaining amino-acids, was freed from Cu with H₂S, and to this was later added the mother-liquor (F2), also freed from Cu, from the reprecipitation of the cysteine as cuprous mercaptide (see Flow Sheet 1).

The crude mercaptide was washed with water and with 0.1N-H₂SO₄ as described in the previous paper; these extracts (AW1) were freed from Cu⁺ and SO₄²⁻ and added to the main solution. Because the work on H5 had shown that much arginine remained with the cysteine even in the reprecipitated mercaptide, a slightly greater volume of water was used for the reprecipitation in the analysis of H6, and the resulting mercaptide was not only washed more thoroughly with water but was also washed with acid (450 ml. 0.1N-H₂SO₄/100 g. original keratin). In spite of this, some arginine (about 500 mg./100 g. keratin) still remained in the cysteine fraction, as shown by subsequent isolation. The washings (AW2) from the reprecipitated mercaptide, after removal of Cu⁺ and SO₄²⁻, were added to the main amino-acid solution.

Cysteine was isolated as hydrochloride, as previously described, the yield (calculated as cystine) from H6 being 13.7% (Table 1). Crop III, which was grossly impure, was taken up in water, made to 50 ml., and 5 ml. of this were used for nitrogen, sulphur and colorimetric arginine determinations; the remainder was fractionated by oxidizing the cysteine, which was then removed and weighed as cystine; arginine was removed as monofluoranate. During an examination of the final filtrate of this fraction, methionine was unexpectedly found to be present. The separations which led to this discovery were effected as follows.

To the remaining 45 ml. were added 20 ml. 6N-HCl, the solution was cooled to –5° and oxidized by dropwise addition of 1N-iodine in ethanol until the yellow colour

FLOW SHEET 1 **HUMAN HAIR HYDROLYSATE**

FLOW SHEET 2

Table 1. *Crystalline cysteine hydrochloride isolated from human-hair hydrolysate (Sample H 6)*

The sample used contained 90.3 g. keratin.

Crop	Weight (g.)	N content (%) [*]	Percentage yield (calculated as cystine)
I	10.86	9.10	9.2
II	1.73	8.93	1.5
III Crude	7.55†	9.63	
Purified	2.70†	11.38	3.0
		Total	13.7

^{*} Theoretical value for cysteine hydrochloride = 8.89; for cystine = 11.65.

† Isolated and weighed as cystine.

persisted (actual volume of 0.97*N*-iodine used = 27.5 ml.; corrected for the sample removed, this is equivalent to 3.56 g. cystine in crop III). The solution was concentrated *in vacuo* to remove ethanol and excess HCl and HI, taken up in 15 ml. water and then almost neutralized by slow addition of cold saturated Ba(OH)₂ to pH 4.8–5.0. After standing for some hours, the cystine was filtered off (corrected wt. = 2.70 g.). Barium was removed from the filtrate with H₂SO₄, the solution made up to 50 ml. and treated with flavianic acid. The small crop of arginine flavianate, after recrystallization, gave typical glistening crystals (corrected wt. = 1.31 g. corresponding to 0.52% of the keratin). The filtrate from the arginine flavianate, freed of flavianic acid and inorganic ions, contained 210 mg. N (corrected for samples removed).

The solution was made alkaline to thymophthalein with Ba(OH)₂, warmed to drive off ammonia and 2 vol. of 95% (v/v) ethanol added. After standing for 3 hr. in the refrigerator, the alcohol-insoluble barium salts were centrifuged down and washed once with 66% (v/v) ethanol. The barium salts redissolved in water with difficulty; barium was taken out exactly with sulphuric acid and the solution was evaporated to dryness (wt. residue = 493 mg., corrected). When redissolved in water and made up to 50 ml. it was found to contain N = 56 mg., arginine = trace only, and cystine (colorimetrically, Shinohara, 1936) = 217 mg. The nitrogen content of this residue (11.35%) was very close to the theoretical value for cystine, but the colorimetric estimation indicated that only about one-half of the fraction consisted of this amino-acid. No further compounds were isolated from this fraction.

The filtrate, consisting of the barium salts soluble in ethanol, was evaporated to drive off the ethanol, freed from barium with sulphuric acid, and made up to 100 ml. (N = 81.5 mg., corrected). The solution was evaporated to dryness (wt. = 510 mg. corrected) and redissolved in exactly 50 ml. water. Colorimetrically it was found to contain 78 mg. cystine and 18 mg. arginine.

