Immunology, 1959, **2**, 384.

Studies on the Physico-Chemical Properties of Reagin to Horse Dandruff

By D. R. Stanworth

Department of Experimental Pathology, University of Birmingham

Summary. Human allergic serum has been fractionated by:

(1) Chromatography on diethyl amino ethyl cellulose columns.

(2) Ultracentrifugation in buffered sucrose gradients.

Passive transfer tests with the fractions showed skin sensitizing activity to horse dandruff allergen to be associated with an electrophoretic gamma$_1$-globulin component possessing a 7S sedimentation constant.

The difficulties encountered in the use of the chromatographic procedure for obtaining potent reagin preparations are discussed.

INTRODUCTION

Physico-chemical studies of skin sensitizing antibodies have been mainly confined to the electrophoretic fractionation of allergic human serum and the subsequent association of reagin activity with one or more of the fractions thus obtained. Such an approach has not led to consistent findings as to the nature of the sensitizing antibody. For instance, free-solution electrophoresis (Cooke *et al.*, 1951) and electrophoresis-convection (Campbell *et al.*, 1954) studies indicated that reagins were not characteristically associated with the (major) serum γ-globulin component. On the other hand, zone electrophoresis has been used by Brattsten *et al.* (1955) and Kuhns (1954) to demonstrate the association of skin sensitizing antibodies with a 'fast γ-globulin' component ('γ_1').

More recently Humphrey and Porter (1957) have applied a relatively new technique of protein separation, developed by Peterson and Sober (1956), to the fractionation of allergic serum. This method, which involves chromatography on an anion-exchange resin derived from cellulose, has the special attraction of separating the γ globulin into four fractions which are eluted ahead of the other serum constituents. As in the electrophoretic procedures, proteins are separated mainly according to differences in the electrical charge on their molecules, but it is likely that additional factors influence chromatographic separation. Humphrey and Porter (1957) were able to show that reagin activity to both mixed grass pollens and horse serum was chiefly localized in the third fraction—electrophoretically a 'fast γ globulin'—obtained by chromatographic fractionation of allergic sera.

The work to be described was undertaken in an attempt to repeat these findings and examine the suitability of the chromatographic procedure for the production of preparations rich in reagin to horse dandruff allergen. In addition, ultracentrifugal and immunological analyses have been carried out on the chromatographic fractions from allergic serum in order to obtain some idea of the molecular size of the serum constituent(s) with which the skin sensitizing antibodies are associated. Independent evidence on this point has been acquired by application of a much milder technique to the fractionation of allergic serum, namely preparative ultracentrifugation in buffered sucrose gradients.

In order to reduce the risk of transmitting plasma jaundice during the biological assay

of skin sensitizing antibodies in humans, these investigations have been confined so far to serum from only one hypersensitive individual.

EXPERIMENTAL METHODS

MATERIALS

Blood was kindly donated by a female laboratory assistant aged 25 who has a long history of hay fever and who consistently gives strong wheal reactions when pricked with extracts of grass pollens, animal danders and many other common inhalants.

After allowing the blood (1 pint) to clot in an incubator at 37° C. for 4 hr. the serum was separated aseptically and stored frozen at −10° C. in 10 ml. portions. Judging from subsequent passive transfer tests, carried out over a period of 9 months, serum stored under such conditions retained its activity fully.

CHROMATOGRAPHY

The technique employed was essentially that described by Peterson and Sober (1956). Diethyl amino ethyl cellulose was prepared from 60 g. portions of Solka-Floc BW 100. The resultant batches of resin were characterized by determination of their titration curves in 0·15 M NaCl solution, from which the extent of incorporation of ionizing groups was calculated.

Five ml. samples of serum, equilibrated with starting buffer, were fractionated in columns (20 cm. long and 1·1 cm. in diameter) at 1° by stepwise elution with phosphate buffers of decreasing *p*H and increasing ionic strength. Three ml. eluates were collected at the rate of about one an hour.

ULTRAFILTRATION

Combined chromatographic eluates were concentrated by ultrafiltration through Visking dialysis tubing (inflated diameter = $\frac{3}{8}$ in.) by a small-scale modification of the technique evolved by Grant (1957), designed for the simultaneous concentration of several solutions (Grant, Rowe and Stanworth, 1958).

DETERMINATION OF PROTEIN CONCENTRATION

Owing to the scarcity of materials this was usually carried out by optical density measurement at 280 mμ in a 2 mm. cell in a Unicam SP 600 spectrophotometer. Protein concentrations were calculated from a calibration graph obtained by similar measurements on human serum γ-globulin solutions prepared from the freeze-dried solid.

ULTRACENTRIFUGATION

(*a*) *Preparative Ultracentrifugation*

One ml. samples of cooled allergic serum were fractionated in phosphate buffer (*p*H 6·9, I = 0·06 containing 0·15 M NaCl)–sucrose density gradients according to a modification of the technique described by Edelman *et al.* (1958). Ultracentrifugation was carried

out in a Spinco model L machine*. Sedimentation was continued for eight hours at 4° C. in lusteroid tubes (5 ml. capacity) in a swing-out rotor (SW 39) at 39,000 r.p.m. As soon as the rotor had stopped the tubes were removed without disturbing their contents and sliced in a mechanical slicer, similar to that developed by Randolph and Ryan (1950) and made by Dr. E. Schuster†. Four fractions, comprising about 85–90 per cent of the starting liquid volume, were usually recovered.

Selection of the positions at which the tubes were to be sliced was guided by including, in one tube of each run, serum containing a trace of Evan's Blue (to label the albumin) and 0·1 ml. of a human serum γ-globulin solution (1 g./100 ml.) labelled with the fluorescent dye lissamine rhodamine B.200 (Chadwick *et al.*, 1958).

(*b*) *Analytical Ultracentrifugation*

was carried out at 20° C. in a Spinco model E machine with a Philpot-Svensson optical system incorporating a phase-plate. Relative compositions were determined from enlarged tracings of the schlieren patterns. After 'fitting' the base line obtained from analysis of the appropriate solvent the areas under the curves were calculated by the method of 'counting squares'.

ASSAY OF SKIN SENSITIZING ACTIVITY

Test solutions were sterilized by centrifugal filtration through Oxoid‡ membranes in Hemming filters. Sterility checks were made on the filtrates.

Passive transfer tests by the Prausnitz-Kustner technique (P-K reaction) were mainly carried out on a single subject—a medical volunteer—who showed no hypersensitivity to the allergenic test solution. 0·1 ml. volumes of serum or serum fractions were injected intradermally into duplicate sites on the back or chest and after 24 hours these were challenged by pricking in a solution of horse dandruff allergen (prepared by the method previously described; Stanworth, 1957) containing about 0·5 protein per 100 ml. or commercial horse dandruff extract (Bencard). After 10 and 20 min. intervals the wheal outlines were traced on celluloid and their mean diameters calculated from the areas of enlarged tracings.

Control prick tests with the allergen solution and 1/100 histamine solution were carried out each time.

A consistency plot of the log (diameters) of 70 pairs of wheals against the log (mean diameters) gave a linear chart indicating that the coefficient of variation of the mean wheal diameter was independent of the size of the wheal. Analyses of variance on 10 and 20 min. wheal diameters (70 pairs) after logarithmic transformation indicated a coefficient of variation of $\pm$ 26 per cent in each case. A similar order of variation was observed by Squire (1950) in an analysis of the results of pricking histamine solutions into the arms of human subjects.

