Immunology, 1965, **8**, 323.

Quantitative Studies on the Assay of Human Skin-Sensitizing Antibodies (Reagins)

I. AN EXAMINATION OF FACTORS AFFECTING THE ACCURACY OF THE PRAUSNITZ-KÜSTNER (P-K) TEST

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(*Received* 13*th April* 1964)

Summary. A quantitative examination of various factors influencing the accuracy of the P–K test was undertaken. The skin site on the back of most recipients tested did not contribute significantly to the variance observed. Coefficients of variation of a single P–K test of the order of 10–20 per cent were frequently demonstrable, for weals exceeding 50 mm^2 in area. Linear log(dose)–response relationships were observed below dose levels corresponding to a 1/5 dilution of the sensitizing serum. A ten-fold increase in dose produced an approximately four-fold increase in weal area. Studies of the influence of the timing and siting of allergen-challenge provided a quantitative indication of the rapid and firm attachment of transferred reaginic antibodies to passively sensitized skin sites.

INTRODUCTION

Although it is now over 40 years since Prausnitz passively sensitized himself to fish by intradermal injection of Küstner's serum (Prausnitz and Küstner, 1921), reagins are still assayed by methods based on this classical experiment (termed the 'P–K test' after the two participants). Attempts to devise a more practicable and accurate *in vitro* technique, involving for example the agglutination of allergen-coated red cells (e.g. Gordon Rose and Sehon, 1958) or some other sensitive indicator of antigen–antibody combination have so far met with little success.* Hence, investigations of the properties of reagins have depended solely on the biological method for assay of skin sensitizing activity.

Modifications of the P–K test have, of course, been employed. Prominent amongst these are a method involving neutralization of reagin with allergen *in vitro* prior to passive transfer (Cooke, 1947) and a reverse procedure where challenging allergen is injected into the normal recipient before the transfer of the allergic serum or fraction (Wright and Hopkins, 1941). All these procedures, however, have provided results of considerable variability. For this reason many investigators have refrained from expressing the results of their P–K tests in anything more accurate than a semi-quantitative form (involving the conventional '+' system of scoring). Where quantitation has been attempted, there has hardly ever been any assessment of the degree of accuracy attained.

* Recent reports (Van Arsdel and Sells, 1963) of measurement of the *in vitro* release of histamine by the addition of specific allergen to passively sensitzed normal human leucocytes suggest, however, that the *in vitro* assay of reagins will soon be feasible.

The studies to be described were undertaken in order to establish the contribution of various factors to the variability of the P–K test, and so to attempt an assessment of its potential accuracy. The development of a reliable method of assay, of known variability, is considered an essential requirement in the chemical and physico-chemical characterization of reagins. Moreover, the eventual development of satisfactory *in vitro* methods of assay (or even of superior alternative biological techniques) will depend intially on an accurate comparison of results with those obtained by means of the classical *in vivo* procedure.

Measurements have been made with similar allergic serum to that used in previous studies, on the physico-chemical characterization of reagins to horse dandruff (Stanworth, 1959). The allergen was introduced into passively sensitized skin sites by the prick method, which has been found to have several advantages (outlined by Harley, 1953) over the more common practice of challenging by intradermal injection. Squire (1950) has made a valuable critical assessment of the accuracy of the prick procedure in a study involving the multiple testing of histamine solutions of varying concentration in the skin of normal individuals. A similar statistical analysis, of the quantitative response to the passive transfer of varying dilutions of allergic serum into normal recipients, has been undertaken here. In addition, the contribution of skin site to the variance of these results has been ascertained. The influence of the timing and siting of the allergen prick has also been investigated.

The quantitative P–K procedure evolved has been applied to an investigation of the stability of reagins under various conditions. The results of this study will be described in a subsequent paper.

MATERIALS AND METHODS

MATERIALS

Allergic Serum

As already mentioned, serum from one horse-sensitive individual was used throughout. The donor (a female laboratory assistant aged 28 years) had a long history of hypersensitivity, being markedly sensitive to horse dandruff and to other common inhalants such as grass pollens and house dust. Although she had been hyposensitized with a mixed pollen extract (Group A12, supplied by C. L. Bencard Ltd.) on several occasions (the last time being about 2 years before the blood was taken) she has never been hyposensitized to horse dandruff allergen.