It was noticed at this point that the solution possessed a cabbage-like odour reminiscent of methionine solutions. A qualitative test for methionine (Sullivan & McCarthy, 1940) was found to be positive. This was a most unexpected finding since this fraction was derived from a reprecipitated and acid-washed mercaptide. This result throws some suspicion upon the universal applicability of Cu₂O as a specific reagent for cystine-S in protein hydrolysates. It should be pointed out, however, that neither the procedure used by

Vickery & White (1933) nor that of Graff, Maculla & Graff (1937) was followed.

Kassell & Brand's (1938) modification of Baernstein's method (1932), applied directly to a sample of the carefully cleaned hair used in preparing H6, gave a figure of 0.98% for methionine by the volatile iodide method, and 0.80% by the thiolactone method. The total S, calculated from the methionine (0.98%) and cystine (15.1%) plus that evolved as H₂S, SO₂, etc. in Baernstein's method (1936) of determining S distribution, was 4.92%. This figure is in fair agreement with the total S value obtained directly on the hair keratin (5.05%). Methionine has not previously been shown to be present in human hair but it has been found in other keratins: wool, 0.2% (Mueller, 1923), 0.5% (Barritt, 1934), steer horn 0.02% (Abderhalden & Heyns, 1932).

Preliminary removal of tyrosine. The main solution of amino-acids (freed from Cu) was concentrated *in vacuo* to a volume of approximately 3 ml./g. of hair. The pH of this solution was ideal for direct crystallization of tyrosine. During the final stages of the evaporation a crop of perfectly formed tyrosine crystals separated, which were almost pure (N found = 7.6%, theoretical = 7.74%). On longer standing, or further concentration, a less pure crop of tyrosine containing some leucine usually crystallized out. The bulk, although not all, of the tyrosine was isolated at this stage (yield 2.1%). Sufficient was removed to reduce contamination of the dicarboxylic acid fraction to the vanishing point (negative Millon's test on the once reprecipitated barium salts).

Determination of arginine. As was noted in a previous paper (Lucas & Beveridge, 1940) a large part (about 30%) of the arginine is precipitated along with cystine by Cu₂O. Arginine was isolated as monoflavianate (Kossel & Gross, 1924) from a number of fractions derived from the original cuprous mercaptide precipitate of H5. No detailed account will be given of the methods used in the fractionation of H5, but the following data show the distribution of arginine at various stages in the fractionation:

Main filtrate from 1st mercaptide precipitate (F1)	= 7.77%
Acid washings of 1st mercaptide precipitate (AW1)	= 0.78%
Filtrate from 2nd mercaptide precipitate (F2)	= 1.83%
Mother liquor of cysteine hydrochloride	= 0.32%
Total	10.70%

Although all the main crops of crystals had satisfactory decomposition points (260–261°), the combined flavianates were purified by leaching with a large volume (about 35 ml./g. flavianate) of hot water for 2 hr. The solution was allowed to cool overnight. The weight of the main crop of monoflavianate corresponded to a yield of 8.86%. The filtrate was concentrated and several small crops of crystals were removed. They were progressively more impure and appeared to be mixtures of arginine flavianate with ammonium flavianate, traces of histidine flavianate, and unidentified material. The value of 10.7% is therefore too high for the arginine content of human-hair keratin, while 8.86% is obviously a minimal figure, possibly as much as 1% low.

About the time that fractionation of H6 was begun, Vickery (1940) published his diflavianate procedure for the determination of arginine and it was decided to utilize this hydrolysate for a comparison of the mono- and diflavianate procedures.

To the main amino-acid solution (from which the bulk of the tyrosine had been crystallized) was added the mother liquor (F2) from the reprecipitation of the cuprous mercaptide and the acid washings (AW1 and AW2) of the two mercaptide precipitates. All had been freed from Cu^{++} and SO_4^{--} . The concentration of the mixture was adjusted to be about 20% with respect to the original amount of keratin used. Four 100 ml. portions were diluted to 150 ml. Two portions were treated by the original monoflavanate procedure and two by the new diflavanate method. The following results were obtained:

	Monoflavanate procedure		Diflavanate procedure	
	Yield (%)	Temp. of decomposition	Yield (%)	Temp. of decomposition
(1)	7.85	261–262°	7.85	261–263°
(2)	7.83	261–263°	7.68	261–262°

The recorded temperature of decomposition of arginine is 258–260°.