RESULTS

(A) CHROMATOGRAPHIC FRACTIONATION OF ALLERGIC SERUM

Typical elution patterns of allergic serum and a normal serum control are compared in Fig. 1. It will be seen that the two patterns, which are readily reproducible, are very

* Beckman Instruments Inc., Spinco Division, Palo Alto, California. † Address: 12 Chadlington Road, Oxford.
‡ Manufactured by Oxo Ltd., London.

similar. A larger fraction (I) of non-adsorbed material followed closely by a minor fraction (II) was eluted by the first buffer solution (*p*H 7·6). Two further fractions (III and IV) were obtained with the second buffer (*p*H 6·2), the concentration peak representing the first of these fractions being sharp though asymmetric, whilst the fourth peak was usually much broader.

The eluates comprising each of these four peaks were combined and concentrated by ultrafiltration. Owing to lack of clear-cut fractionation on further elution with the third solvent (0·05 M NaH_2PO_4) combination of these eluates, after consideration of the corresponding part of the curve, was more arbitrary as indicated by the short vertical lines. These provided, on ultrafiltration, fractions V–XI.

Results obtained by assaying the skin sensitizing activities of the chromatographic fractions separated from 5 ml. of allergic serum are shown in the form of a histogram in

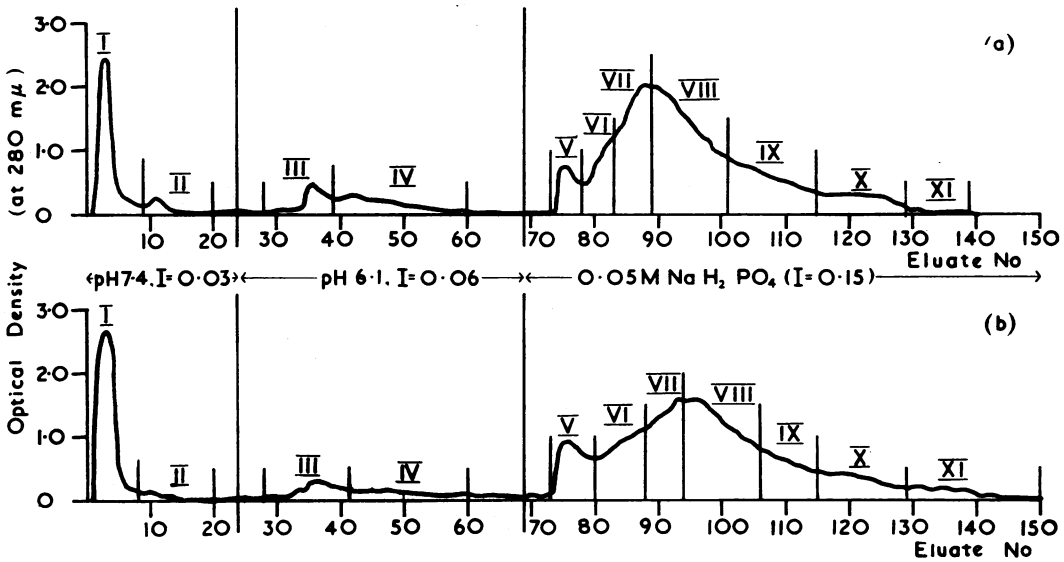

FIG. 1. Chromatographic patterns of allergic (*a*) and normal (*b*) human serum. In each case 5 ml. serum was fractionated on a column (20·0 × 1·1 cm.) of DEAE cellulose containing 0·82 m.moles ionizing groups/g.resin. Eluates (3 ml.) were combined as indicated by the short vertical lines to give fractions I–XI.

Fig. 2. 1·5 ml. concentrates of fractions I–X and five-fold dilutions of each were tested in duplicate, together with neat allergic serum and its dilution.

A comparison of the distribution of reagin activity shows fraction III to be clearly the most active, although there is some spread of activity into other fractions. When, however, one takes into consideration the protein contents of the test solutions it becomes obvious that fraction I is relatively unreactive and the spread of activity from fraction V onwards is almost negligible. Moreover, the results of the tests with the dilute solutions indicate that the activities of fractions II and IV are considerably lower than the activity of fraction III. This becomes apparent by comparing the mean wheal diameters of the wheals elicited by the dilute fraction III solution, containing approximately 100 mg. protein per 100 ml., with the diameters of the appreciably smaller wheals elicited by the concentrated solutions of fractions II and IV (containing 200 mg. and 500 mg. protein per 100 ml. respectively).

Several chromatographic fractionations of allergic serum were carried out, but it was not always easy to reproduce the results described above. This was due partly to difficulty in obtaining complete control of the many factors affecting chromatography. Thus, although the chromatographic pattern shown in Fig. 1 was readily repeatable, a clear-cut fractionation of skin-sensitizing antibodies was not always achieved. In some cases, for instance, there was a shift of the activity maximum to a fraction adjacent to fraction III. The other main difficulty was the substantial loss in reagin activity which often accompanied the processes of fractionation and subsequent concentration. This factor will be considered in a later section dealing with the stability of skin sensitizing antibodies.

The results given in Fig. 3 illustrate the variability of the chromatographic procedure for fractionating skin sensitizing antibodies. On this occasion 3 ml. of allergic serum was

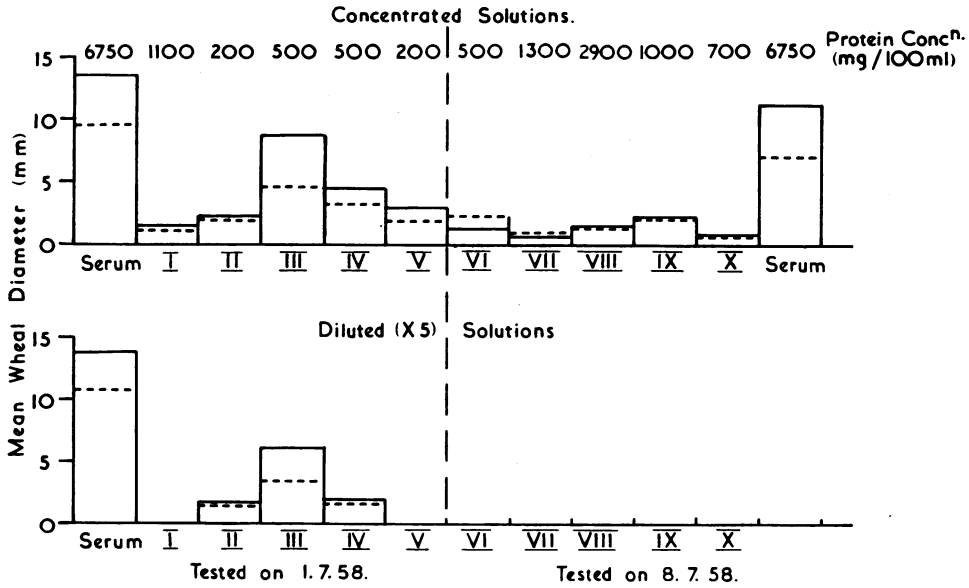

FIG. 2. Distribution of reagin to horse dandruff amongst allergic serum chromatographic fractions I–X. Ten-minute wheal diameters are represented by the horizontal dotted lines and 20-minute diameters by the heights of the blocks.

chromatographed on a shorter column (15 × 1·0 cm.) than that used in the run described earlier. Another difference was the elution with buffers saturated with toluene, a practice adopted by previous users of the chromatographic technique. As usual 0·1 ml. of each solution was injected into duplicate sites on the back of a 'normal' recipient, but this time only one site of each pair was challenged with horse dandruff allergen solution. The other was tested with a commercial grass pollen extract (Bencard) to which the test subject was found to show a slight sensitivity.

As will be seen from Fig. 3, a clear-cut fractionation of reagin to horse dandruff allergen was not achieved on this occasion. Fraction III and IV solutions both appeared to possess the same activity, but only one of these, namely fraction III, showed activity on 5-fold dilution. On the other hand, more consistent results were obtained from the tests with mixed grass pollen extract. In this case the distribution of reagin amongst the

concentrated solutions of fractions I–IV was parallel to that obtained from the transfer of the diluted (5-fold) solutions. Fraction III showed maximal activity to the pollen allergen, although the relative activity of fraction I was higher than that usually recorded in the distributions of reagin to horse dandruff allergen.