The freshly taken blood from the horse-sensitive donor was incubated for 4 hours at 37° to enhance retraction of the clot. The serum was separated aseptically after centrifugation. Aliquots each of 5 ml. were freeze-dried in ampoules which were afterwards sealed under nitrogen and stored at 4°. Reconstitution was effected by the addition of sterile distilled water. Under such conditions of storage, reaginic activity was found to be retained. Hence, the freeze-dried allergic serum provided a valuable standard of reference in P–K testing.

Allergens

Solutions of total horse dandruff protein (2 g. per cent (w./v.) in 0·15 M saline) were used for challenging passively sensitized skin sites. (It had been previously established that this

concentration of allergen solution was in excess of the amounts required to exhaust—by a single prick—sites passively sensitized with the undiluted allergic serum.) The freshly prepared allergen solutions were stored at 4° for short periods prior to use.

All preparations were sterilized by filtration through Swinny filters, before their introduction into human skin.

METHODS

The adopted mode of assaying reagin activity was essentially that first described by Prausnitz and Küstner (1921), and extensively developed by Coca and Grove (1925), except that prick testing with the solution of challenging allergen was preferred to its intradermal injection. Allergic serum (or a dilution) in 0·1 ml. volumes was injected intradermally by means of a 1 ml. tuberculin syringe into the back of a suitable normal recipient (eight were selected from a group of healthy young male medical student volunteers who acted as recipients throughout the present study). The outlines of the injection 'weals' were marked in Indian ink, their distribution being noted and also photographed in colour (Kodak-Ektachrome).

The backs of the recipients were also photographed immediately prior to challenge of the sites and any premature reactions were noted. Challenge was usually made 24–48 hours after passive sensitization. It was accomplished by placing a drop of allergen solution at the centre of the outlined site and pricking the epidermis once with a sterile sewing needle, if possible at the needle mark left by the initial sensitizing injection. After 10 seconds had elapsed the excess allergen solution was wiped off each site with cotton wool. The outlines of the resultant weals were traced, usually after 20 minutes, in Indian ink on a superimposed transparent plastic sheet (Mylar*). The appearances of the flares were also noted and a photographic record in colour was always obtained. The areas of the weal tracings were determined by superimposition on graph paper (mm., $\frac{1}{2} \times 1$ cm.) followed by the 'counting of squares'. These values were taken as indicators of skin reactivity rather than the weal diameters, calculated by assuming the weals to be of ideal circular form (a practice adopted in previous studies). There did not seem to be any advantage in converting the weal areas to diameters in the statistical analysis of P–K test results.

The appropriate control tests were carried out in each recipient each time. These always included direct prick tests with the challenging allergen solution. (Tests with a standard histamine solution were only performed when selecting suitable recipients for passive transfer.)

The protein concentrations of test solutions were checked by a micro-Folin-Ciocalteu procedure (Lowry, Rosebrough, Farr and Randall, 1951), employing a human serum albumin solution as standard. For the studies of quantitative response the allergic serum was usually diluted with buffered phosphate (pH 7·5, 0·01 M)–saline (0·15 M), except on the occasions where a serum protein solution was employed as diluent.

RESULTS

1. DETERMINATION OF THE CONTRIBUTION OF SKIN SITE TO VARIANCE IN P–K TESTS IN DIFFERENT RECIPIENTS

Constant volumes (0·1 ml.) of horse dandruff sensitive allergic serum, diluted five times with buffered phosphate (pH 7·5, 0·01 M)–saline (0·15 M), were injected into thirty-six

* Obtainable from Du Pont (U.K.) Ltd., 76 Jermyn Street, London, S.W.1.

sites arranged approximately 1½ in. apart in a square distributed symmetrically over the backs of three normal recipients (as shown in Fig. 1). The sites were challenged 24 hours later by pricking in horse dandruff protein solution. In addition to the tracing of weal outlines, a photographic record in colour (an example is shown in Fig. 2) was obtained to augment the area measurements. The results are expressed in site order in Tables 1–3 together with an analysis of their variance, according to the method described by Brownlee (1948).

TABLE 1

RESULTS OF MULTIPLE P–K TESTS WITH DILUTED (1/5) HORSE DANDRUFF SENSITIVE ALLERGIC SERUM

Weal areas (mm²) after 20 minutes

Back—left side			*Back—right side*		
102	56	50	72	96	50
80	88	58	70	89	78
71	90	94	63	66	76
58	92	76	51	103	90
114	57	76	35	70	80
73	102	50	74	70	82

The arrangement of the areas corresponds to the positioning of the weals on the recipient's back, the sites nearer the neck being at the top of each column.

