- Arnstein, H. R. V. & Keglević, D. (1956). *Biochem. J.* **62**, 199.
- Arnstein, H. R. V. & Neuberger, A. (1951). *Biochem. J.* **50**, 154.
- Arnstein, H. R. V. & Neuberger, A. (1953). *Biochem. J.* **55**, 271.
- Arnstein, H. R. V. & Stanković, V. (1956). *Biochem. J.* **62**, 190.
- Borsook, H. & Dubnoff, J. W. (1940). *J. biol. Chem.* **132**, 307.
- Bray, H. G., Clowes, R. C., Thorpe, W. V., White, K. & Wood, P. B. (1952). *Biochem. J.* **50**, 583.
- Bray, H. G., Neale, F. C. & Thorpe, W. V. (1946). *Biochem. J.* **40**, 134.
- Bray, H. G., Thorpe, W. V. & White, K. (1951). *Biochem. J.* **48**, 88.
- Christensen, H. N., Cooper, P. F. jun., Johnson, R. D. & Lynch, E. L. (1947). *J. biol. Chem.* **168**, 191.
- Christensen, H. N. & Lynch, E. L. (1946). *J. biol. Chem.* **166**, 87.
- Christensen, H. N., Riggs, T. R. & Ray, N. E. (1951). *Analyt. Chem.* **23**, 1521.
- Christensen, H. N., Streicher, J. A. & Elbinger, R. L. (1948). *J. biol. Chem.* **172**, 515.
- Cohen, P. P. & McGilvery, R. W. (1946). *J. biol. Chem.* **166**, 261.
- Donaldson, H. H. (1924). *The Rat. Data and Reference Tables*, 2nd ed. pp. 209-26. *Amer. anat. Mem.* no. 6.
- Griffith, W. H. (1929). *J. biol. Chem.* **82**, 415.
- Griffith, W. H. (1930). *J. biol. Chem.* **85**, 751.
- Hamilton, P. B. & Archibald, R. M. (1944). *Industr. Engng Chem. (Anal.)*, **16**, 136.
- Henriques, O. B., Henriques, S. B. & Neuberger, A. (1955). *Biochem. J.* **60**, 409.
- Hubbell, R. B., Mendel, L. B. & Wakeman, A. J. (1937). *J. Nutr.* **14**, 273.
- Krueger, R. (1950). *Helv. chim. acta*, **33**, 429.
- Reiner, J. M. (1953). *Arch. Biochem. Biophys.* **46**, 53.
- Schachter, D. & Taggart, J. V. (1953). *J. biol. Chem.* **203**, 925.
- Shemin, D. & Rittenberg, D. (1944). *J. biol. Chem.* **153**, 401.
- Sheppeck, M. & Griffith, W. H. (1936). *J. biol. Chem.* **114**, xcii.
- Vries, A. de & Alexander, B. (1948). *J. clin. Invest.* **27**, 665.
- White, A. (1941). *Yale J. Biol. Med.* **13**, 759.
- Winnick, T., Friedberg, F. & Greenberg, D. M. (1948). *J. biol. Chem.* **173**, 189.

The Isolation and Identification of Horse-Dandruff Allergen

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Although much work has been carried out on the characterization of the agents which elicit the symptoms of human hypersensitivity, a chemically pure allergen has not yet been isolated. This can be attributed to various difficulties encountered in fractionating the complex mixtures obtained by extraction of allergenic materials, as well as to the lack of accurate methods for assaying biological activity. Consequently, it is not known whether the activity of extracts is confined to a single constituent. Until this is established it is impossible to ascertain whether there is any underlying chemical structure peculiar to all types of allergen molecules.

Extracts of inhalant-type allergens, such as pollens, house dust and animal danders, have been found to contain multiple proteins, pigment and a wide range of substances of low molecular weight. From such starting materials, numerous attempts have been made to isolate pure constituents (see, for instance, the reviews of Augustin, 1952, 1955). In all cases, the active fractions (often several were isolated from one system) proved to be of protein nature, although most showed an unusual stability to heat treatment and some appeared even to resist proteolysis. The part played by prosthetic groups in the biological reactions of these materials is still

uncertain. Most active fractions of plant origin have been found to contain 'bound' carbohydrate and, in some cases, pigment, but removal of these 'conjugated' substances often failed to reduce skin reactivity.

The work described here was undertaken with a view to identifying the allergen (or allergens) of horse dandruff and to obtaining a pure preparation which might prove useful in biochemical studies of the allergic reaction. Only the mildest of chemical procedures have been adopted in an attempt to preserve the native structure of the sensitizing substance. The drastic treatment applied to many allergenic extracts in the past has probably contributed to the difficulty of isolating pure constituents. In many cases the sole aim appears to have been the preparation of skin-reactive materials regardless of the deleterious action of the reagents employed (strong acid, strong alkali, organic solvents, etc.). Although such treatments sometimes apparently did not decrease the potency of the allergen, as indicated by prick testing of patients, they would tend to alter its chemical structure. This is particularly undesirable where the preparation of the allergen is required for immunochemical studies.

Squire (1950), using saline extracts of horse dandruff, achieved a partial purification of the protein constituents by fractionation with sodium sulphate. In addition, quantitative skin-testing procedures were defined. The solubility and serological characteristics of the active protein suggest that the dandruff allergen might be a modified form of horse-serum albumin. This possibility has been borne in mind throughout the present study and, where possible, the properties of the dandruff antigens have been compared with those of crystalline horse-serum albumin in the hope of providing a chemical explanation for this intra-species relationship.

MATERIALS AND METHODS

Preparation of horse-dandruff extract. A suspension of horse brushings in acetone at 1° was poured through a large Büchner funnel to separate the hair from the dandruff (mainly epidermal scales). After standing in cold acetone for 1 hr. to remove fats, the dandruff was separated by filtration with suction and dried over P_2O_5 in a vacuum desiccator. A soft grey powder was obtained. Portions (100 g.) were extracted by trituration with 0.85% (w/v) saline (1 l.), toluene (100 ml.) being added as preservative. The resultant thick cream was stored, with occasional stirring, at 1° for 3 days, after which insoluble material (mainly keratin) was removed by centrifuging (10 000 rev./min.) at 5°, the dark-brown supernatant being used for many tests and termed 'crude dandruff extract'.

As a preliminary purification process, substances of low molecular weight such as peptides, amino acids, urea, sugars and salts (comprising about 40% of the total solids) were removed from the crude extract by dialysing portions (100 ml.) in Vico cellophane sacs at 1° for 3 days against large volumes (5 l.) of tap water and then for 1 day against distilled water. 'Dialysed extracts' obtained in this way were light brown and opalescent, containing about 1% (w/v) of protein.

More protein (about 25% of that initially extracted) could be obtained by a second extraction of the defatted powder.

Determination of protein concentration. Total nitrogen was estimated by the micro-Kjeldahl technique of Ma & Zuazaga (1942), distillations being carried out in a Markham (1942) type of apparatus. Protein solutions were digested overnight.

Non-protein nitrogen was estimated after precipitation of the protein by the addition of an equal volume of 10% (w/v) trichloroacetic acid.

In calculating protein concentrations from the nitrogen contents of solutions, the conversion factor 6.25 was always used.

Total solids were estimated by heating duplicate portions of solution in a hot-air oven at 110° to constant weights.

Ultraviolet-absorption measurements. Cells 1 cm. in width were used, measurements being made in an SP. 500 spectrophotometer (Unicam Instruments Ltd.).

Electrophoretic analysis

Free-solution electrophoresis. Barbitone buffer, pH 8.6; I, 0.1 (sodium diethylbarbiturate, 0.1 M; diethylbarbituric

acid, 0.2 M), was mainly used. Specific conductivities of diffusates were measured at 0.5°.

Analyses were carried out at 0.5° in a Tiselius apparatus, incorporating a Philpot-Svensson optical system. Monochromatic light ($\lambda = 546$ m μ .) was used, schlieren patterns being analysed by the method of dropping perpendiculars from minima separating the peaks (Tiselius & Kabat, 1939) and estimating the enclosed areas by the method of 'counting squares'.

In calculating the potential gradient within the cell the specific conductivity of the protein solution was assumed to be equal to that of the diffusate. The small correction theoretically required for volume change in the electrode vessels was neglected.