Obviously it is not wise to draw any firm conclusions from the results of single PK tests, particularly as the subject undergoing such tests showed some reactivity to one of the allergens used. It seems unlikely, however, that there is any differentiation between the fractionation of the horse dandruff and pollen reagins on the DEAE-cellulose column.

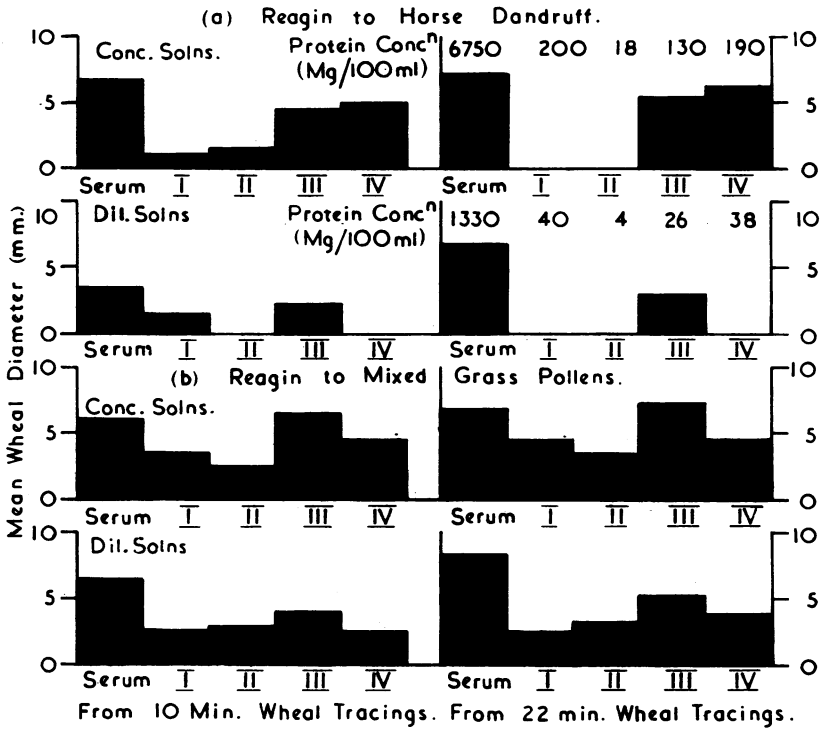

FIG. 3. Distribution of reagins to horse dandruff (*a*) and to mixed grass pollens (*b*) amongst the same allergic serum chromatographic fractions (I–X).

Electrophoretic Analysis of Chromatographic Fractions

The electrophoretic compositions of chromatographic fractions I–X (see Fig. 1) were compared by analysis on wide sheets of Whatman 3 MM paper in barbitone buffer (pH 8·6, $I = 0{\cdot}05$). A similar distribution to that reported by Peterson and Sober (1956), who analysed each fraction separately, was revealed.

As is seen in Fig. 4, there was a progressive increase in mobility of the γ-globulin component from fraction I–III. From fraction IV onwards this electrophoretic component was present in only minor amounts as compared with the predominant faster-moving globulins. It was difficult, therefore, to compare the relative mobilities of the γ-globulin component of fractions IV–X by paper electrophoresis. This difficulty was partially overcome by means of comparative immunoelectrophoretic analysis employing specific

anti-human γ globulin serum prepared in rabbits. The results of such an analysis on chromatographic fractions I–X are shown in Fig. 5.

The relative mobilities of the γ-globulin components in the different fractions were

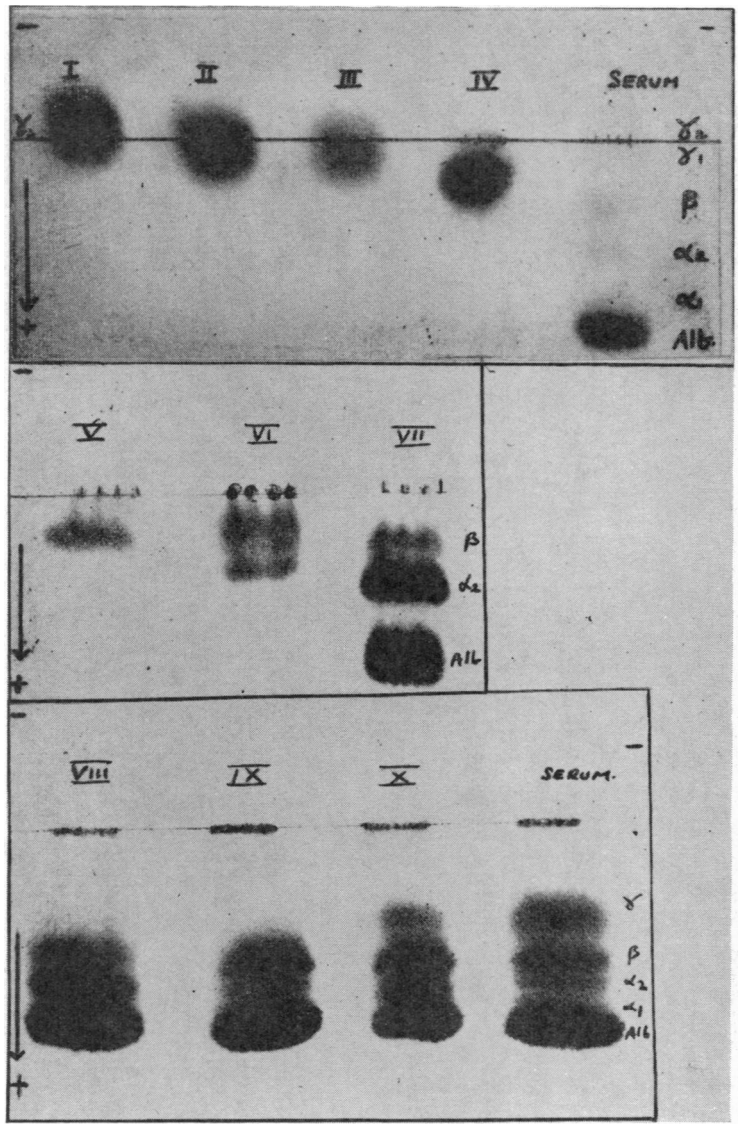

FIG. 4. Comparative paper-electrophoresis of allergic serum chromatographic fractions (I–X) on wide sheets (47 × 22 cm.) of Whatman 3 MM paper. Electrophoresis was carried out in barbitone buffer (pH 8·6, I = 0·05) under a current of 15 ma. for 6 hours and the paper stained with bromophenol blue.

estimated by comparing the distances of the precipitin lines from the starting point. This method is somewhat inaccurate owing to difficulty in establishing the mid-positions of the precipitin arcs, some of which were asymmetric or incomplete. This was attempted, however, by constructing perpendiculars to the tangents of the arcs on a tracing of the

precipitin lines (Fig. 5b). Measurements were made from these perpendiculars to a common line (o—o) transversing the back edges of the antigen cups at right angles to the direction of electrophoresis.