Analysis of variance

Source of variance	*Sum of squares*	*Degrees of freedom*	*Mean squares*	*Significance*
Between columns	2474	5	495	$P>0{\cdot}20$
Between rows	262	5	52	$P>0{\cdot}20$
Residual	13068	25	523	
Total	15804	35		

Mean weal area = 73 mm². Standard deviation = 18 mm².
Coefficient of variation = 25 per cent.
Recipient G.W. Date of tests: 5th–6th September 1961. Interval between sensitization and challenge, 24 hours.

As will be seen, by comparison of Tables 1 and 2, recipients G.W. and M.D. showed very similar responses. The mean area of the weals elicited in the two subjects was of similar magnitude, being 73 ± 18 mm² in recipient G.W. and 65 ± 21 mm² in recipient M.D. In neither recipient was the skin site (whether considered in rows or columns) found to make a significant contribution to the variance of the weal areas. A combined analysis of variance, however revealed that the variation between the results of tests in the two recipients was significantly greater ($0{\cdot}05 > P \gg 0{\cdot}01$) than the variation of the weal areas elicited in a single individual. This underlines the importance of carrying out all comparative P–K tests in the *same* recipient.

The third recipient (S.K.) proved to be most unsatisfactory. He showed a much lower reactivity, producing a mean weal area of only 22 mm², and considerable variability (±85 per cent). A significant vertical site to site variation ($0{\cdot}05 > P > 0{\cdot}01$) appeared to be a contributory factor to this high variance. It should be stressed, however, that the