These determinations indicate that the two procedures are equally effective on this type of amino-acid mixture. However, in view of the greater speed with which the monoflavanate procedure may be carried out, it appears preferable. The monoflavanate procedure has another advantage, if one proposes to do further work on the filtrate, since the removal of excessive amounts of flavianic acid is avoided.

From the combined mother liquors of the four determinations, a further small quantity of arginine diflavanate was obtained, which on recrystallization corresponded to 0.20% arginine in the keratin. In addition to the arginine isolated from the main amino-acid solution, 0.52% was obtained from the mother liquor of the cysteine hydrochloride. The total yield was thus 8.57%. The discrepancy between our figures (8.86, 8.57%) and that of Vickery (9.4%) may possibly be due to losses by adsorption of the arginine on Cu_2S and CuS in the separation of cystine, for, in spite of thorough washing, this precipitate retained considerable N (about 5–6 mg./g.).

Precipitation of the dicarboxylic acids. The excess flavianic acid was removed by acidifying with 10N- H_2SO_4 (0.15 ml./g. hair) and extracting repeatedly with butanol. The extracts were combined and shaken several times with very dilute H_2SO_4 to recover any amino-acids which might have been removed. These were returned to the main solution. Slightly more $\text{Ba}(\text{OH})_2$ was added than would be required to precipitate the added H_2SO_4 . The precipitate (BaSO_4 and traces of Ba flavianate) was filtered off and washed repeatedly by suspension in boiling water, which was allowed to cool before filtration with suction. This was done to minimize losses, particularly of aspartic acid. The combined filtrates were made strongly alkaline to thymolphthalein with $\text{Ba}(\text{OH})_2$, the mixture was concentrated *in vacuo* to remove NH_3 , diluted to give a 15–20% concentration of (remaining) amino-acids and treated with 3 vol. of ethanol. By addition of the ethanol dropwise, with vigorous mechanical stirring, over a period of several hours, the precipitates obtained were usually granular. The gummy precipitates described by others and at first obtained by us appear to be due to too hasty addition of the ethanol; they are more difficult to work with than are the granular ones and carry down more extraneous N.

After two washings with 80% (v/v) ethanol, the crude dicarboxylates were redissolved in water—five small portions, each about double the bulk of the precipitate, being used. The insoluble residue was set aside. The solution of barium salts was taken down *in vacuo* to about two-thirds of the volume used for the first precipitation with ethanol, and 2 vol. of 95% (v/v) ethanol were added slowly, with vigorous stirring. The product, which at this stage was always granular, was washed thrice with 80% (v/v) ethanol. Unless arginine and tyrosine had been removed previous to this step, the product of the first reprecipitation was always contaminated with significant quantities of these amino-acids. Under the conditions used, one reprecipitation sufficed to give a fairly satisfactory dicarboxylic acid fraction.

The filtrates from these precipitates were always acidified at once with H_2SO_4 to minimize racemization of the remaining amino-acids.

Separation of glutamic acid. The reprecipitated barium dicarboxylates and the water-insoluble residue mentioned above were dissolved in dilute HCl and freed from Ba with H_2SO_4 . The solution was evaporated *in vacuo* to a small volume (1 ml./g. hair) and while being cooled to -10° was saturated with dry HCl gas. After several days' standing at 0° the mixture, which always became very dark in colour, was filtered on a fritted glass funnel. The residue was washed twice with cold concentrated HCl and finally with cold, saturated ethanol-HCl. The product was dried to constant weight *in vacuo* over NaOH and conc. H_2SO_4 . By concentration of the mother liquor a further very small crop of glutamic acid hydrochloride was usually obtained. The total yield was equivalent to 10.6% glutamic acid in human-hair keratin. (Found: N, 7.62. Calc. for $\text{C}_5\text{H}_9\text{O}_4\text{N} \cdot \text{HCl}$: N, 7.64%.)

Only a very small amount of glutamic acid escaped precipitation at this stage. Even after removal of aspartic acid and resaturation of the mother liquor with HCl no appreciable amount of glutamic acid hydrochloride was obtained. However, during the course of attempts to isolate hydroxyglutamic acid, a small residue insoluble in glacial acetic acid (see below) proved to be glutamic acid. The amount obtained accounted for less than 0.1% of the protein.