Whilst recognizing the arbitrary nature of this procedure, and ignoring any effects differences in protein concentration might have on the shape of the lines, certain con-

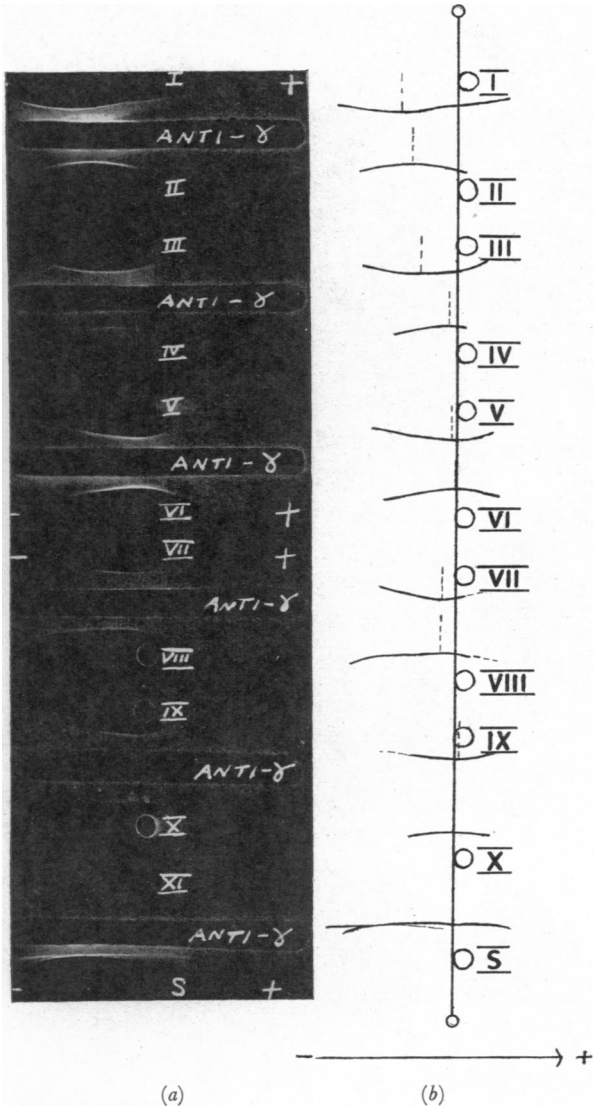

FIG. 5. Immunoelectrophoresis of allergic serum and its chromatographic fractions (I–X) in 0·8 per cent agar in barbitone buffer (*p*H 8·6, I = 0·05). A current of 5 ma. was passed for 18 hours.
(*a*) Precipitin lines obtained with rabbit antiserum specific to human serum γ-globulin.
(*b*) Tracing of precipitin lines constructed for direct comparison of their positions relative to a common starting line (o—o).

clusions have been drawn from the results obtained. One point that emerged was that the progressive increase in mobility of the γ-globulin component of fractions I–III revealed by paper electrophoresis was not continued throughout the whole series of chromatographic fractions. It will be noticed, for instance, that the γ-globulins of fractions VII and VIII are slower than those of adjacent fractions VI and IX.

According to the degree of displacement of their precipitin lines (in the direction of endosmotic flow) the γ-globulins could be classified in the following order of increasing electrophoretic mobility:

Fraction I <II <III <VIII <VII <IV <V <VI <IX
X

Hence by comparing the skin sensitizing activities of the fractions referred to in Fig. 2 with the above classification (obtained by analyses on a different series of chromatographic fractions) and by taking into consideration the results of the paper electrophoretic analyses of the early fractions (I–IV), it could be inferred that reagins to horse dandruff allergen—concentrated in fraction III—are associated with relatively slow γ_1-globulin*. (Fraction I, which moves much more slowly than all the other γ-globulin components on paper electrophoresis, is considered to be a γ_2 component.) The elongation and asymmetry of some of the precipitin lines were interpreted as an indication of heterogeneity of the corresponding γ-globulin components—possibly due to contamination with fraction III component.

Gel-diffusion precipitin analyses were also carried out with a mixed anti-(α+β)-globulin serum. Fractions I and II were shown to be completely free from α- and β-globulin contaminants. A trace of β-globulin was detected, however, in Fraction III and considerably more of the same component was found in Fraction IV (as would be expected from the comparative paper-electrophoretic analyses). A specific rabbit antiserum was used to identify this antigen as siderophilin. It seemed unlikely, therefore, that skin sensitizing activity was associated with a β-globulin contaminant rather than with the γ_1-globulin in the chromatographic fractions.

Ultracentrifugal Analysis of Chromatographic Fractions

The distributions of the S7 (γ-globulin-containing) component and the S19 component in the allergic serum chromatographic fractions were determined by ultracentrifugal analysis in phosphate buffer (pH 7·2, I = 0·3). Some of the patterns obtained are shown in Fig. 6.

Fractions I and II proved to be entirely composed of S7 type component, a finding which was substantiated even when solutions of protein concentrations as high as 1·4 g./100 ml. were analysed. Fraction III was almost entirely composed of this component together with a trace of more slowly sedimenting material. The S7 component content of the other fractions analysed decreased sharply from 10 per cent in fraction IV, which was mainly composed of S5 material (probably siderophilin, which was detected immunologically) to 3 per cent in fraction X. The $S_{20,w}$ value of the major component ('S7') of fraction III, at a protein concentration of 660 mg./100 ml., was found to be 6·75. This value was the same as the $S_{20,w}$ value obtained from measurements of the sedimentation constant of fraction I at a similar protein concentration, suggesting that the electrophoretic components γ_1 and γ_2 (comprising fractions III and I respectively) were of similar molecular weights.

No S19 component was detected in fractions I–IV, in which the bulk of the reagin activity was eluted. This component first appeared in fraction V (as shown in Fig. 6), which was not obtained until elution with the third solvent (0·05 M NaH_2PO_4) had

* The distinction between γ_1- and γ_2-globulins is made solely on the basis of electrophoretic mobility without any implication with regard to possible other differences.

started. This finding was confirmed by application of a more sensitive technique for estimating S19 material, namely immunological analysis with specific rabbit antisera to both α_2 glycoprotein and iso-agglutinin—the two main constituents of the high mol. wt. ultracentrifugal component of human serum.