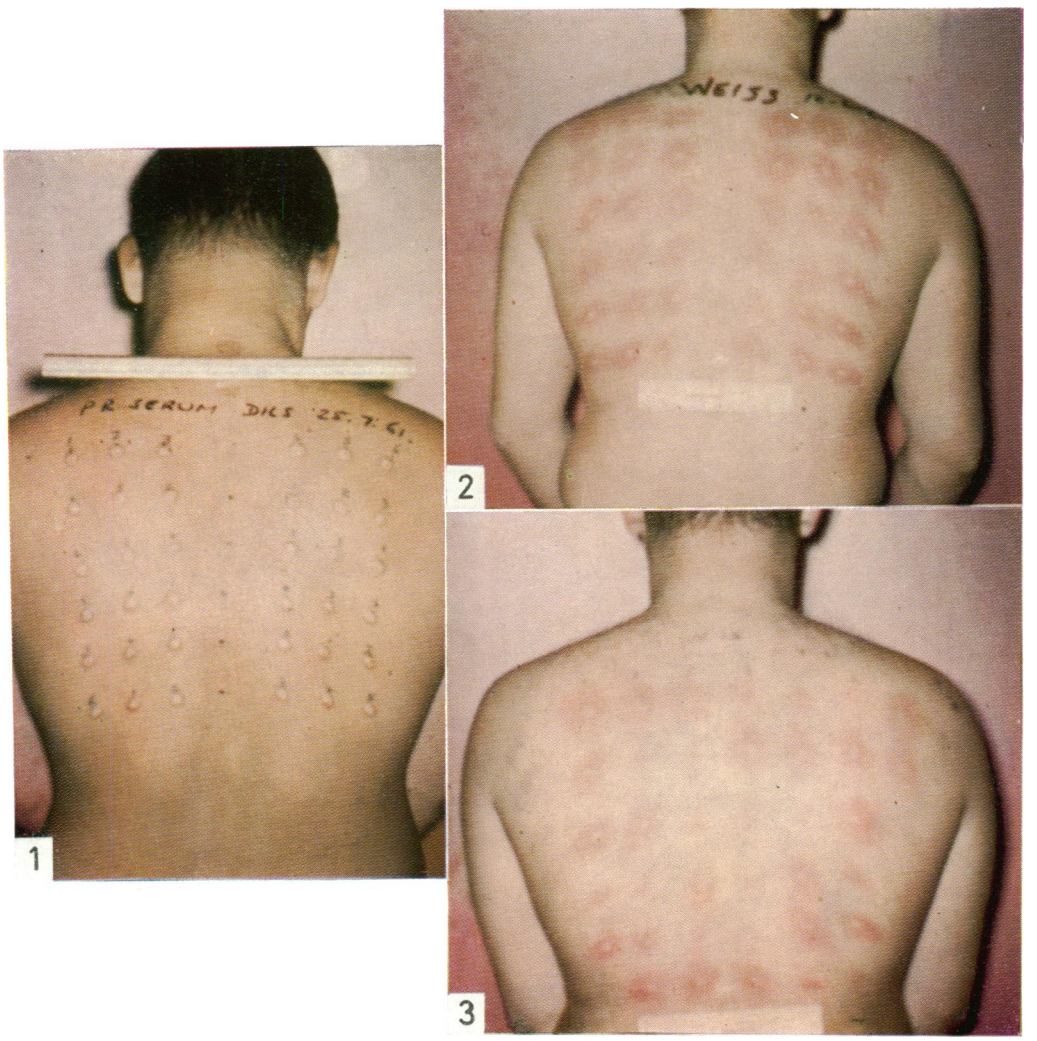

FIG. 1. Arrangement of injection weals in multiple P–K testing of horse dandruff allergic serum (diluted 1 in 5) on the backs of normal recipients. (Print taken from Ektachrome transparency.)

FIG. 2. Typical reactions obtained in the test series illustrated in Fig. 1, 30 minutes after challenge with allergen. (Print taken from Ektachrome transparency.)

FIG. 3. Photographic record of quantitative response of recipient G.W. to P–K testing with varying dilutions of sensitizing serum. See Table 4 for site layout. (Print taken from Ektachrome transparency.)

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TABLE 2

RESULTS OF MULTIPLE P-K TESTS WITH DILUTED (1/5) HORSE DANDRUFF SENSITIVE ALLERGIC SERUM

Weal areas (mm²) after 20 minutes

Back—left side			*Back—right side*		
38	96	85	27	20	74
74	65	62	68	0	65
88	43	45	27	76	66
55	72	71	88	73	73
77	111	75	41	79	75
58	44	102	91	66	52

The arrangement of the areas corresponds to the positioning of the weals on the recipient's back.

Analysis of variance

Source of variance	*Sum of squares*	*Degrees of freedom*	*Mean squares*	*Significance*
Between columns	2072	5	414	$P>0{\cdot}20$
Between rows	2420	5	484	$P>0{\cdot}20$
Residual	15221	25	609	
Total	19713	35		

Mean weal area = 65 mm². Standard deviation = 21 mm².
Coefficient of variation = 32 per cent.
Recipient M.D. Date of tests: 5th–6th September 1961. Interval between sensitization and challenge, 24 hours.

TABLE 3

RESULTS OF MULTIPLE P-K TESTS WITH DILUTED (1/5) HORSE DANDRUFF SENSITIVE ALLERGIC SERUM

Weal areas (mm²) after 20 minutes

Back—left side			*Back—right side*		
0	0	0	14	0	0
6	4	0	29	41	63
33	54	36	38	47	34
18	16	12	62	31	0
15	15	45	23	17	6
13	37	50	13	17	6

The arrangement of the areas corresponds to the positioning of the weals on the recipient's back.

Analysis of variance

Source of variance	*Sum of squares*	*Degrees of freedom*	*Mean squares*	*Significance*
Between columns	891	5	178	$P>0{\cdot}20$
Between rows	4389	5	878	$0{\cdot}05>P>0{\cdot}01$
Residual	7413	25	287	
Total	12683	35		

Mean weal area = 22 mm². Standard deviation = 19 mm².
Coefficient of variation = 85 per cent.
Recipient S.K. Date of tests: 5th–6th September 1961.

statistical analysis (see Table 3) of the results of P–K tests performed in recipient S.K. cannot be accepted as very meaningful, owing to the unsuitability of his skin for passive sensitization. In this connection, it is worth mentioning that the recipient's back was covered with a copious growth of hair which had to be removed by shaving prior to performing the P–K tests. He would probably fall into the class of 'poor reactors' which, according to Coca and Grove (1925), comprise 5 per cent of normal individuals.

2. ANALYSIS OF VARIANCE OF MULTIPLE P–K TESTS WITH VARYING DILUTIONS OF ALLERGIC SERUM IN DIFFERENT RECIPIENTS

(a) *Tests with Horse Dandruff Sensitive Allergic Serum Diluted with Buffered Saline*

Each of six different dilutions of allergic serum was tested six times in the backs of three normal recipients. The sites were allocated on the basis of a self-conjugate standard 6×6 Latin Square (Fisher and Yates, 1948), distributed symmetrically over the back (in a similar arrangement to that shown in Fig. 1). After 24 hours, challenge of the sensitized sites was effected by pricking in the allergen solution in the usual manner. A photographic record of the response of one of the recipients (G.W., who was also used in tests described previously in Section 1), is reproduced in Fig. 3. The areas of the weals evoked in each of the three recipients are arranged in site order in Table 4.