Separation of aspartic acid. The filtrate from the glutamic acid hydrochloride was freed of inorganic ions, and aspartic acid was precipitated with freshly prepared and thoroughly washed copper hydroxide in the usual way (Ritthausen, 1869; Foreman, 1914). In different preparations the water of crystallization varied from 2 to 4.5 moles, as other workers have found (cf. Jones & Moeller, 1928). A value of 3.50% aspartic acid in human-hair keratin was obtained (calculated from the N content of the Cu salt). The product was dried *in vacuo* at room temperature, over P_2O_5 , for analysis. (Found: N, 5.79; Cu, 26.9%. Calc. for $\text{C}_4\text{H}_5\text{O}_6\text{NCu} \cdot 2\text{H}_2\text{O}$: N, 6.08; Cu, 27.6%.)

Hydroxyglutamic acid (?) Considerable N remained in the mother liquor from the copper aspartate, but no more than traces of glutamic acid hydrochloride could be obtained, nor could more aspartic acid be isolated. The fraction, freed of inorganic ions, gave a yellowish green fluorescence when treated with β -naphthol in concentrated H_2SO_4 , a reaction described by Dakin (1918) and confirmed by Harington & Randall (1931) as one given by β -hydroxyglutamic acid. The isolation of this substance was therefore attempted.

The fraction was evaporated to dryness *in vacuo* and extracted with glacial acetic acid. The resulting solution was taken to dryness and the residue submitted to the same procedure, these procedures being repeated until the material was completely soluble in glacial acetic acid. The insoluble residue, which was very small, did not give the fluorescence; its N content corresponded with that of glutamic acid.

The acetic acid solution was evaporated, and the residue taken up in water and converted to the silver salt, as described by Dakin (1918). A considerable amount of N was left unprecipitated, which indicated the presence of other amino-acids in this fraction. The insoluble silver salt was decomposed with H_2S ; the resulting solution gave the typical yellowish green fluorescence with β -naphthol and H_2SO_4 . On boiling with zinc carbonate no N was precipitated, apparently indicating the absence of glutamic acid. When the solution was freed of inorganic ions and allowed to evaporate slowly, an amorphous, hygroscopic residue was obtained, equivalent to 1% of the hair. When dried to constant weight *in vacuo* over P_2O_5 it contained 8.59% N; calc. for $C_5H_9O_4N$: 8.59%.

One portion was converted to the silver salt and analyzed:

	Content (%) of	
	Ag	N
Found	57.8	3.85
Calc. for $C_5H_9O_4NAg$	57.3	3.71
Calc. for $C_5H_9O_4NAg$	59.8	3.88

The Ag and N contents of the silver salts of glutamic and hydroxyglutamic acids are rather close to one another, but in view of the above indication as to the absence of the former, the figures assume added significance as evidence that the product is the latter compound.

Another portion (300 mg.) of the fraction was oxidized with chloramine-T, as described by Dakin (1918), and the product treated with *p*-nitrophenylhydrazine (740 mg.) on a boiling water-bath. Red needles (80 mg.) were filtered off, washed repeatedly with hot absolute ethanol and recrystallized from nitrobenzene. The product melted at 297–299°; accepted for *p*-nitrophenylosazone of malic semialdehyde 297–299°. However, analysis for N gave 24.5%, whereas the theoretical value is 21.6%. The N content is closer to that of the *p*-nitrophenylosazone of glyoxal, a product which could have arisen had any serine been present in the fraction. Had any appreciable amount of serine been present, the N content of the free amino-acid would have been considerably higher than was found. Unsuccessful attempts were made to form the carbobenzoxy derivative (Abderhalden & Murke, 1937), which has been recommended for the identification of β -hydroxyglutamic acid. The attempted formation of the diethyl ester hydrochloride likewise met with failure. All of the material isolated was expended in further unsuccessful attempts to prove unequivocally that it consisted of hydroxyglutamic

acid. Unfortunately none of the fraction remained to which the recently described characterization of Nicolet & Shinn (1942) could be applied.

Histidine. The filtrate from the barium dicarboxylates, acidified at once with H_2SO_4 , was set aside until it was convenient to proceed with it. After removal of $BaSO_4$, the histidine was precipitated as the silver salt at pH 7.4 and finally obtained as the difluorinate (Vickery & Leavenworth, 1928*b*) after a mercuric sulphate precipitation. Two crops were isolated corresponding to a total yield of 0.63% histidine. Decomposition point 243°; recorded value 250°.