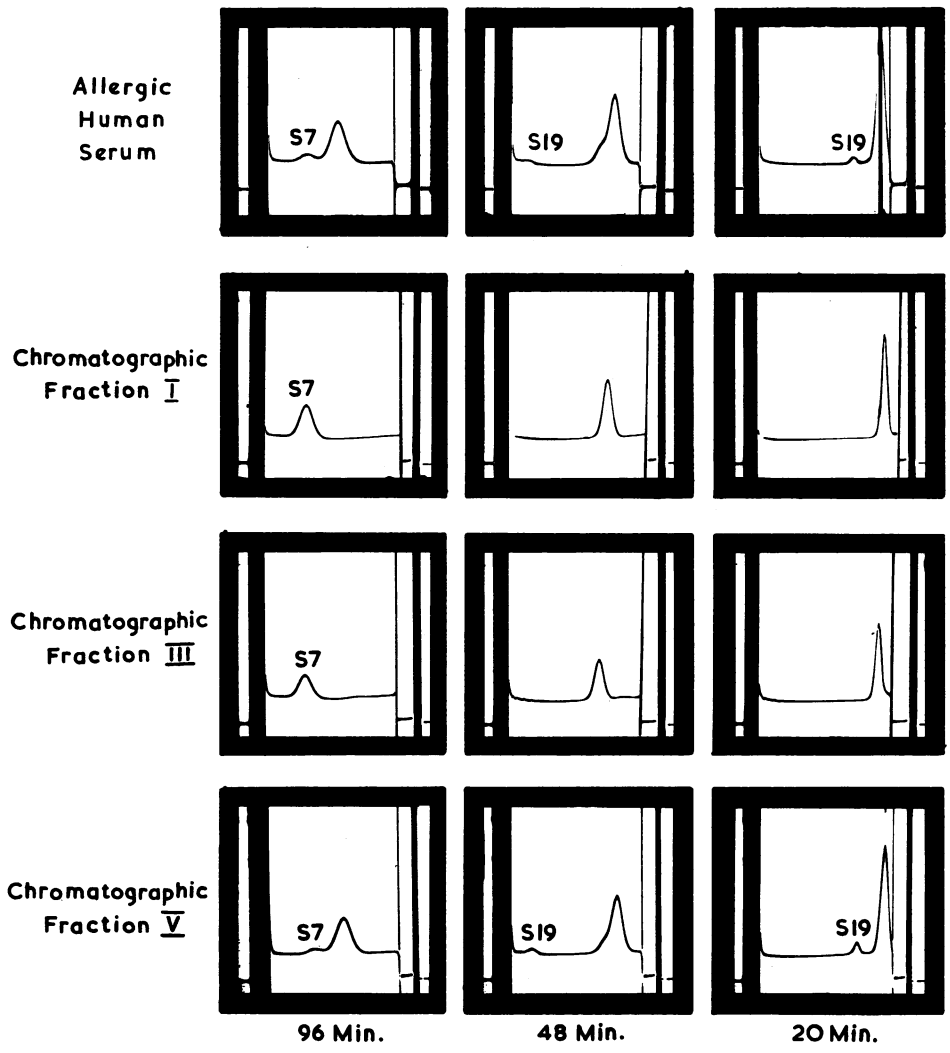

FIG. 6. Ultracentrifugal patterns of allergic serum (diluted to contain 1·4 g. protein/100 ml.) and chromatographic fractions I, III and V (in solutions containing 0·7 g. protein/100 ml.). Solvent: phosphate buffer (*p*H 7·2, I = 0·3); speed: 60,000 r.p.m.; temp. 20° C.

More recently these observations have been confirmed by the results of full-scale ultracentrifugal and quantitative gel-diffusion precipitin analyses of all 13 fractions obtained in another complete chromatographic elution of the allergic serum. This particular investigation was undertaken as part of an extensive study of the nature of human serum isoagglutinins (Ellis *et al.*, 1959). The results obtained are summarized in

Fig. 7, which shows the distribution of S7-γ-globulin, S19-α_2-glycoprotein, and S19-isoagglutinin (termed 'Iota' protein) components amongst the 13 fractions. (It was judged to be worth while combining eluates to give extra fractions on this occasion because of better resolution on elution with the third solvent.) Each of these fractions was concentrated by freeze-drying rather than by ultrafiltration and resuspended in buffered saline to provide as concentrated solutions as possible.

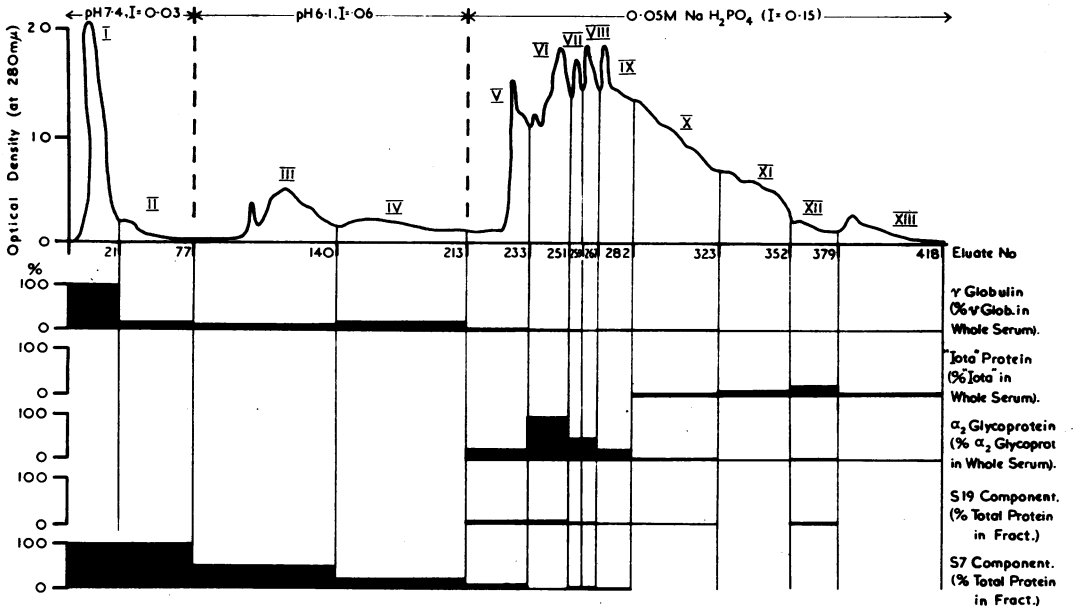

FIG. 7. Distribution of gamma globulin, α_2-glycoprotein and 'Iota' protein amongst chromatographic fractions I–XIII obtained by fractionation of 5 ml. allergic serum on a column (18 × 1·1 cm.) of DEAE cellulose resin (containing 0·72 m.moles ionizing groups/g.). The concentrations of these components, estimated immunologically, are expressed as fractions of their concentrations in whole serum. (See Ellis *et al.* 1959)

Fig. 7 also demonstrates that once again no S19 component was detectable, either by ultracentrifugal or immunological analysis, in the fractions (I–IV) eluted by the first two solvents (pH 7·6 and pH 6·2).

Stability of Skin Sensitizing Antibodies

The instability of the activity of reagin-containing chromatographic fractions was apparent throughout this work. For instance, although the activity of whole serum was found to be unaffected by prolonged storage at −10° C. in the frozen state and by storage at least for a few days in the freeze-dried form, isolated reagin-containing fractions appeared to be much more susceptible to these treatments. Another factor found to be contributing to the appreciable losses of skin sensitizing activity recorded during PK tests on most series of chromatographic fractions was the technique used for concentrating these fractions. This was established by diluting a sample of the allergic serum with 50 volumes of 0·15 M NaCl solution and concentrating it back to its original volume by ultrafiltration through Visking dialysis tubing. Comparison of the reaginic activities of this material with untreated serum revealed that about 50 per cent of the activity of whole

serum was lost during the concentration process. On the other hand, this loss was reduced to about 30 per cent by simultaneous dialysis with 0·15 M NaCl solution during ultrafiltration.

It is conceivable that isolated reagin-containing fractions, possibly deprived of some protective factor, would be even more susceptible than whole serum to the ultrafiltration process. This fact has yet to be established. It is also necessary to determine whether any serious losses in activity occur during the actual chromatographic process.

Recovery of γ-globulin from Chromatographic Columns

The approximate recovery of γ-globulin in the chromatographic fractionation illustrated in Fig. 7 was calculated from both the quantitative immunological and ultracentrifugal estimates of the protein in the various fractions. These values are listed in Table 1 together with the recoveries of total protein.

TABLE 1

RECOVERY OF PROTEIN FROM A CHROMATOGRAPHIC COLUMN (18·0 × 1·1 CM.) OF DEAE CELLULOSE RESIN (CONTAINING 0·72 M.MOLES IONIZING GROUPS/G. RESIN)

	Total protein		*Gamma globulin (mg.)*	
	Solute (mg)	*Vol. of soln. (ml.)*	*From ultracentrifugal analysis*	*From immunological analysis*
Amount of protein applied to column (as serum) ..	290	5	45	46
Amount of protein eluted	160	500	19	20
% recovery ..	55		42	43

Both methods of estimation revealed a rather high loss of γ-globulin (about 57 per cent), which was partly due to losses occurring during the freeze-drying. Two difficulties were encountered in this process, which involved the drying of relatively large volumes of dilute solutions. First there was the loss of some dried protein into the vacuum line and, secondly, it was not easy to obtain complete recovery of the small yields of dried material which were left spread over large areas of the flasks.