TABLE 4

TWENTY MINUTE WEAL AREAS (MM^2) AFTER P–K TESTING THREE RECIPIENTS WITH VARIOUS DILUTIONS OF HORSE DANDRUFF ALLERGIC SERUM (DILUTED WITH BUFFERED SALINE)

Dates of tests: 25th–26th July 1961

Recipient G.W.						*Recipient J.F.*						*Recipient H.S.*					
Left side			*Right side*			*Left side*			*Right side*			*Left side*			*Right side*		
A	B	C	D	E	F	A	B	C	D	E	F	A	B	C	D	E	F
116	105	74	54	4	4	86	48	55	5	1	0	111	91	111	76	12	0
D	E	F	A	B	C	D	E	F	A	B	C	D	E	F	A	B	C
63	13	0	116	94	56	15	2	0	110	81	49	38	8	0	183	129	72
B	C	D	E	F	A	B	C	D	E	F	A	B	C	D	E	F	A
93	92	33	12	8	116	92	47	6	0	0	76	78	96	90	6	0	159
E	F	A	B	C	D	E	F	A	B	C	D	E	F	A	B	C	D
8	0	139	67	56	63	2	0	106	70	51	3	8	0	178	93	100	113
C	D	E	F	A	B	C	D	E	F	A	B	C	D	E	F	A	B
67	47	7	0	120	70	75	6	0	0	108	68	103	78	6	0	130	93
F	A	B	C	D	E	F	A	B	C	D	E	F	A	B	C	D	E
0	148	118	84	52	6	0	101	69	42	2	0	0	219	106	93	60	0

Serum diln. used	*Mean weal area (mm^2)*	*Coeff. of variation (%)*	*Serum diln. used*	*Mean weal area (mm^2)*	*Coeff. of variation (%)*	*Serum diln. used*	*Mean weal area (mm^2)*	*Coeff. of variation (%)*
A = Undiluted	126	10	A = Undiluted	98	13	A = Undiluted	163	22
B = 1/5	91	20	B = 1/5	71	19	B = 1/5	98	17
C = 1/10	72	19	C = 1/10	53	20	C = 1/10	96	13
D = 1/25	52	20	D = 1/25	6	70	D = 1/25	76	31
E = 1/50	8	40	E = 1/50	1		E = 1/50	7	31
F = 1/100	2	153	F = 1/100	0		F = 1/100	0	

In general, the coefficient of variation of weal area in each of the recipients was fairly constant at 10–20 per cent, providing the mean area was greater than about 50 mm². Substantially greater variations were shown, however, by the smaller weal areas produced by the higher dilutions of test serum. This finding exposes a disadvantage of the dilution P–K technique which is not often appreciated.

The contribution to variance made by the skins of the different recipients, and by site variation within the skin of individual recipients, was ascertained by means of a combined analysis of variance (recorded in Table 5). As was found previously, in two out of three

TABLE 5

COMBINED ANALYSIS OF VARIANCE OF WEAL AREAS GIVEN IN TABLE 4

Source of variance	*Sum of squares*	*Degrees of freedom*	*Mean squares*	*Significance*
Site (rows)	798	5	160	$P>0{\cdot}20$
Recipient (columns)	22,365	2	11,183	$P<0{\cdot}001$
Serum dilution (treatments)	222,355	5	44,471	$P<0{\cdot}001$
Residual	33,784	95	356	
Total	279,302	107		

of the recipients receiving thirty-six tests with only one dilution of serum (i.e. 1 in 5), there was no significant contribution to the variance of the results by the skin site of any of the three recipients. On the other hand, there was a highly significant variation ($P < 0{\cdot}001$) in the size of weal evoked by a given serum dilution in the skin of the different recipients. As was to be expected, the treatment (i.e. variation of the dilution of the allergic serum transferred) also made a very great contribution to variance in every case. After elimination of these sources of variance, the standard deviation of a single determination of weal area *in any recipient* was not greater than 23 mm². This corresponds to a coefficient of variation of 40 per cent, on an *average* weal area of 57 mm².

An additional factor contributing to the variance of the measurements in recipient H.S. was the difficulty in establishing the true outlines of the weals—often substantially larger than the weals elicited by a corresponding dose in the other recipients—owing to their failure to protrude clearly above their surrounding flares even after palpation. This increases the error inherent in the practice of quantitating oedema by measurement of weal area.