Horizontal paper-strip electrophoresis. The technique adopted was a development of that originated by Turba & Enenkel (1950) described fully by Hardwicke (1954). In most experiments, strips (39 cm. $\times$ 6–9 cm.) of Whatman no. 3 MM paper were used. Portions (0.01 ml.) of 1% (w/v) protein solutions were applied to the strip. Barbitone buffer (pH 8.6; I, 0.05) was most commonly used as solvent and a current of about 2 mA/strip was passed for about 16 hr. at room temperature. Protein was located by staining as described by Hardwicke.

Relative protein concentrations were determined by eluting the dye from segments of paper containing the separated components with 0.01 N-NaOH (10 ml.) for 30 min. (according to the method of Kunkel & Tiselius, 1951) and then measuring the optical densities of the eluates at 600 m μ . in a Hilger Spekker absorptiometer. The fading of the dye in this eluting fluid reported by Franglen & Martin (1954) was not encountered, provided that measurements were made within 1 hr. after elution.

Only narrow side strips (1 cm. wide) of paper, each carrying approx. 15% of the total protein, were stained in preparative runs to locate the proteins on the unstained centre section. Appropriately cut segments were eluted with 0.85% saline for 24 hr.

Preparative zone electrophoresis. The vertical starch-column method of Flodin & Porath (1954) was used to prepare sufficiently large quantities of electrophoretic components for chemical, immunological and biological analyses. Commercial potato starch, thoroughly washed with 0.85% saline and then with barbitone buffer (pH 8.6; I, 0.05) at 1° was used as the solid supporting medium. Samples containing about 300 mg. of protein in 6 ml. of buffer were separated under a potential of 600 v d.c. (giving a current of 20 mA) for about 16 hr.

The column was eluted with buffer and eluates (2.5 ml.) were collected by an automatic fraction collector (Towers and Co. Ltd.). Distribution of electrophoretic zones was revealed by measuring the absorptions ($\lambda = 277.5$ m μ .) of portions (0.2 ml.) of the eluates diluted with water (3 ml.) against a solvent blank consisting of barbitone buffer similarly diluted.

Carbohydrate estimations. The Molisch reaction, with 1% (w/v) ethanolic α -naphthol solution and conc. H_2SO_4 , was used as a rapid preliminary test for carbohydrate.

Carbohydrate bound to proteins separated electrophoretically on paper was revealed by staining with periodic acid-Schiff's reagent, according to the method of Kōiw & Grönwall (1952). For recording the results it was necessary to photograph almost immediately the pink bands obtained, as background colour rapidly developed.

Quantitative measurements of carbohydrate. A modified orcinol colorimetric technique, applied by previous workers (Rimington & Van den Ende, 1940; Friedmann, 1949) to the estimation of hexose in horse-serum glycoproteins, was used to determine the hexose contents of horse-dandruff protein fractions. Absorption measurements were made at 520 m μ . in a Unicam spectrophotometer against the appropriate reagent blank. Equimolecular proportions of galactose and mannose were used for construction of a standard curve.

Phosphorus estimation. The Fiske & Subbarow (1925) colorimetric method was applied to the estimation of protein-bound phosphorus.

Chromatographic analysis. One-dimensional ascending-partition chromatography was employed, pyridine-amyl alcohol-water (35:35:30, by vol.) and pyridine-ethyl acetate-water (20:40:40, by vol.) proving to be satisfactory solvent systems.

Samples (10 mg.) of glycoprotein were hydrolysed by heating with conc. H₂SO₄ (1 ml.) in sealed tubes in a steam oven for 4 hr., according to the method of Werner & Odin (1949). Sulphate was removed from the hydrolysates by addition of Ba(OH)₂.

Chromatograms were run on Whatman no. 1 paper at 27° for 1-3 days and then sprayed with aniline hydrogen oxalate (Horrocks & Manning, 1949). A modified Morgan-Elson reagent (Partridge, 1948) was used to reveal hexosamines and *N*-acetyl hexosamines.

Molecular-weight determination. The apparatus used was similar to that employed by Adair (1949) in the determination of the osmotic pressures of haemoglobin solutions, involving measurement of the osmotic head in terms of toluene coloured with dye. Semi-permeable collodion membranes were prepared according to the method of Alexander & Johnson (1949). Osmotic pressure measurements were made at 1° on protein solutions of varying concentrations (up to 1%, w/v) in buffer solutions (*I*, 0.2) at the isoelectric pH of the protein. Latterly, however, osmotic pressure measurements were made in an electronic colloid osmometer (Rowe, 1953).

Immunological techniques. Antisera were prepared by injecting rabbits intravenously with alum-precipitated protein from concentrated [3% (w/v) protein] crude horse-dandruff extract (Kabat & Mayer, 1948). Five days after the last injection of the 3 weeks' course of immunization, the rabbits were bled from the ear.

'Ring' tests, performed in narrow glass tubes (2 mm. internal diam.), proved useful in selection of suitable antisera for subsequent gel-diffusion analyses.

The horizontal gel-diffusion technique, evolved by Ouchterlony (1948) and Elek (1949), was used for comparing the protein components in horse-dandruff fractions. The distances between the edges of the antigen and antiserum agar was usually 11 mm., and the plates were incubated at 37° for 1-2 days or, in some cases, kept at room temperature. To obtain a permanent record of the precipitin lines, the agar slabs were fixed in buffered (pH 7.4) formal saline and stained with chlorazol black E (Gell, 1955).

Further information was provided by a modification of the Grabar & Williams (1953) immuno-electrophoretic technique. Solutions (1%, w/v) of agar in barbitone buffer (pH 8.6; *I*, 0.05) were used to form the gels, through which a current of about 3 mA was passed for 10 hr. Antiserum was then placed in a trough cut at a suitable distance from, and parallel with, the line of electrophoretic separation.

Preparation of crystalline horse-serum albumin reference substance. A crude crystalline albumin fraction was prepared by the method of Adair & Robinson (1930). After three recrystallizations it was dialysed until salt-free, and freeze-dried. The product was found to contain 0.3% of hexose and showed a major and one minor precipitin line in an agar-diffusion test of a 1% (w/v) solution with rabbit antiserum raised against whole horse serum.

Assay of allergenic activity. The biological activities of centrifugally Seitz-filtered dandruff preparations were determined by quantitative prick testing of horse-sensitive human patients. Duplicate tests were made by pricking drops of protein solutions (in 0.85% saline), suitably diluted after estimation of their nitrogen contents, into selected sites on the patient's forearm. After an interval of 10 min., the outlines of the wheals were traced in ink on stiff celluloid. The area of the enlarged tracings were determined by the method of 'counting squares' and used for calculating accurate mean wheal diameters.

Squire (1950) demonstrated the reliability of this method by repeated testing with histamine and subjecting the results to an analysis of variance, which indicated a coefficient of variation of about 16% for a single measurement (provided that suitable precautions in the selection of body site and dose were observed). He showed also that over a wheal size range of 1-6 mm. the relation between wheal size and the logarithm of the histamine concentration is approximately linear.

Subjects. A group of six male subjects (aged 9-13) were selected, from patients suffering with bronchial asthma of sufficient severity to warrant regular attendance at a schools' asthma clinic, because of their marked skin reactivities to horse-dandruff extract (Bencard reagent). In addition, they gave positive skin tests to other common allergens such as pollens, dust, etc.

RESULTS

Preliminary studies on the chemical composition of horse-dandruff extract

Protein. Dialysed dandruff extract exhibited the opalescence of a typical protein solution and was found to have the usual type of protein ultraviolet-absorption spectrum (Fig. 1A). It was possible to

Fig. 1. Ultraviolet-absorption spectrum of dialysed horse-dandruff extract (approx. 50 mg. %, protein solution) before (A) and after (B) deproteinization with 10% (w/v) trichloroacetic acid.

remove all detectable absorbing material by precipitation with 10% (w/v) trichloroacetic acid, the supernatant showing no specific absorption (Fig. 1B).

Of the common precipitants, phosphotungstic acid was found to be the most effective in removing protein from dialysed horse-dandruff extract. Sulphosalicylic acid precipitated 95% and trichloroacetic acid 92% of the protein nitrogen precipitated by phosphotungstic acid. There was good agreement between the amount of protein in the phosphotungstic acid precipitate (a 16% nitrogen content being assumed), namely 1.28 g./100 ml. of extract, and the total solids content of dialysed dandruff extract (1.31 g./100 ml.) when allowance was made for an ash content of 2.3%. Hence it was inferred that most of the non-dialysable material in horse dandruff was of protein nature.