Better recoveries of γ-globulin were recorded in fractions which were concentrated by ultrafiltration. Calculations of recoveries in fractions I–V from several fractionations, obtained from optical density and ultracentrifugal measurements on the ultrafiltrates, indicated losses of about 30 per cent γ-globulin on the DEAE cellulose columns. Such a finding, which might also reflect losses of protein on the dialysis tubing during ultrafiltration, must be taken into consideration in attempts to account for poor recoveries of reagin activity by the chromatographic procedure.

(B) ULTRACENTRIFUGAL FRACTIONATION OF ALLERGIC SERUM

Ultracentrifugal fractionation of the allergic serum was first attempted in a salt gradient. The design of the experiment is shown in Fig. 8 and the results obtained are given in

Table 2. Although good recoveries of reaginic activity were recorded there was no clear-cut resolution of the S19 component from the S7 component in the three serum fractions analysed. It was not possible, therefore, to decide with which of these two ultracentrifugal components the skin sensitizing antibodies were associated.

With a view to increasing the resolution of S19 from S7 component a smaller depth of serum relative to the depth of solvent was used in the next fractionation, which was carried

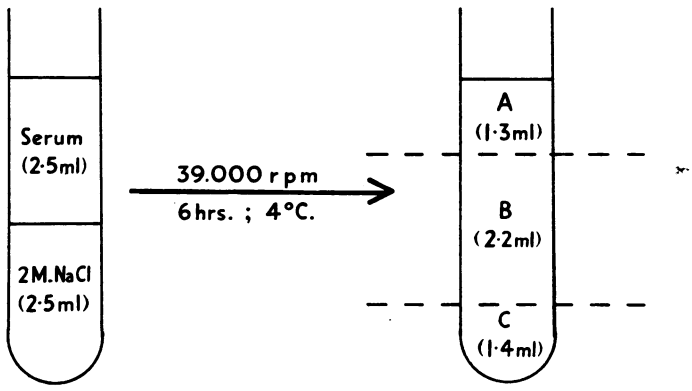

FIG. 8. Scheme for preparative ultracentrifugal fractionation of allergic serum in a salt gradient in lusteroid tubes (scale = 1 : 1).

TABLE 2

ULTRACENTRIFUGAL COMPOSITIONS AND REAGIN CONTENTS OF FRACTIONS OBTAINED BY DIFFERENTIAL SEDIMENTATION OF ALLERGIC SERUM IN A SALT GRADIENT (AS ILLUSTRATED IN FIG 8).

Fraction	*Volume of soln. (after dialysis) ml.*	*Ultracentrifugal composition*			*Mean wheal diameter (mm.)*	
		S19 Comp. mg./100 ml.	*S7 Comp. mg./100 ml.*	*S4.5 Comp. mg./ 100ml.*	10 *min.*	20 *min.*
Serum (neat)	—	270	1,013	5,467	7·7	9·3
A ..	1·4	0	101	1,019	2·7	3·1
B ..	2·6	81	295	1,414	7·3	7·0
C ..	1·7	112	318	1,440	8·2	10·0
Diluted solutions (Dil. 1 in 5)		54	203	1,098	4·7	5·5
A/10 ..		0	10	102	0·0	0·6
B/10 ..		8	30	141	1·7	2·7
C/10 ..		11	32	144	2·8	2·8

out in a sucrose density gradient according to a modification of the method used by Edelman *et al.* (1958) to obtain samples of S19 component from rheumatoid arthritis sera. The design of a preliminary experiment using this technique was similar to that finally adopted (illustrated in Fig. 9), except that aqueous solutions of sucrose were first employed. Unfortunately, however, although this procedure resulted in good resolution of S19 from S7 component, almost the whole of the skin sensitizing activity of the serum was destroyed.

This problem was overcome by employing phosphate buffer (*p*H 6·9, $I = 0{\cdot}06$) containing 0·15 M NaCl solution instead of distilled water as the solvent for the preparation of the 10 per cent and 30 per cent sucrose solutions. One ml. portions of sera were fractionated by ultracentrifugation in buffered sucrose gradients as illustrated in Fig. 9. After 8 hours at 39,000 r.p.m. the lusteroid tubes were sliced in the positions shown. The resultant fractions (A–D) were dialysed together with whole serum controls against 1 litre of phosphate buffer (*p*H 6·9, $I = 0{\cdot}06$) containing 0·15 M.NaCl at 1° C. overnight. Removal of sucrose from fractions B, C, and D by this process was, of course, accompanied by dilution of the solutions in the dialysis sacs. Distribution of skin sensitizing antibody to horse dandruff allergen amongst the fractions is shown in the form of a histogram in Fig. 10, which also includes the ultracentrifugal compositions for comparison. The results of analysis of the bottom fraction (D) from a duplicate tube are included, this fraction being referred to as fraction 'D''.

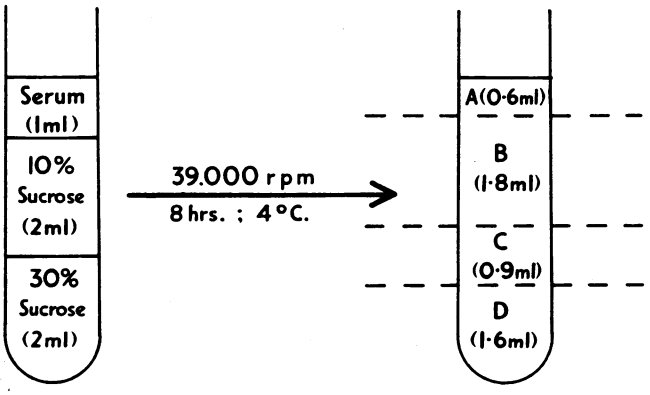

FIG. 9. Scheme for preparative ultracentrifugal fractionation of allergic serum in a buffered sucrose gradient in lusteroid tubes (scale = 1 : 1).

In addition to neat serum, controls were prepared by diluting portions of serum (1 : 1) with phosphate buffer, buffered 10 per cent sucrose and buffered 30 per cent sucrose and dialysing (as described above) in order to check the effect of the solvents on skin sensitizing activity. Passive transfer tests were also carried out with 5-fold dilutions of these controls and of the solutions of the ultracentrifuge fractions.

The contents of the S7 and S19 components in the serum and fractions were calculated from enlarged tracings of the ultracentrifuge patterns, taking the optical densities at 280 mμ of the solutions recovered from the ultracentrifuge cell to be representative of total protein concentration. Although this procedure does not take into account differences in the extinction coefficients of the constituent proteins, it revealed distributions of the serum ultracentrifugal components that were confirmed by quantitative gel-diffusion precipitin analysis (Gell, 1957) with specific antisera to γ-globulin, α_2-glycoprotein and 'isoagglutinin' referred to earlier.

Moreover, microkjeldahl N estimations (by the method of Ma and Zuazaga, 1942) carried out on one series of ultracentrifugal fractions (A–D), obtained in a repeat experiment, showed a similar distribution of total proteins to that provided by the optical density measurements, although these appear to have underestimated the protein contents

of fractions B and C (as is shown by the results of estimations on the tube 2 fractions, recorded in Table 3).