Log(dose)–response curves plotted from the data given in Table 4 are shown in Fig. 4. The standard deviations of the mean weal areas are also indicated on the graphs. The dose–response relationships shown by the different recipients were approximately linear up to a limiting concentration of sensitizing serum corresponding to a 1/5 dilution. Above this level, however, the linear curves showed a tendency to flatten out—an effect which was more pronounced in recipients G.W. and J.F. than in H.S. Log(dose)–response curves obtained by the testing of other recipients, in experiments to be described later, exhibited a similar flattening out with doses above those corresponding to about a 1/5 dilution of allergic serum. Tests involving the rechallenge of passive transfer sites failed to provide evidence that the effect was due to lack of sufficient allergen to interact with all reagin available at sites injected with undiluted allergic serum. It is possible that it reflects an overloading of the recipient's skin sites with reagin, excess sensitizing antibody being lost by diffusion from the transfer site.

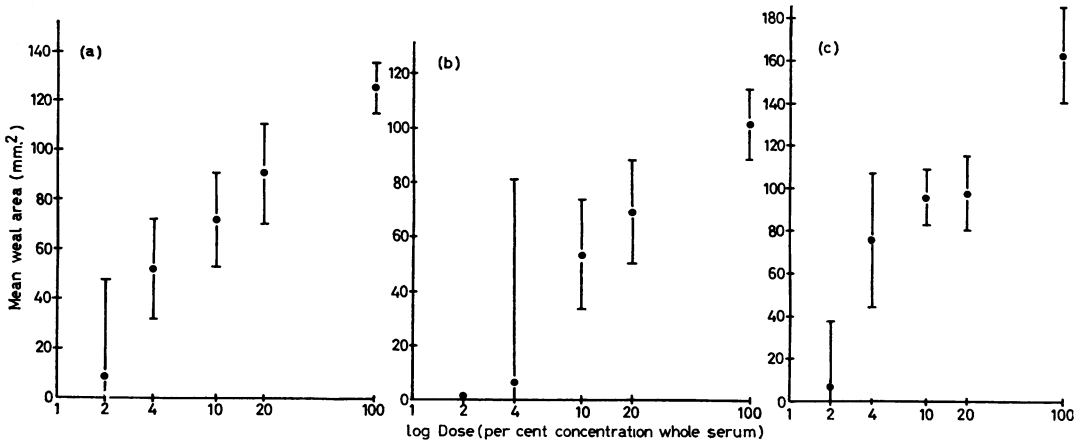

FIG. 4. Quantitative responses of recipient G.W. (a), J.F. (b) and H.S. (c) to sensitizing serum diluted with buffer.

A composite log(dose)–response curve, obtained by plotting the averages of the mean weal areas evoked in the three recipients tested and fitting the best straight line by the method of least squares is shown in Fig. 5 (curve a). Above weal areas of 50 mm² (where

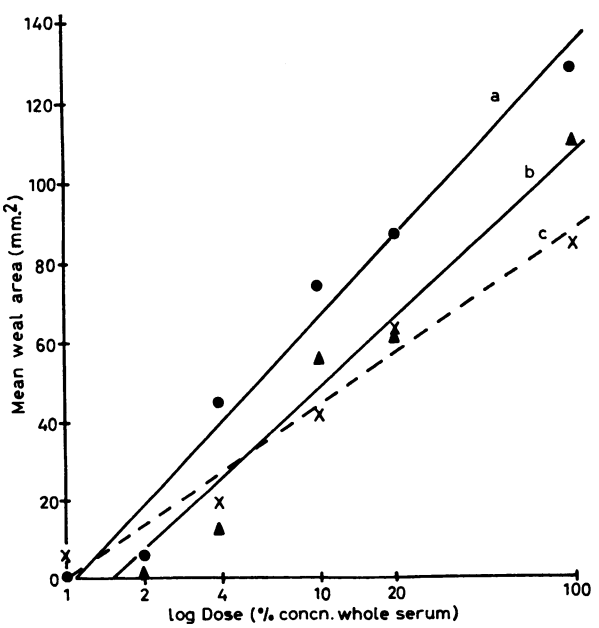

FIG. 5. Averaged quantitative responses of recipients G.W., J.F. and H.S. (a), recipients G.W. and I.P. (b) and recipients A.K. and R.D. (c).

the coefficient of variation of a single determination is usually between 10 and 20 per cent), a ten-fold increase in the dose produces an approximate four-fold increase in response. Similar quantitative responses (curves b and c in Fig. 5) were revealed by plotting the averages of weal areas induced into two pairs of recipients in other series of tests.