Lysine. The filtrate from the silver histidine precipitate was freed from silver with H_2S , and to it was added the mother liquor from the mercuric sulphate precipitation from which the Hg had been removed. Lysine was precipitated with phosphotungstic acid in the usual way and isolated as the picrate (Vickery & Leavenworth, 1928*a*). The weight of the product corresponded to 2.4% of lysine in the keratin. Decomposition point, 243°; recorded value, 250°.

SUMMARY

1. The influence of the order of separation of the amino-acids from a human-hair hydrolysate upon their quantitative isolation has been investigated. A suitable order of separation of the dicarboxylic and dibasic amino-acids is reported.

2. Aspartic acid (3.5%) has been isolated for the first time from human hair.

3. Glutamic acid has been isolated from human hair in higher yields than previously reported.

4. The quantities of basic amino-acids isolated agree essentially with those reported in the literature.

5. Methionine has been found in human hair, and its amount determined as 1.0%.

6. The unexpected finding of methionine in a fraction derived from a carefully washed and reprecipitated cuprous mercaptide precipitate casts some doubt upon the universal applicability of the method of estimating cystine by the organic S content of this precipitate.*

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* After the present paper was written, the issue of the *Biochemical Journal* for September 1943, containing three papers by Chibnall and co-workers (Bailey, Chibnall, Rees & Williams, 1943; Chibnall, Rees & Williams, 1943) reached us. Several points mentioned in our paper are also reported by these investigators.

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The Analysis of Hair Keratin

3. ISOLATION OF PROLINE FROM HUMAN HAIR

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Previous communications from this laboratory have described the attempted quantitative isolation of the amino-acids of human-hair hydrolysates. To date, the separations of cystine (Lucas & Beveridge, 1940), and the dicarboxylic and basic amino-acids (Beveridge & Lucas, 1944) have been reported. This paper gives details of further treatment of the amino-acids remaining in the hydrolysate, which has led to the isolation of proline and has indicated the probable absence of hydroxyproline. No data concerning these amino-acids in human hair have as yet appeared in the literature.

In the preceding papers comment was made upon the importance of the order of separation of the amino-acids. Without going into the experimental details, it may be mentioned that four attempts to isolate proline quantitatively were made, with variations in the reagents used and in the stage of the analysis at which they were applied. Precipitation with ammonium reineckate (Käpfhammer & Eek, 1927), after removal of the cystine, the dicarboxylic acids and arginine, was quickly and easily carried out, but subsequent removal of the reineckate from the proline fraction and from the filtrate was a laborious and time-consuming procedure. The scheme described below gave excellent results and appeared more suitable for a systematic separation of the amino-acids such as was being attempted.

EXPERIMENTAL

The filtrate from the phosphotungstic acid precipitation of lysine (Lucas & Beveridge, 1940; Beveridge & Lucas, 1944) was freed from inorganic ions in the usual way. The mother

liquors from the purification of lysine, as picrate, were freed from picric acid and returned to the main solution. The resulting solution was therefore a human-hair hydrolysate from which melanin, cystine, arginine, tyrosine, ammonia, dicarboxylic acids, histidine and lysine had been removed, in that order, and in as quantitative a manner as possible.

The solution was concentrated *in vacuo* and allowed to stand in the room for some days, during which one large crop and several smaller ones of leucine crystallized and were filtered off. (Found: N, 10.67. Calc.: N, 10.69%.) These accounted for about 7% of the hair keratin. No attempt has been made as yet to distinguish between leucine, norleucine and isoleucine in the material isolated.

The materials removed from the hydrolysate up to this point account for 58% of the original weight of hair keratin, i.e. about one-half of the products of hydrolysis have been removed and identified at this stage. The N accounted for amounts to 66% (including 7% lost by adsorption on inorganic precipitates).

The remaining amino-acids were converted to copper salts by repeated evaporation with an excess of copper carbonate. (The commercial preparation of copper carbonate available here contained small amounts of soluble inorganic salts which were leached out with warm water before use.) The resulting copper salts were separated into water-insoluble (fraction A) and water-soluble salts (fraction B) as described by Town (1928), Brazier (1930) and Damodaran (1931).

Fraction A was relatively small, since most of the amino-acids which form water-insoluble copper salts had already been removed. It has not yet been examined.

The aqueous extract (fraction B) was evaporated, and salts were granulated and dried with acetone in the usual manner (Town, 1928). By repeated extraction with absolute methanol the copper salts more soluble in this solvent (fraction B-2) were separated from those insoluble