The results illustrated in Fig. 10 and also those of the repeat experiment (given in Table 3) reveal a striking correlation of skin sensitizing activity with the distribution of

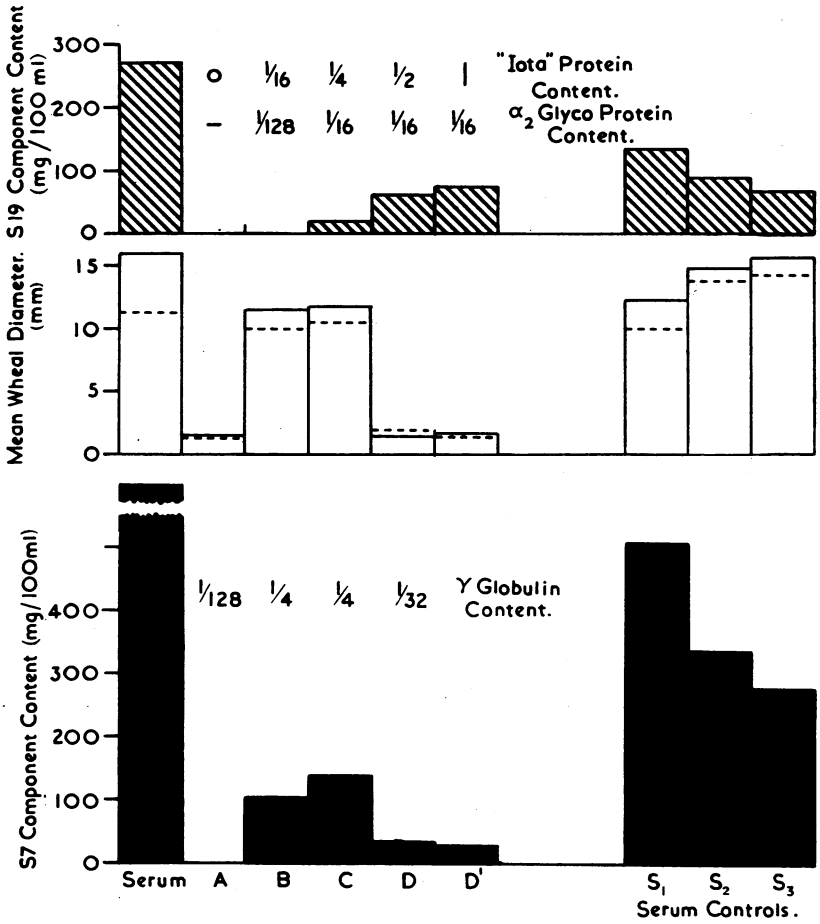

FIG. 10 Comparison of the reaginic activities (to horse dandruff) of allergic serum ultracentrifuge fractions (A–D) with their concentrations of S19 and S7 components. Fraction 'D' was the bottom fraction from a duplicate tube. Ten-minute wheal diameters are represented by the horizontal dotted lines and 20-minute diameters by the heights of the blocks. The concentrations of γ-globulin, 'Iota' protein and α_2-glycoprotein, estimated immunologically, are expressed as fractional concentrations of whole allergic serum.

The results of similar analyses on portions of serum diluted (1 : 1) with phosphate buffer (S_1), buffered 10 per cent sucrose (S_2) and buffered 30 per cent sucrose (S_3) are included. All solutions (ultracentrifuge fractions and controls) were dialysed against phosphate buffer (*p*H 6·9, I = 0·06) containing 0·15 M NcCl before being tested.

S7 (γ-globulin) component in the preparative ultracentrifuge fractions. There is no such correlation with either of the other two serum ultracentrifugal components. For instance, the S19 component is concentrated in fraction D, which shows extremely low reagin activity. Similarly it is quite impossible to assign reagin activity to the S4.5 component.

These findings were confirmed by testing diluted (5-fold) solutions of fractions A–D. As with the concentrated solutions, 0·1 ml. portions were injected into duplicate sites on the back of a normal recipient. On one occasion, however, only one site of each pair was later challenged with horse dandruff allergen solution, the other being tested with mixed grass pollen extract (Bencard). The results obtained (also shown in Table 3) indicated that the reagins to grass pollen possessed similar sedimentation properties to the reagins to horse dandruff in being associated with the S7 component. (The test subject's slight hypersensitivity to grass pollen must, of course, be taken into account in comparing the activities of the fractions given in Table 3.)

TABLE 3

RESULTS OF A REPEAT FRACTIONATION OF HUMAN ALLERGIC SERUM (1 ML./TUBE) BY DIFFERENTIAL SEDIMENTATION IN A BUFFERED-SUCROSE GRADIENT (AS ILLUSTRATED IN FIG. 9)

Fraction	*Volume recovered (ml.)*	*Volume after dialysis (ml.)*	*Protein conc. from OD measurement mg./100 ml.*	*Protein conc. from N. estimation mg./100 ml.*	*Ultracentrifugal composition (mg./100 ml.)*			*Mean wheal diameter (mm.)*		*Wheal diameter of diluted (×5) solutions at 20 min. (mm.)*	
					S19	*S7*	*S4·5*	*At 10 min.*	*At 20 min.*	*v. Horse dander*	*v. Grass pollen*
SERUM (dil. 1 : 1 with buffered saline)	0·5	0·7		(2210)	(88)	(332)	(1790)	9·7	11·1	8·4	9·3
TUBE 1											
A	0·7	0·8	(250)		0	0	250	0·6	0·7	0·7	2·2
B	1·8	2·6	950		6	128	816	10·8	11·5	7·0	5·3
C	0·7	1·4	450		47	121	282	6·5	9·3	5·7	3·8
D	1·0	2·4	80		55	25	0	1·4	0·2	0·0	2·9
CONTROL PRICK TESTS										0·2	2·9
TUBE 2											
A	0·7	0·7	250	250							
B	2·0	2·3	1100	1640							
C	0·3*	0·6	350	430							
D	0·7*	1·5	90	90							

* Unusually large proportion of these fractions lost (during slicing of tube).

Recovery of total protein from ultracentrifugal fractionations of allergic serum (1 ml. portions) was calculated to be of the order of 80–85 per cent, after allowing for the loss of liquid occurring during the slicing of the tubes after sedimentation. Assuming the S7 component was entirely composed of γ-globulin, about 90 per cent of this protein was recovered. The assumption seems justified since the other main serum electrophoretic components, demonstrated in the ultracentrifugal fractions (A–D) by paper electrophoresis, are known to sediment with the S19 and S4.5 components.

DISCUSSION

The results obtained by the chromatographic fractionation of reagin to horse dandruff tend to confirm the findings of Humphrey and Porter (1956), who studied reagins to grass

pollens and horse serum, in showing that reagin activity is confined mainly to the earlier fractions eluted from DEAE cellulose columns and is maximal in fraction III. From immunoelectrophoretic studies it is possible to explain such a distribution of activity in terms of the association of reagin with a γ_1-globulin component. This component has been classified as a 'slow' γ_1-globulin to distinguish it from the faster γ_1-globulin detected by immunoelectrophoretic analysis of other relatively inactive serum fractions.

On the other hand, there is no convincing evidence of the association of reagin to horse dandruff with more than one individual electrophoretic component. The spread of small amounts of activity into chromatographic fractions composed chiefly of faster moving electrophoretic components (β-globulin, α-globulin, etc.) is probably due to contamination with traces of γ_1-globulin only detectable by a sensitive immunological technique.

This is not surprising when one considers that the γ-globulin precipitin line in immunoelectrophoretic patterns of whole human sera extends through the β-globulin region (Grabar and Williams, 1955). It seems possible, therefore, that the claims made by some earlier workers of association of reagin activity with more than one serum electrophoretic component were due to the failure of the insensitive techniques available at that time to reveal the spread of small amounts of a single active constituent into several fractions.

As mentioned earlier, it was not easy to achieve reproducible fractionations of reagin by the chromatographic procedures, because the distribution of activity appeared to be readily susceptible to comparatively slight changes in the conditions of elution. It was possible, for instance, to shift the activity maximum to earlier fractions than fraction III by using solvents containing 0·03 M NaCl solution. As this shift was not accompanied by any discernible change in the chromatographic pattern it was assumed that the reagin molecules formed a minor component of the γ_1-globulin fraction (or possibly they combine with the globulin molecules). Insufficient immunoelectrophoretic analyses have been carried out yet to establish whether shifts in the reagin distribution accompany shifts in the distribution of 'slow' γ_1-globulin amongst the early chromatographic fractions.