(b) *Tests with Horse Dandruff Sensitive Serum Diluted with Recipient's Serum*

It is sometimes the practice to stabilize isolated reagin fractions by the addition of whole human serum or a solution of a serum protein fraction. Hence, it was considered possible that dilution of the sensitizing serum with such protein solutions, rather than with buffered saline, might eliminate some of the variance of the P–K test results reported in the previous section.

Similar dilutions of the allergic serum to those adopted previously (using buffered saline as diluent) were made with freshly taken recipient's serum. Tests sites were again allocated in a Latin Square arrangement on the backs of two recipients, one of whom (G.W.) had been used in the two series of tests described previously. As before, the weal areas evoked by the different dilutions of allergic serum are arranged in site order, in Table 6. The results of separate analyses of variance of the determinations made in the two recipients are given in Table 7, which also includes a combined analysis to establish the variance between the responses of the two recipients.

TABLE 6

TWENTY MINUTE WEAL AREA (MM^2) AFTER P-K TESTING TWO RECIPIENTS WITH VARIOUS DILUTIONS OF HORSE DANDRUFF ALLERGIC SERUM (DILUTED WITH RECIPIENT'S SERUM)

Dates of tests: 8th–9th November 1961

Recipient G.W.						*Recipient I.P.*					
Left side			*Right side*			*Left side*			*Right side*		
A	B	C	D	E	F	A	B	C	D	E	F
105	78	49	9	4	0	99	40	18	2	0	0
D	E	F	A	B	C	D	E	F	A	B	C
15	0	0	77	78	69	4	0	0	90	42	32
B	C	D	E	F	A	B	C	D	E	F	A
48	52	16	0	0	74	57	47	8	0	0	98
E	F	A	B	C	D	E	F	A	B	C	D
4	0	128	56	57	18	0	0	143	60	54	5
C	D	E	F	A	B	C	D	E	F	A	B
85	48	6	0	127	83	49	8	0	0	123	46
F	A	B	C	D	E	F	A	B	C	D	E
0	148	96	97	20	0	0	106	74	60	4	0

Serum diln. used	*Mean weal area (mm^2)*	*Coefficient of variation (%)*	*Serum diln. used*	*Mean weal area (mm^2)*	*Coefficient of variation (%)*
A = Undiluted	110	25	A = Undiluted	110	16
B = 1/5	73	22	B = 1/5	51	17
C = 1/10	67	26	C = 1/10	43	33
D = 1/25	21	60	D = 1/25	5	44
E = 1/50	2	123	E = 1/50	0	
F = 1/100	0		F = 1/100	0	

In contrast to the previous findings (see Tables 1 and 4), the skin site (in a vertical direction) was found to make a significant contribution to the variance. This effect was more pronounced in recipient G.W. ($0{\cdot}01 > P > 0{\cdot}001$) than in recipient I.P.

TABLE 7

ANALYSIS OF VARIANCE OF WEAL AREAS GIVEN IN TABLE 6

Source of variance	*Sum of squares*	*Degrees of freedom*	*Mean squares*	*Significance*	*Sum of squares*	*Degrees of freedom*	*Mean squares*	*Significance*
Vertical site (rows)	951	5	190	$P>0{\cdot}20$	362	5	72	$P>0{\cdot}20$
Horizontal site (columns)	3739	5	748	$P\begin{cases}>0{\cdot}001\\<0{\cdot}01\end{cases}$	1412	5	282	$P\begin{cases}>0{\cdot}01\\<0{\cdot}05\end{cases}$
Serum dilution (treatments)	61376	5	12275	Very great	54579	5	10916	
Residual	2551	20	128		1826	20	91	
Total	68617	35			58179	35		

Combined analysis of variance

Source of variance	*Sum of squares*	*Degrees of freedom*	*Mean squares*	*Significance*
Site (rows)	3664	5	733	$P\begin{cases}<0{\cdot}01\\>0{\cdot}001\end{cases}$
Recipient (columns)	1985	1	1985	$P\begin{cases}<0{\cdot}01\\>0{\cdot}001\end{cases}$
Serum dilution (treatments)	112158	5	22432	$P<0{\cdot}001$
Residual	10973	61	180	
Total	128780	72		

($0{\cdot}05>P>0{\cdot}01$). It is an interesting observation as far as G.W. is concerned because the skin site of the same recipient made no significant contribution to the variance of the previously described tests, performed 64 days (Table 1) and 104 days (Table 4) earlier. Possibly previous P–K tests performed 44 days earlier in these recipients had caused some impairment of tissue responsiveness, because Bowman and Walzer (1953) have shown that such an effect can persist for as long as 4 weeks after P–K testing and limits the reliability of P–K tests on the same site.