The work of Squire (1950), who showed that whereas no appreciable loss of allergenic activity was caused by prolonged dialysis of dandruff extract all activity was removed by precipitation with trichloroacetic acid and other precipitants, indicated that skin reactivity was associated with this protein material.

Carbohydrate. Dialysed dandruff extract gave a positive Molisch reaction, contrary to the findings of Squire (1950). The problem was to decide whether this carbohydrate was conjugated with protein, present as free polysaccharide or to be found in both forms.

Carbohydrate was also detected in extract deproteinized with trichloroacetic acid but was precipitated completely by phosphotungstic acid. Attempts were made to obtain some quantitative measure of this constituent by application of a method used by Winzler, Devor, Mehl & Smyth (1948) to estimate the 'mucoprotein' in human

plasma. In Table 1, the 'mucoprotein' contents of crude and dialysed dandruff extracts are compared with their total hexose contents, the differences giving the amounts of carbohydrate associated with protein precipitated by perchloric acid (or presumably by trichloroacetic acid). Although the results obtained were only semi-quantitative, owing to the difficulty of separating mucoprotein from the less soluble protein, it was evident that the major portion of the carbohydrate (determined as hexose) in dandruff extract was associated with the perchloric acid-precipitated (or heat-coagulated) protein.

Practically none of the so-called 'mucoprotein', which was found to have a hexose/nitrogen ratio of 1.38 (in crude extract), appeared to be removed from crude extract by dialysis. The possibility of this constituent possessing appreciable allergenic activity appeared unlikely, however, on account of Squire's finding that all activity was removable from dandruff extract by trichloroacetic acid precipitation. Moreover, this was later confirmed by testing a sample obtained from a full-scale fractionation of dialysed extract.

Approximately 19% of the total carbohydrate of crude dandruff extract was removed by dialysis (probably in the form of free sugars).

Phosphorus. Quantitative colorimetric analyses on acid digests of 1% (w/v) dandruff protein solution (reconstituted freeze-dried dialysed extract) revealed a protein-bound phosphorus content of less than 2 mg./100 g. On the basis of a low phosphorus content Squire (1950) had ruled out the possibility of dandruff extract containing significant amounts of nucleoprotein. This was confirmed by the ultraviolet-absorption measurements, which failed to indicate any specific absorption at the 260 m μ . wavelength (Fig. 1, curve A).

Pigment. The properties exhibited by the pigment in crude dandruff extract suggested that it might be melanin (probably originating from the horse's melanophores and removed from the dandruff powder during saline extraction). Approximately 40% was removed by the dialysis process.

Electrophoresis

Early workers (Longcope, O'Brien & Perlzweig, 1926) on the horse-dandruff-extract system found that a major allergenic fraction showed maximum precipitation between pH 3.2 and 3.8. Later Squire (1950) demonstrated maximal precipitation of the active material at pH 4.0. Hence alkaline buffers, in which the proteins would be expected to be of sufficiently high charge, were used in preliminary electrophoretic analysis in a Tiselius apparatus.

As phosphate buffer (pH 7.7) has proved better than barbitone (pH 8.6) for the electrophoretic

Table 1. *Carbohydrate content of horse-dandruff extract*

Dandruff extract (2 ml.) was deproteinized by the addition of 0.75M perchloric acid (8 ml.). After 10 min. the precipitates were separated by filtration through Whatman no. 540 paper and mucoprotein was precipitated from portions (5 ml.) of the filtrate by the addition of 5% (w/v) phosphotungstic acid in 2N-HCl (1 ml.) in centrifuge tubes. The precipitates were separated after 15 min. by centrifuging (15 000 rev./min.), washed and dissolved in 80% sat. Na₂CO₃ (5 ml.) for analysis of their hexose contents.

Extract	Total hexose (mg./100 ml.)	Mucoprotein hexose (mg./100 ml.)	Difference (mg./100 ml.)
Crude	88.0	7.6	80.4
Dialysed	71.0	7.1*	63.9

* A value of 5.2 mg./100 ml. was obtained for mucoprotein precipitated from extract which had been deproteinized by heat coagulation at pH 4.0

separation of horse-serum proteins, it was anticipated this would also prove superior in the analysis of horse-dandruff extract. Results of comparable analyses in buffers of similar ionic strength (0.1) showed that the converse was true. The index of symmetry (i.e. the ratio of the height of the major descending component) determined from the schlieren patterns obtained after 198 min. electrophoresis in barbitone buffer was 0.81 as compared with a value of 0.44 after only 48 min. separation in the phosphate buffer. Thus anomalous boundary effects appeared to be much more pronounced in the phosphate system, nor were these effects reduced appreciably by carrying out the analysis in phosphate buffer of twice the original ionic strength (i.e. 1, 0.2).

These results were, somewhat surprisingly, analogous to those obtained by Longworth (1942) on electrophoretic analysis of human serum, barbitone (pH 8.6) giving a better separation than phosphate buffer (pH 7.7) of equivalent ionic strength. Longworth was of the opinion that the most suitable solvent for the analysis of an animal serum was species-dependent.

Electrophoretic analysis in barbitone buffer (pH 8.6; I , 0.1) resulted in the separation of three

components, lettered arbitrarily as a , b and c in order of decreasing mobility in the schlieren patterns shown in Fig. 2A. It will be noticed that the pigment moved with the slowest (c) component.

The relative concentrations of the components, which were all assumed to have the same refractive-index increment, were calculated from the ascending schlieren pattern as separation of the c component was far from complete in the descending limb. Paper-strip electrophoresis in barbitone buffer (pH 8.6; I , 0.05) also failed to separate this component (Fig. 2B). In some analyses, however, the major b component was split in the paper medium (Fig. 2C). Indeed, the slight asymmetry of the major peak noticed in free-solution patterns had suggested that the b component was heterogeneous. The faster of the two components was designated ' b_1 ' and the slower ' b_2 '. The concentrations of the paper-strip components ($a = 5.6\%$; $b = 85.3\%$; $c = 9.1\%$) showed good agreement with the corresponding values calculated from the ascending schlieren pattern ($a = 6.0\%$; $b_1 = 18.8\%$; $b_2 = 64.4\%$; c , etc. = 10.8%) which suggest that the dandruff proteins did not differ greatly in their contents of non-protein prosthetic groups.

Determination of the isoelectric point of the major component

Mobilities of the b component were calculated from descending schlieren patterns obtained by electrophoresis of dandruff extract in three buffers of different pH. The mobility-pH curve intersected the zero-mobility line at pH 4.1, indicating an isoelectric value near to that obtained by the 'maximum-precipitation' method mentioned earlier. The horse-dandruff b component was found to have a mobility of $-8.03 \times 10^{-5} \text{ cm}^2 \text{ v}^{-1} \text{ sec}^{-1}$ in barbitone buffer (pH 8.6; I , 0.1); the corresponding value for horse-serum albumin (five times recrystallized) was -6.49 .

Solubility studies

Quantitative salt fractionations of horse-dandruff extract were undertaken with a view to determining the solubility limits of the constituent proteins, a necessary preliminary to their efficient separation.

Both phosphate buffer (pH 6.5) and saturated ammonium sulphate solution (adjusted to pH 7.0 with concentrated ammonia) were used as precipitants at controlled temperature. Use of phosphate enabled the protein contents of the supernatants to be determined by direct nitrogen estimations as well as affording a control on pH. The solubility curve obtained is shown in Fig. 3. Included, for comparison, in this figure is the curve obtained by plotting the ultraviolet absorptions ($\lambda = 277.5 \text{ m}\mu$) of the supernatants against ionic strength of precipitants. Both curves revealed points of inflexion

Fig. 2. Electrophoresis patterns of horse-dandruff extract [1% (w/v) protein] in barbitone buffer (pH 8.6); (A) free solution analysis in buffer of ionic strength 0.1 (potential gradient = 7.31 v/cm., after 148 min.); (B) paper-strip analysis in buffer of ionic strength 0.05 (current = 2 ma for 16 hr.); (C) paper-strip analysis under conditions obtaining in previous experiment, illustrating the splitting of the major (b) component.

at the same ionic strengths, indicating fractionation of three constituents.