The main disadvantage in using the chromatographic procedure for the preparation of skin sensitizing antibodies has been, however, losses of activity, revealed in subsequent passive transfer tests. It is unfortunately necessary to concentrate the chromatographic fractions before their testing and it is at this stage that most of the losses seemed to have occurred. Experiments are now in progress to find ways of eliminating these losses, and also any losses which might be found to occur during the actual chromatographic process. As mentioned earlier, the activity of the skin sensitizing antibody in the serum appears to be much more stable than that of isolated fractions. This could be due to the loss of a serum protective factor during fractionation.

It seems probable, too, that any abrupt change in the ionic environment of the skin sensitizing antibody proves deleterious. This was revealed by the drastic losses of activity of the serum fractions which had been sedimented into sucrose solutions prepared by dissolving the sugar in distilled water. A similar, less deleterious effect might be operative during elution of serum fractions from the chromatographic columns with solutions of low ionic strength. It was interesting to find, however, that sedimentation of the allergic serum in buffered sucrose gradients lead to good recoveries of reagin activity.

The ultracentrifugal analyses on both the chromatographic and preparative ultracentrifuge fractions have clearly demonstrated reagin activity to horse dandruff allergen to be associated with the S7 component and not with the S19 components. In the analysis of the chromatographic fractions, S19 component could not be detected in fractions I–IV

which contained the bulk of the reagin activity; a finding supported by employing an extremely sensitive immunological technique for detecting high molecular weight protein constituents. This means that even if the reagin molecule, probably present in the allergic serum and fractions as only a trace component, were to be an S19 type component, it was being eluted from the chromatographic column far in advance of the other high molecular weight protein which also moves in the γ_1-globulin position on electrophoresis, namely the isoagglutinin type protein (see Fig. 7).

These findings contradict those recently reported by Sehon (1958), who studied the sedimentation of reagins to ragweed pollen extract in a partition ultracentrifuge cell and found the active molecules to have a sedimentation constant of approximately 16·5S. Although reagins to a pollen allergen rather than a dandruff allergen were involved in this case it seems unlikely that the size of the sensitizing antibody is dependent on the nature of the allergen, particularly as reagins to both horse dandruff and to mixed grass pollen were shown to be associated with the S7 (γ-globulin) component (see Fig. 3 and Table 3). In other words, there is no evidence against the skin-sensitizing antibodies being of similar size to the precipitating antibodies found in human serum.

Although the preparative ultracentrifuge fractions, obtained by a mild process, retain their activity well they are very heterogeneous, even the most active fractions being contaminated with albumin and other serum constituents. The processing of such fractions in the preparative ultracentrifuge could, however, in theory lead to the isolation of mainly pure S7 component. On the other hand, the chromatographic procedure not only provides relatively homogeneous γ-globulin but it is capable of further subdivision of this constituent. The present evidence seems to suggest that reagin is a γ_1-globulin so that, provided the chromatographic process can be made less deleterious, there is hope of obtaining this protein in relatively concentrated form free from the many other components of very similar physico-chemical properties which make up the complex γ-globulin family of molecules.

ACKNOWLEDGMENTS

The author is indebted to Professor J. R. Squire for his most helpful advice and criticism and to Dr. G. Holti for performing the passive transfer tests. Thanks are due to Dr. P. G. H. Gell and Dr. J. F. Soothill for carrying out the immunological analyses. Dr. K. W. Walton kindly supplied a sample of fluorescent gamma globulin. Miss P. Ratcliff and Miss J. Burns provided valuable technical assistance and Mr. R. Crockson kindly prepared the diagrams. I am grateful, also, to Mr. J. Barson (Department of Bacteriology, University of Birmingham) for performing sterility tests on the solutions used for human injection. Finally, I wish to thank the donor of the blood and the recipient of the passive transfer tests, who made this work possible.

REFERENCES

BRATTSTEN, I., COLLDAHL, H. and LAURELL, A. H. F. (1955). 'The distribution of reagins in the serum protein fractions obtained by continuous zone electrophoresis.' *Acta Allerg.*, **8**, 339–48.

CAMPBELL, D. H., CANN, J. R., FRIEDMAN, T. B. and BROWN, R. A. (1954). 'The relation of allergy reagins to electrophoretic components of serum.' *Science*, **119**, 289–91.

CHADWICK, C. S., MCENTEGART, M. G. and NAIRN, R. C. (1958). 'Fluorescent protein tracers. A trial of new fluorochromes and the development of an alternative to fluorescein.' *Immunology*, **1**, 315–27.

COOKE, R. A., SHERMAN, W. B., MENZEL, A. E. O., CHAPIN, H. B., HOWELL, C. M., SCOTT, R. B., MYERS, P. A. M. and DOWNING, L. M. (1951). A.C.T.H. and Cortisone in allergic diseases. Clinical, serological (electrophoretic), and immunologic studies.' *J. Allergy*, **22**, 211–36.

EDELMAN, G. M., KUNKEL, H. G. and FRANKLIN, E. C. (1958). 'Interaction of the rheumatoid factor with antigen antibody complexes and aggregated gamma globulin.' *J. exp. Med.*, **108**, 105–20.

ELLIS, H. A., GELL, P. G. H., ROWE, D. S., SOOTHILL, J. F., STANWORTH, D. R. and WALTON, K. W.

REFERENCES (*continued*)

(1959). 'The iota protein of normal and pathological human serum.' In preparation.

GELL, P. G. H. (1957). 'The estimation of the individual human serum proteins by an immunological method.' *J. clin. Path.*, **10**, 67–71.

GRABAR, P. and WILLIAMS, C. A. (1955). 'Méthode immuno-électrophoretique d'analyse de mélanges de substances antigeniques.' *Biochim. biophys. Acta.*, **17**, 67–74.

GRANT, G. H. (1957). 'The proteins of normal urine.' *J. clin. Path.*, **10**, 360–7.

GRANT, G. H., ROWE, D. S. and STANWORTH, D. R. (1958). 'The concentration of protein solutions by ultrafiltration through dialysis tubing.' *Biochem. J.*, **69**, 13P.

HUMPHREY, J. H. and PORTER, R. R. (1957). 'Reagin content of chromatographic fractions of human gamma-globulin.' *Lancet*, **1**, 196–7.

KUHNS, W. J. (1954). 'Immunochemical studies of antitoxin produced in normal and allergic individuals hyperimmunised with diphtheria toxoid. IV. Differences between human precipitating and non-precipitating skin-sensitizing diphtheria antitoxin as shown by electrophoresis.' *J. exp. Med.*, **99**, 577–88.

MA, T. S. and ZUAZAGA, G. (1942). 'MicroKjeldahl determination of nitrogen. A new indicator and an improved rapid method.' *Industr. Engng Chem. (Anal.)*, **14**, 280–2.

PETERSON, E. A. and SOBER, H. A. (1956). 'Chromatography of proteins. I. Cellulose ion-exchange adsorbents. II. Fractionation of serum protein on an ion-exchange cellulose.' *J. Amer. chem. Soc.*, **28**, 751–5, 756–63.

RANDOLPH, M. L. and RYAN, R. R. (1950). 'A slicer for sampling liquids.' *Science*, **112**, 528.

SEHON, A. H. (1958). 'Biophysical and immunochemical problems in allergy.' *Proc. of Third Int. Cong. of Allergology (Paris)*, 290–30.

SQUIRE, J. R. (1950). 'The relationship between horse dandruff and horse serum antigens in asthma.' *Clin. Sci.*, **9**, 127–50.

STANWORTH, D. R. (1957). 'The isolation and identification of horse-dandruff allergen.' *Biochem. J.*, **65**, 582–98.