Although the use of phosphate permitted the direct quantitative determination of precipitated protein by micro-Kjeldahl nitrogen analysis, ammonium sulphate has been more widely used as a protein precipitant in previous work and has become a standard against which to assess the solubilities of unknown proteins. A further quantitative fractionation therefore was carried out with ammonium sulphate, the amounts of proteins

Fig. 3. Solubility curve of dialysed horse-dandruff extract in phosphate buffer, pH 6.5, at 25°. Varying amounts of 3M phosphate buffer (prepared from KH_2PO_4 and 4N-KOH) and 0.85% saline were added to portions (1 ml.) of dandruff extract to a final volume of 20 ml. in each case. After being heated in a water bath at $25 \pm 0.05^\circ$ for 24 hr., and shaken occasionally, the precipitates were separated through Whatman no. 542 paper. Nitrogen contents (●) of the filtrate (diluted 10 times) were estimated by the micro-Kjeldahl technique, and optical densities (▲) measured in 1 cm. cells.

Fig. 4. Solubility curve of dialysed horse-dandruff extract in ammonium sulphate, pH 7.0, at 1°. Varying amounts of saturated ammonium sulphate solution (adjusted to pH 7.0 with concn. ammonia) and 0.85% saline were added to portions (1 ml.) of dandruff extract to a final volume of 20 ml. Mixtures were stored in a cold room for 24 hr., shaken occasionally, and precipitates separated by filtration (at 1°) through Whatman no. 542 paper. After the first portions of filtrates had been discarded, portions (1 ml.) were diluted (10 times) and optical densities measured in 1 cm. cells.

precipitated being determined by ultraviolet-absorption measurements on the supernatants. By allowing the protein-salt mixtures to come to equilibrium at 1° before filtration through Whatman no. 542 paper at room temperature, the possibility of further precipitation occurring in the spectrophotometer cell during measurement was avoided.

The solubility curve is shown in Fig. 4. Again, fractionation of three dandruff protein constituents is indicated. None of these showed a precipitation limit corresponding to 50% saturation with ammonium sulphate, whereas a similar experiment on horse serum indicated that the albumin was beginning to precipitate at this salt concentration.

Salt-fractionation of horse-dandruff extract

The conditions adopted in initial fractionations were similar to those commonly used in the salt-fractionation of horse serum. After separating the material precipitated by 50% saturated ammonium sulphate the soluble proteins were further fractionated by either lowering the pH or increasing the salt concentration. Precipitates and supernatants were analysed electrophoretically in free solution and, where possible, the relative concentrations of the separated components calculated from the enlarged schlieren patterns (Stanworth, 1953). A difficulty, particularly in cases of incomplete separation, was the identification of the various electrophoretic components in the different fractions.

As was to be expected, better fractionations were achieved by adopting the ammonium sulphate-concentration limits indicated by the quantitative fractionation with this salt (Fig. 4). A detailed scheme of a typical fractionation is shown in Fig. 5. The wet weights of the precipitates (after one washing with precipitant) are included in order to provide some indication of the distribution of protein amongst the various fractions. The largest portion of protein (about 50% of the total) was precipitated in fraction III between 55 and 80% saturation with ammonium sulphate.

The precipitates were taken up in tap water and dialysed at 1°, against repeated changes of tap water and finally against distilled water, until all the salt had been removed. Protein contents of the dialysed solutions were determined by micro-Kjeldahl nitrogen estimations and the concentrations adjusted to 1% (w/v), by dilution or evaporation in cellophan sacs in a stream of cool air, in preparation for subsequent analyses. All such solutions were stored at 1° for short periods and eventually freeze-dried.

Isolation of crystalline fraction

After numerous attempts, a specimen of crystalline dandruff protein was obtained, during the purification of crude fraction II (initially precipitated

between 42.5 and 55.0 % saturation with ammonium sulphate).

Twice-precipitated crude material (wet wt. 20 g.) was taken up in water (100 ml.), the solution being clarified by filtration through Whatman no. 542 paper. Saturated ammonium sulphate solution (72 ml.) was then added slowly to the solution at 1°, the sides of the beaker being scratched with a glass rod, until precipitation commenced. After standing for 3 hr. in the cold room the pigmented precipitate (wet wt. 10 g.) was separated by centrifuging. This was redissolved in water (100 ml.). Addition of saturated ammonium sulphate (76 ml.) at 1° brought down a slight precipitate, which was left standing overnight in the hope of adsorbing crystallization-inhibiting pigment. The precipitate was separated by filtration through Whatman no. 1 paper, leaving a pale-brown opalescent filtrate, on the surface of which there tended to form what

appeared to be crystals. Storage of this solution (containing 43 % saturated ammonium sulphate) at 1° for another day resulted in the formation of a slight precipitate, which proved to be entirely crystalline on microscopic examination.

The crystals appeared to be of hexagonal form, thus differing from the trigonal horse-serum albumin crystals. They were shown to be anisotropic by examination under a polarizing microscope, and an aqueous solution gave a positive biuret reaction.

Further crystalline precipitates were obtained from the mother liquor. These were taken up in water, dialysed until salt-free and freeze-dried. More recently, a similar crystalline protein has been isolated from the ammonium sulphate solution washings (43 % saturated) of crude fraction I cooled to 1°, during a full-scale fractionation of horse-dandruff extract.

Saturated ammonium sulphate (adjusted to pH 7.0 with concentrated ammonia) was used as precipitant except in the precipitation of fraction IV, when solid salt was added. All precipitations were carried out in the cold room at 1°. The wet weights refer to the weights of the centrifuged precipitates after being washed once with approx. equal volumes of precipitant solutions of appropriate salt concentrations.

Electrophoretic analysis of fractions

The paper-strip technique was used in the electrophoretic analysis of the salt-precipitated protein fractions, enabling side-by-side comparison of mobilities to be made and thus overcoming the difficulty of identifying individual components, which was encountered in earlier free-solution analyses.

Results of a comparison of the electrophoretic patterns of total dialysed extract and its crude protein fractions (I-IV) on a wide sheet of Whatman no. 3 MM paper (22 cm. $\times$ 47 cm.) are illustrated diagrammatically in Fig. 6. (Paper-strip analysis of

Fig. 6. Diagrammatic illustration of results obtained on comparison of the electrophoretic compositions of salt-precipitated dandruff protein fractions and total dialysed extract. Protein solutions (1%, w/v) were analysed on a wide (22 cm. $\times$ 47 cm.) sheet of Whatman no. 3 MM paper in barbitone buffer (pH 8.6; I , 0.05; current 10 ma for 8 hr.).

fraction V under similar conditions was not accomplished.) The system of nomenclature adopted in lettering the electrophoretic components of the other fractions was consistent with that applied to the components separated from whole extract (Fig. 2). The *c* and *e* components referred to in the diagram were only revealed by analysis of 6% (w/v) solutions of certain fractions (notably fraction I), their mobilities relative to the other components being established by comparative electrophoresis on narrower (9 cm. wide) strips. It was evident that the two *b* components, which appeared to be closely related and often inseparable by electrophoresis of whole extract, had been separated by the ammonium sulphate fractionation. The difference between these constituents was further emphasized by staining paper-strip electrophoretic patterns of the various fractions with periodic acid-Schiff's reagent (Köiw & Grönwall, 1952), which showed the more soluble *b*₂ component to be rich in protein-bound carbohydrate (Fig. 7). On the other hand, no carbohydrate could be detected in the less-soluble *b*₁ component (predominant in fractions I and II). The faster *a* component contained a small amount of carbohydrate.

The crystalline protein proved to be electrophoretically homogeneous as indicated by paper-strip analysis of a 3% (w/v) solution. Its mobility on paper in barbitone buffer (pH = 8.6; I , 0.05) was identical with that of the *b*₂ component, although it differed from this protein in not containing any carbohydrate detectable by the periodic acid-Schiff's reagent.

Staining of paper-strip electrophoretic patterns with a saturated solution of oil red O in 60% (w/v) ethanol (Durrum, Paul & Smith, 1952) failed to detect protein-bound lipid in any of the components.

Fig. 7. Distribution of protein-bound carbohydrate in paper-electrophoresis patterns of horse-dandruff extract and protein fractions. (The *b* component is not split in the crude and dialysed extract patterns.) The dried strips were divided lengthwise, one section (P) being stained with bromophenol blue and the other (C) with periodic acid-Schiff's reagent.

Analysis of protein-bound carbohydrate

Paper-partition chromatographic analysis of the acid hydrolysates of crude glycoprotein fractions showed the protein-bound carbohydrate to be composed of galactose, mannose and glucosamine. In addition to these sugars, the highly soluble fraction V was found to contain a trace of glucose and another constituent, which was probably fucose.

It seemed significant that the sugar constituents of the horse-dandruff glycoprotein fractions were similar to those found in horse-serum proteins. Moreover, results obtained by application of a colorimetric method used by Friedmann (1949) in determining the relative concentrations of the hexose constituents of horse-serum proteins suggested that the galactose and mannose were present in similar proportions (namely 1:1) in the proteins from the two different sources. This involved measurement of the colour developed with orcinol reagent at two wavelengths, 470 and 520 m μ . The ratio of absorption at 470 m μ . to that at 520 m μ . then gave a measure of the galactose:mannose ratio in the mixture. Ratios obtained by measurements on solutions of most dandruff-protein fractions showed reasonable agreement with the mean ratio determined from similar measurements on varying dilutions of a standard solution containing equimolecular proportions of galactose and mannose (Table 2). Conclusive evidence from quantitative chromatographic analyses is, however, required.

Table 2. *Hexose contents of horse-dandruff proteins*

Hexose (galactose + mannose) contents of duplicate portions (1 ml.) of 0.25–1.00 % (w/v) protein solutions were estimated by the orcinol technique, optical density measurements being made at 520 m μ . Measurements were also made at 470 m μ ., the 470/520 m μ . absorption ratio providing an estimate of the relative concentrations of galactose and mannose comparable with the Ilford filter ratio 604/602 employed by Friedmann (1949).

Standard sugar solution (galactose + mannose, 1:1)	Total hexose content (γ /ml.)	Absorption ratio 470/520 m μ . (orcinol reagent)	Hexose/nitrogen ratio
1	200	1.03	—
2	150	1.02	—
3	100	1.03	—
4	50	1.02	—
Protein fraction			
Dialysed extract	—	1.03	0.312
Fraction I	—	1.10	0.010
Fraction II	—	1.04	0.115
Fraction III	—	1.01	0.376
Fraction IV	—	1.00	0.245
Fraction V	—	—	8.620
Crystalline protein	—	1.10	0.013

The total hexose contents of the dandruff-protein fractions were also determined by the orcinol method, absorptions being measured at the 520 m μ . wavelength against the appropriate reagent blank. A calibration curve was constructed from the absorption values obtained by the measurements on dilutions of the standard galactose-mannose (1:1) solution at this wavelength. The carbohydrate contents expressed as hexose:nitrogen ratios in Table 3 were in the order expected from the results of the qualitative technique applied to the paper-electrophoretic patterns. According to the classification of Stacey (1946), the highly soluble fraction V which contained 48.0 % of hexose and 5–6 % of nitrogen would be termed a 'mucopolysaccharide'. Although it failed to react with biuret reagent, this could not be considered an indication of complete lack of peptide as polypeptide-polysaccharide complexes having low amino acid contents often show no reaction, particularly in the presence of contaminating pigment.

Assay of allergenic activities of dandruff fractions

The relative allergenic activity of dandruff extracts and fractions was assayed by prick testing patients with solutions containing a standard (0.1 g. %) concentration of protein. In Table 3, some results are set out for comparison of these activities with the composition of each specimen as determined by paper electrophoresis. It will be seen that the crude extract gave rise to wheals measuring from about 17 to 6 mm. in mean diameter, mainly according to the degree of sensitivity of the subjects tested. The results obtained with each of the other specimens have therefore been expressed as percentages of the mean diameter of the wheals elicited in duplicate tests with crude extract for each individual.

An analysis of variance in terms of patients, sites and fractions revealed that there was no significant variation ($P > 0.20$) between the reactions of the different patients expressed in this way. This was interpreted as meaning (a) that the expression of results as percentages of the reaction size with a standard (crude extract) was valid, and (b) that the five individuals tested did not show significant variation in specific hypersensitivity to any particular dandruff-protein constituent. The contribution of the site to variance ($0.10 > P > 0.05$) did not quite reach the conventional level of significance. Nevertheless, as the sample analysed was small and as Squire (1950) has demonstrated a significant site variation by analysis of a larger number of prick tests with histamine on normal subjects, standard errors of the combined means were calculated by combining the variances due to sites 1 and 2. There was a marked variation in the mean reactivities of the various protein fractions.

Table 3. *Electrophoretic compositions and allergenic activities of salt-precipitated horse-dandruff protein fractions*

Relative concentrations of the electrophoretic constituents separated on filter paper were determined by quantitative elution of bromophenol blue in 0.01 N-NaOH (assuming all components bound dye to the same extent). Wheel diameters were calculated from tracings made 10 min. after pricking 0.1% (w/v) Seitz-filtered protein solutions into duplicate sites on hypersensitive patients' right forearms. The results, expressed as percentages of the mean wheel diameters elicited by crude dandruff extract, are arranged in order of decreasing reactivity of the patients. There was a time interval of 14 days between the two series of tests performed on patient M. H. The standard errors of the combined means were obtained by combining the variances due to sites 1 and 2 (also in Tables 4 and 5).

Protein fraction	Relative concentrations of electrophoretic constituents (%)				Wheel diameters elicited on duplicate sites						
	a	b ₁	b ₂	Remainder (c, d, etc.)	Site	Patient			Patient M.H. (1st test) (mm.)	Patient R.V. (mm.)	Standard error of mean
						D.P. (mm.)	M.H. (2nd test) (mm.)	A.P. (mm.)			
Crude extract	6.0	18.8	64.4	10.8	1 2	18.2 15.3	16.8 13.5	14.5 13.4	11.9 6.9	8.7 5.6	—
% mean diameters elicited by crude extract											
Dialysed extract	4.9	25.3	56.7	13.1	1 2	121.4 67.9	89.7 61.4	108.4 145.4	103.4 62.1	84.6 63.1	14.0
Fraction I	0.0	74.6	0.0	25.4	1 2	27.4 48.2	37.2 42.1	26.9 42.0	0.0 34.5	47.7 60.0	8.5
Fraction II	0.0	71.8	13.4	14.8	1 2	32.7 50.0	34.5 40.7	47.9 62.2	0.0 17.2	81.5 86.2	16.5
Fraction III	6.8	8.7	78.3	6.2	1 2	52.4 128.0	89.7 104.1	84.0 75.6	118.4 67.8	95.6 60.0	14.5
Fraction IV	52.3	0.0	40.0	7.7 (4.2=d)	1 2	74.4 69.6	86.8 86.9	83.0 84.6	69.0 71.3	55.4 84.6	5.3
Fraction V	—	—	—	—	1 2	0.0 0.0	— —	0.0 0.0	— —	— —	—

Comparison of the potencies of fractions I-IV (in terms of mean wheal diameter which is proportional to log dose of allergen) with their hexose/nitrogen ratios (Table 2) and electrophoretic compositions (Table 3) shows that the most potent fraction (III), possessing 96% of the activity of the whole dialysed extract, contained the most hexose and a preponderance of the carbohydrate-rich b_2 constituent. (The mucopolysaccharide fraction V gave negative results in the two patients tested.) Summarizing,

the fractions (III and IV) containing a high proportion of b_2 component were the most potent, whereas those (I and II) deficient in this protein were comparatively unreactive. (It should be mentioned that the value, 13.4%, given for the relative concentration of b_2 component in fraction II can only be considered very approximate owing to the failure to effect a 'clean' electrophoretic separation of this component from the b_1 constituent predominant in this fraction.) There appears to be

Table 4. *Allergenic activities of partially purified horse-dandruff protein constituents*

Dialysed eluates containing each of the three main constituents of salt-precipitated (80-100% saturated ammonium sulphate) fraction IV, separated by electrophoresis both on paper and in a starch column, were used in the prick testing of horse-sensitive patients.

A. Paper electrophoretic components. The low protein content of most eluates, even after concentration by evaporation in cellophane sacs in a current of air, prevented the use of the usual 0.1% solutions in the biological testing.

Predominant electrophoretic component	Protein concn. of soln. tested (mg./100 ml.)	Site	Wheal diameters elicited in duplicate sites			Combined mean (mm.)
			Patient A.P. (mm.)	Patient D.P. (mm.)	Patient M.H. (mm.)	
Total fraction IV	100	1	12.4	13.3	8.7	12.6
		2	15.2	17.2	8.7	
a	20	1	4.5	4.0	1.6	3.5
		2	3.8	3.5	3.6	
b_2	22	1	8.4	8.3	6.4	7.8
		2	9.2	9.9	4.2	
d	118	1	1.8	0.0	4.0	1.7
		2	1.2	0.0	3.3	
'Tail'	20	1	1.3	2.5	0.0	0.7
		2	0.0	0.0	0.0	
Blank paper eluate	—	1	0.0	3.6	0.0	0.6
		2	0.0	2.8	0.0	

B. Starch electrophoretic components. The results of skin testing dialysed eluates from a starch column, diluted to contain 0.1% of protein, are expressed as percentages of the mean wheal diameters elicited by the parent fraction (IV). A solution of the crystalline dandruff protein, of similar concentration, was tested at the same time.

Eluate no.	Predominant electrophoretic component	Site	Wheal diameters elicited on duplicate sites				Mean	Standard error of mean
			Patient K. B.	Patient B. F.	Patient D. P.	Patient A. P.		
Total fraction IV	$a + b_2 + d$	1	4.2	10.3	15.3	6.5	—	—
		2	14.3	16.2	20.6	2.2		
% mean diameters elicited by total fraction								
5-7	a	1	25.8	53.9	14.0	0.0	24.3	17.7
		2	23.7	75.9	0.0	0.0		
8-11	a	1	36.6	65.4	25.1	61.4	42.7	8.9
		2	37.6	53.4	23.5	38.6		
15	b_2	1	186.0	90.2	115.1	197.7	123.3	30.8
		2	143.0	112.8	79.9	161.4		
16-19	b_2	1	146.2	122.6	70.9	238.6	128.4	30.9
		2	110.8	106.0	83.8	152.3		
30	d	1	—	—	—	0.0	—	—
		2	—	—	—	0.0		
Crystalline protein	Same paper-electrophoretic mobility as b_2 component	1	36.6	79.7	53.1	36.4	47.2	10.2
		2	32.3	61.9	36.9	38.6		

no similar relationship between allergenic activity and the relative content of any of the other (*a*, *b*, *c*, *d*, etc.) electrophoretic components. For instance (cf. Table 3), if the *a* component were mainly responsible for allergenic activity, fraction IV (containing 52 % of this substance) would have been considerably more potent than any of the other fractions. Likewise this fraction would have shown the highest skin reactivity if the *d* component were allergenic. By similar reasoning, the preponderance of the *b*₁ component in the relatively unreactive fraction I rules out this substance as an important allergen. It seemed likely therefore that the *b*₂ electrophoretic component, comprising 64 % of the total protein in crude dandruff extract, was the main allergen. Moreover, the lack of a significant variation between the reactions of the individuals tested, as already mentioned, indicated that there was no evidence of specific hypersensitivity to any other proteins.

More clear-cut evidence in support of this finding was provided by skin testing more purified protein preparations. The results of tests on the electrophoretic components of fraction IV, separated either on filter paper or in a vertical starch column, are given in Table 4*A* and *B*, together with the allergenic activity of the crystalline dandruff protein which was tested at the same time. It was again found in both series of tests that the major part of the biological activity was associated with the *b*₂ constituent. However, the crystalline protein which on electrophoresis showed the same mobility as the *b*₂ component but which was deficient in carbohydrate was relatively unreactive on skin testing. The small amount of activity associated with the other components was probably due to contamination with *b*₂ component, undetected by paper-strip electrophoretic analysis. [Similar contamination might also account for the slight activity of fraction I (Table 3), which contained 75 % of the apparently unreactive *b*₁ electrophoretic component.]

It was interesting to find that the electrophoretic processes and subsequent elution from the supporting media did not reduce allergenic activity, nor did freeze-drying of the active protein prove deleterious.

Immunological analyses

Agar-diffusion techniques, which allow the side-by-side comparison of antigenic composition, provide a more sensitive test of protein purity than the physicochemical techniques already described. By applying these methods, horse-dandruff protein fractions which appeared homogeneous by paper electrophoresis were found to contain more than one antigen. In this way, trace contamination by the *b*₂ component was detected in the fractions of low but measurable allergenic activity.

A typical test is illustrated in Fig. 8, in which the

antigenic compositions of the salt-precipitated dandruff-protein fractions are each compared with those of total extract. Several antigens could be detected in whole extracts tested with homologous antiserum. These were identified, as far as possible, by comparison with relatively pure protein preparations (obtained by preparative electrophoresis, etc.). A faint *b*₂ component line was found in the fraction I antigen pattern, which explained why this preparation possessed some allergenic activity although no *b*₂ component could be detected in this fraction by filter-paper electrophoresis. (The small amount of skin reactivity shown by the crystalline material could also be attributed to contamination with *b*₂ component similarly detected.)

An accurate assessment of antigen concentration cannot be made by this technique. Nevertheless, some measure can be obtained as both the distance of the precipitin line from the centre cup and its density are influenced by antigen concentration. From such considerations, no precipitin line other than that associated with the *b*₂ electrophoretic component could be assigned to the substance responsible for the allergenic activity of the various fractions. Furthermore, the *b*₂ line remained relatively unaffected when a solution of the potent allergenic fraction III was allowed to react with antiserum which had been first absorbed with weakly skin-reactive fraction I, a treatment which eliminated most of the other precipitin lines.

Fig. 8. Agar-diffusion precipitin patterns of dialysed dandruff extract (D) and salt-precipitated protein fractions (I–IV). Protein solutions (0.3 %) were tested with rabbit antiserum raised against crude dandruff extract. A 10 % (w/v) solution of total horse-serum proteins (HS) was also tested.

Purification of allergen

The gel-diffusion precipitin technique provided a sensitive guide to protein purity in subsequent attempts to prepare very pure allergen from the most active salt-precipitated fraction III. Sub-fractionation (both by salting-out and 're-resolution' procedures) with ammonium sulphate achieved some degree of purification, as assessed by paper electrophoresis, although precipitin tests still revealed antigenic heterogeneity. Of six fractions assayed, that precipitated between 70 and 80% saturation with ammonium sulphate was found to be the most pure and proved to be a little more skin-reactive than crude horse-dandruff extract (possessing 115% of the activity of the whole). There was fairly close correlation between the activities of these fractions and their carbohydrate contents (expressed as hexose/nitrogen ratios), in keeping with earlier findings on the cruder salt-precipitated protein fractions.

Purer and more potent preparations were obtained, however, by vertical electrophoresis in starch columns. As much as 300 mg. of freeze-dried material was fractionated at a time by this method. After electrophoresis, the protein contents of the eluates (2.5 ml.) were determined by ultraviolet-absorption measurements against a buffer-solvent blank. The electrophoretic pattern obtained by

Fig. 9. Starch electrophoresis pattern of salt-precipitated horse-dandruff protein fraction III in barbitone buffer (pH 8.6; I , 0.05; current 22 mA for 15 hr.). Protein contents of eluates were determined by optical density measurement (●) before dialysis and by nitrogen estimation (▲) after dialysis.

analysis of salt-precipitated fraction III in barbitone buffer (pH 8.6) is shown in Fig. 9. It will be noticed that the protein contents of selected eluates, determined from micro-Kjeldahl nitrogen estimations on dialysed solutions, followed the optical-density curve closely.

Paper-electrophoretic analysis served as a useful check on the composition of eluates, before immunological analysis and assessment of their skin reactivities. The results of precipitin tests are shown in Fig. 10 (A). The most pure eluate (no. 11) of those tested was further analysed by the immunoelectrophoretic technique, Fig. 10 (B), and found to be considerably more pure than the parent fraction,

Fig. 10. (A) Agar-diffusion precipitin patterns of selected starch electrophoresis eluates (nos. 11, 12, etc., in Fig. 9) from horse-dandruff protein fraction III (1%, w/v, solns.), whole fraction III (1%, w/v, soln.) and crystalline horse-serum albumin (10%, w/v, soln.) tested with antiserum raised against crude dandruff extract. (B) Comparison of the most skin-reactive eluate (no. 11) with its parent fraction (III), by means of the immunoelectrophoretic technique with antiserum raised against crude extract.

Table 5. *Allergenic activities of starch-electrophoresis eluates of salt-precipitated (55–80 % saturated ammonium sulphate) fraction III*

Dialysed solutions containing 0.1 % protein were prick tested on horse-sensitive patients, the results being expressed as percentages of the mean wheal diameters elicited by the parent fraction.

Eluate no.	Predominant electrophoretic component	Site	Wheal diameters elicited on duplicate sites			Mean	Standard error of mean	
			Patient D. P. (mm.)	Patient K. B. (mm.)	Patient B. F. (mm.)			
Total fraction III	$a + b_2$	1	12.6	14.6	5.3	6.8	—	—
		2	16.5		10.4			
% mean diameters elicited by total fraction								
9	a	1	94.5	15.2	100.0	79.9	38.1	
		2	57.5	13.2	158.8			
11	b_2	1	141.1	121.5	170.6	150.3	37.1	
		2	78.8	160.8	219.1			
13	b_2	1	139.7	165.8	91.2	132.5	50.4	
		2	60.3	100.0	238.2			
14	b_2	1	106.8	98.7	132.4	113.8	22.7	
		2	93.2	119.0	(132.4)			
15	b_2	1	89.7	169.6	148.5	120.8	27.1	
		2	66.4	116.5	133.8			
16	b_2	1	80.1	100.0	216.2	117.5	12.0	
		2	97.3	93.7	117.6			

a slower contaminating component (probably c) having been removed by the electrophoretic process, though appearing in increasing amounts in eluates 12–16. This eluate was also found to be the most potent, possessing 150 % of the activity of parent fraction III, although all the eluates corresponding to the main b_2 peak showed high allergenic activity (Table 5).

Preliminary chemical characterization of horse-dandruff allergen

Initial osmotic pressure (O.P.) measurements were made on a partially purified allergen preparation (contaminated with a little a component) at its isoelectric point in acetate buffer (I , 0.2) performed by means of a modified Adair technique. A mean mol.wt. value of about 42 000 (it was assumed that the protein contained 16 % of nitrogen, as micro-Kjeldahl estimations on solutions prepared from dried solid had suggested) was obtained from the results of measurements at varying protein concentrations (below 1 %, w/v) and extrapolating the osmotic pressure/concn.—concn. curve to zero concentration. In comparison a crystalline horse-serum albumin reference substance was found to have a mol.wt. of 71 000.

The relatively pure preparation obtained by starch electrophoresis, however, was found to have a mol.wt. of 34 000 in phosphate buffer (pH 7.4; I , 0.3) as compared with a value of 22 000 found for the crystalline dandruff protein. Each of these results was obtained by osmotic pressure measure-

ments made in an electronic colloid osmometer (Rowe, 1953) on two solutions of different protein content (below 1 %, w/v).

Quantitative carbohydrate analysis, by the orcinol technique, showed the purified allergen preparation to contain 9.2 % of hexose (expressed as a percentage of protein) in the form of galactose and mannose. Its amino acid composition, revealed by preliminary qualitative chromatographic analysis, appeared similar to that of crystalline horse-serum albumin. Details of these and other analyses will be included in a full account of the allergen's chemical properties, to be reported later.

Serological relationship between horse dandruff and horse-serum antigens

The results obtained by application of the gel-diffusion precipitin technique suggested that the serological cross reaction between horse-dandruff protein and horse-serum albumin demonstrated by Squire (1950), who used the more laborious method of optimum proportions, might be due to traces of serum albumin and globulin admixed with the dandruff constituents.

Dandruff and serum antigens were tested with antisera raised against crude dandruff extract, crystalline horse-serum albumin and horse serum. The precipitin line produced by reaction of allergen with antiserum to crude dandruff extract was intersected by the horse-serum albumin precipitin line, which showed that there was no relationship between these two proteins. The serum-albumin

line, however, deviated and continued faintly close to the cup containing dialysed whole dandruff extract, which probably therefore also includes small amounts of this protein (a suggestion which explains why the antiserum to dandruff extract contains antibodies against horse-serum albumin). Conversely, lines due to reactions of concentrated dandruff extract [20 % (w/v) of protein] with antiserum to serum albumin were continuous with the homologous-antigen line. By similarly testing dandruff extract (and fractions derived from it) with antiserum raised against whole horse serum, lines continuous with the serum-albumin and with one of the serum-globulin lines were evident in the dandruff antigenic patterns; the latter line was well marked in the pattern of dandruff fraction I, but absent from fraction III which would seem to include traces of serum albumin.

Until these findings have been substantiated by tests with absorbed antisera, the presence of traces of serum protein in horse dandruff is tentatively concluded.

DISCUSSION

The results presented provide evidence that allergenic activity is confined to only one of the several protein constituents detected in horse-dandruff extract, namely the carbohydrate-rich b_2 electrophoretic component. Where paper electrophoresis fails to reveal this substance in preparations showing low but measurable activity, such as fractions rich in the a and b_1 components, traces of b_2 are detectable by the more sensitive immunological techniques.

There is no reason to suppose that other constituents are important allergens. Indeed, statistical analysis of the skin-test results indicates that there is no significant difference between the reactions of the limited number of subjects tested. Small differences apparent in some subjects' specificities (Tables 4 and 5) can be attributed to the inaccuracy of this method employed for assaying allergenic activity *in vivo*. In this respect, however, it should be noted that the coefficient of variation of the size of the wheals elicited by the b_2 component in the various subjects was of the order of 23 %, a value similar to the 16 % variation shown by Squire (1950) to be expected for a single measurement of the wheal elicited by pricking normal subjects with histamine. This rather wide spread in the size of wheals produced by a particular test solution has been responsible for the reluctance of many workers to express results in quantitative terms. Nevertheless, the use of the skin test as a quantitative assay of allergenic activity is considered to be valuable provided that the standard error of the results obtained with each test solution is given.

The relatively small increase (about 30 %) in skin reactivity achieved by extensive purification of the dandruff allergen explains why crude salt-precipitated protein fractions often proved to be little more reactive than the crude extract from which they were derived. By analogy, it might be possible to account for the failure of investigators of other allergen systems to effect any marked increases in activity by application of similar procedures. It appears that about 36 % of inactive protein (in addition to contaminating pigment) was removed from crude horse-dandruff extract by salt-precipitation and subsequent starch electrophoresis, resulting in the preparation of a white freeze-dried solid. An initial purification was, of course, effected by the prolonged dialysis of the crude extract, which removed about 40 % of the pigment in addition to substances of low molecular weights. The 10 % decrease in allergenic activity accompanying the dialysis process (cf. Table 3) is within the limits of error of the assay.

Ammonium sulphate fractionation of dandruff extract separated the allergenic b_2 component from the b_1 component to which it appeared to be 'combined' in solution. It is possible that the purification procedures adopted failed to free the allergen from other inactive substances. Is, for instance, the carbohydrate (9 % hexose) an essential part of the allergen molecule, or is it contained in a polysaccharide-rich contaminant? A small amount of unreactive mucopolysaccharide (isolated as fraction V from the supernatant obtained after saturation with ammonium sulphate) is present in horse-dandruff extract. It is unlikely, however, that any significant amount of this substance is contaminating the purified allergen preparation. Estimation of the mucopolysaccharide contents of crude dandruff protein fractions (by the method used by Winzler *et al.* in 1948 to estimate serum mucopolysaccharide) indicated that the major portion of the hexose found in such fractions was bound to perchloric acid- (or trichloroacetic acid-) precipitated protein. There was no correlation of skin reactivity with mucopolysaccharide content (Stanworth, 1953). Moreover, an attempt to combine the mucopolysaccharide constituent with the carbohydrate-deficient b_1 component (by adding fraction V to a solution of fraction I) proved unsuccessful, as indicated by paper-electrophoresis, nor did the mixture show any appreciable increase in skin reactivity.

Analysis of the carbohydrate contents of certain starch-electrophoresis eluates from salt-precipitated fractions indicated that the purer and more potent (on a nitrogen basis) the fraction, the higher must be its hexose/nitrogen ratio, which suggested that any contaminant of the allergen is carbohydrate-free protein. Such an assumption is substantiated by

the failure of carbohydrate-deficient crystalline protein, of similar electrophoretic mobility to the b_2 component, to show any appreciable skin reactivity. Furthermore, there is no evidence from precipitin tests that the crystalline protein (molecular weight 22 000) is allergen deprived of carbohydrate.

Hence it is considered that horse-dandruff allergen contains a carbohydrate prosthetic group, in common with many other inhalant-type allergens. On account of its predominant protein-like properties, it would be termed a 'mucoprotein' according to the classification of glycoproteins proposed by Stacey (1946).

It would be tempting to explain the supposed serological cross-reactivity between horse-dandruff allergen and horse-serum albumin (Squire, 1950) in terms of common sugar constituents (galactose, mannose and glucosamine). According to agar-diffusion precipitin tests, however, the two proteins are unrelated. On the other hand, a contaminating antigen in the crystalline horse-serum albumin preparation (containing 0.3 % of hexose) appears to cross-react with the allergen (illustrated in Fig. 10A). Possibly this reaction is due to a serological relationship between the allergen and one of the horse-serum glycoprotein constituents such as seromucoid, which has been shown to contain 10.7 % of carbohydrate in the form of *N*-acetylglucosamine, *D*-galactose and *D*-mannose (Rimington & Van den Ende, 1940). These highly soluble substances tend to be co-precipitated with the albumin during salt-fractionation of serum.

Nevertheless, the apparent admixture of traces of horse-serum albumin and globulin with the dandruff constituents offers an alternative and simpler explanation of the serological relationship between the dandruff and serum antigens. It will be necessary to confirm these preliminary findings by carrying out more extensive precipitin tests with absorbed antisera. Squire (1950) rejected this mechanism on the basis of results obtained from skin tests on horse-sensitive asthmatics, and from the precipitin method of optimum proportions which does not allow the identification of antigens by direct comparison of their precipitin lines.

Although the isolation of an antigenically pure skin-reactive substance from horse dandruff is not claimed (extensive agar-diffusion tests on the most pure preparation revealed faint precipitin lines due to trace contaminants) the allergen preparation obtained is considered to be more pure than any other inhalant-type allergen yet isolated. Furthermore, all tests carried out were consistent with the hypothesis that skin reactivity is confined to this one constituent. In contrast, investigators of other allergen systems have found that activity is associated with several constituents. Augustin

(1953), for instance, has fractionated an ultra-filtrate of crude grass-pollen extract into four skin-reactive protein complexes, differing in electrophoretic mobility and carbohydrate and pigment content but apparently possessing the same qualitative composition. Multiple active peptide-carbohydrate complexes were fractionated chromatographically from dialysed ragweed-pollen extract by Frankel, Johnson, Schiele, Bukantz & Alexander (1955). House-dust extract has also been fractionated, by electrophoresis, into a pigmented and non-pigmented component of similar allergenic activity, both of which were revealed in a 'standard purified allergen' obtained by acid or acetone precipitation (Rimington, Stillwell & Maunsell, 1947). Recently Silver & Bookman (1956) have described, in a preliminary report, the paper-electrophoretic fractionation of acetone-precipitated horse-dandruff material into two major components, the slower of which was associated with skin reactivity. These workers estimated the molecular weight of the dandruff antigen to be approximately 40 000 from sedimentation measurements. In none of these cases, however, has the preparation of a chemically pure allergen been satisfactorily demonstrated.

A series of skin tests with the purified freeze-dried horse-dandruff allergen preparation is being planned in order to assess its suitability as a solid standard for materials used in the skin testing of horse-sensitive asthmatics. It is intended, also, to use this protein for hyposensitization purposes. Until larger numbers of patients have been tested quantitatively, however, it may be wise to carry out skin tests both with crude and with purified allergens in case some individuals are sensitive to some minor component, as is evidently the case with the horse serum-albumin component.

SUMMARY

1. The composition of horse-dandruff extract has been studied by means of physicochemical and immunochemical techniques, which have revealed the presence of at least seven protein or polypeptide constituents containing varying proportions of conjugated carbohydrate in addition to pigment and dialysable substances.

2. Electrophoretic analyses, both in solution and on paper, provided measures of the relative concentrations of the major protein constituents, which were lettered arbitrarily in order of decreasing mobility in veronal buffer at pH 8.6. Staining of the paper-strip patterns with periodic acid-Schiff's reagent revealed the distribution of protein-bound carbohydrate.

3. Fractional precipitation with saturated ammonium sulphate solution, between limits indicated

by preliminary quantitative fractionations, provided a series of precipitates containing varying proportions of the electrophoretic components.

4. All fractions were assayed by prick testing horse-sensitive asthmatics and allergenic activity was found to be associated with a single carbohydrate-rich constituent (designated 'b₂'), predominant in the fraction precipitated between 55 and 80 % saturation with ammonium sulphate and comprising 64 % of the total protein in whole dandruff extract. Additional evidence as to the nature of the allergen was obtained from skin tests with relatively pure constituents isolated by preparative electrophoresis on paper and in a starch column.

5. The precipitin line due to the allergen was identified in antigen patterns obtained by the horizontal gel-diffusion technique and detected in preparations of low skin reactivity, which appeared to be allergen-free according to the less sensitive electrophoretic analysis.

6. A very soluble mucopolysaccharide fraction (containing 48 % of hexose), and a crystalline protein (of similar electrophoretic mobility on paper to the allergen, but containing only 0.2 % of hexose), were found to be inactive.

7. A highly purified allergen preparation was obtained by the starch electrophoresis of the allergen-rich salt-precipitated fraction.

8. Preliminary chemical analyses showed the allergen to contain 9 % of hexose in the form of galactose and mannose, in addition to all the common amino acids. Its molecular weight, determined from osmotic pressure measurements, was found to be 34 000.

9. Evidence is provided that the serological relationship between horse dandruff and horse-serum antigens is probably due to the admixture of serum albumin and globulin with the dandruff constituents.

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REFERENCES

- Adair, G. S. (1949). In *Haemoglobin*, p. 191. Ed. by Roughton, F. J. W. & Kendrew, J. C. London: Butterworth Scientific Publications.
- Adair, G. S. & Robinson, M. E. (1930). *Biochem. J.* **24**, 993.
- Alexander, A. E. & Johnson, P. (1949). *Colloid Science*, vol. 2, append. 2. Oxford: Clarendon Press.
- Augustin, R. (1952). *Quart. Rev. Allergy*, **6**, 290.
- Augustin, R. (1953). *Biochem. J.* **54**, x.
- Augustin, R. (1955). *Quart. Rev. Allergy*, **9**, 504.
- Durum, E. L., Paul, M. H. & Smith, E. R. B. (1952). *Science*, **116**, 428.
- Elek, S. D. (1949). *Brit. J. exp. Path.* **30**, 484.
- Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
- Flodin, P. & Porath, J. (1954). *Biochim. biophys. Acta*, **13**, 175.
- Franglen, G. T. & Martin, N. H. (1954). *Biochem. J.* **57**, 626.
- Frankel, S., Johnson, M. C., Schiele, A. W., Bukantz, C. & Alexander, H. L. (1955). *J. Allergy*, **26**, 33.
- Friedmann, R. (1949). *Biochem. J.* **44**, 117.
- Gell, P. G. H. (1955). *J. clin. Path.* **8**, 269.
- Grabar, P. & Williams, C. A. (1953). *Biochim. biophys. Acta*, **10**, 193.
- Hardwicke, J. (1954). *Biochem. J.* **57**, 166.
- Horrocks, R. H. & Manning, G. B. (1949). *Lancet*, **1**, 1042.
- Kabat, E. A. & Mayer, M. M. (1948). *Experimental Immunochimistry*, p. 543. Springfield, Illinois: Thomas.
- Köiw, E. & Grönwall, A. (1952). *Scand. J. clin. Lab. Invest.* **4**, 244.
- Kunkel, H. G. & Tiselius, A. (1951). *J. gen. Physiol.* **35**, 89.
- Longcope, W. T., O'Brien, D. P. & Perlzweig, W. A. (1926). *J. Immunol.* **11**, 253.
- Longworth, L. G. (1942). *Chem. Rev.* **30**, 323.
- Ma, T. S. & Zuzaga, G. (1942). *Industr. Engng Chem. (Anal.)*, **14**, 280.
- Markham, R. (1942). *Biochem. J.* **36**, 290.
- Ouchterlony, O. (1948). *Acta path. microbiol. scand.* **25**, 186.
- Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
- Rimington, C., Stillwell, D. E. & Maunsell, K. (1947). *Brit. J. exp. Path.* **28**, 309.
- Rimington, C. & Van den Ende, M. (1940). *Biochem. J.* **34**, 931.
- Rowe, D. S. (1953). *J. Physiol.* **123**, 18 P.
- Silver, D. Z. & Bookman, R. (1956). *J. Allergy*, **27**, 127.
- Squire, J. R. (1950). *Clin. Sci.* **9**, 127.
- Stacey, M. (1946). *Advanc. Carbohydr. Chem.* **2**, 161.
- Stanworth, D. R. (1953). Ph.D. Thesis: University of Birmingham.
- Tiselius, A. & Kabat, E. A. (1939). *J. exp. Med.* **69**, 119.
- Turba, F. & Enenkel, H. J. (1950). *Naturwissenschaften*, **37**, 93.
- Werner, I. & Odin, L. (1949). *Uppsala LäkFören. Förh.* **54**, 69.
- Winzler, R. J., Devor, A. W., Mehl, J. W. & Smyth, I. M. (1948). *J. clin. Invest.* **27**, 609.