It is also of interest to note, from a comparison of the results given in Tables 4 and 6, that the mean areas of the weals elicited in recipient G.W. by sensitizing serum diluted to varying extents with recipient's serum were *in all cases* less than those elicited with serum diluted with buffered saline. Before attributing this finding to a specific effect of the diluent however, it would be necessary to exclude more likely causes such as 'day to day' variation of the recipient's skin reactivity or even to a loss in activity of the test serum during storage. Nevertheless, the results of the experiment to be described next—in which aliquots of sensitizing serum diluted with serum albumin solution or buffered saline were tested *simultaneously* in the same recipient—suggest that the nature of the diluent can influence the weal response in P–K testing.

Although the magnitude of the response shown by recipient G.W. to the various dilutions of sensitizing serum was reduced when the recipient's serum was used as diluent, a similar log(dose)–response relationship to that shown previously to buffer-diluted sensitizing serum (Fig. 4a) was obtained (as is shown by Fig. 6, in which the previous quantitative response of recipient G.W. is plotted for comparison).

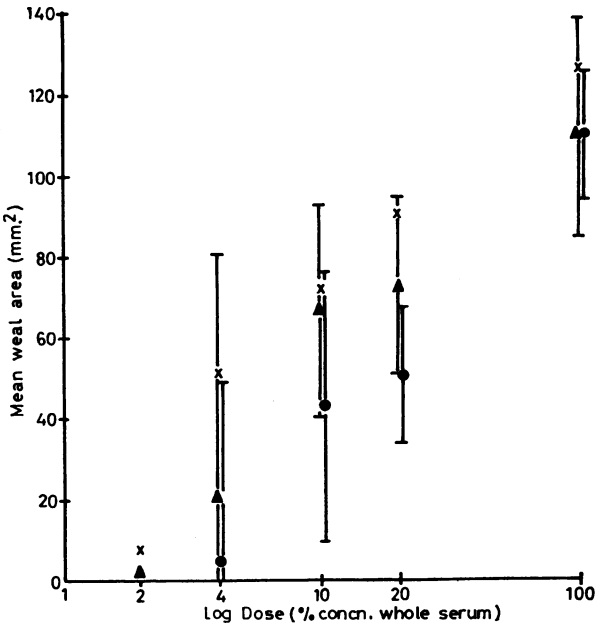

FIG. 6. Quantitative responses of recipients G.W. (▲) and I.P. (●) to sensitizing serum diluted with recipient's serum. The previous log(dose)–response relationship shown by recipient G.W. (when tested with sensitizing serum diluted with buffer) is indicated by the points marked with a cross.

The combined analysis of variance (Table 7) of the results of determinations made in recipients G.W. and I.P. (given in Table 6) again revealed a significant 'between recipient' variation ($0{\cdot}01 > P > 0{\cdot}001$). This indicates that the use of recipient serum as diluent (instead of buffered saline) fails to eliminate the variance of P–K tests contributed by the use of different recipients (revealed by the previous combined analysis of variance described in Table 5).

(c) *Tests with Horse Dandruff Sensitive Serum Diluted with Human Serum Albumin Solution*

The usefulness of human serum albumin as an alternative to buffered saline in the dilution of sensitizing serum for quantitative response studies was also investigated, by performing simultaneous P–K tests in the *same* recipient. The tests with allergic serum diluted with a 6 per cent (w./v.) solution of human serum albumin (HuSA) (Cutter, Batch G-3312) were carried out in the back of a single recipient alongside tests with serum diluted with buffered saline. The layout of the sensitized skin sites is indicated in Table 8. Sites arranged in a 5×5 self-conjugate Latin square on the left side of the spinal column were sensitized with the albumin-diluted serum, whilst sites forming a mirror image of this arrangement on the right side were sensitized with buffer diluted serum. The areas of weals evoked after 24 hours challenge are arranged in site order in Table 8.

Application of Student's '*t*' test to a comparison of the responses elicited at corresponding sites on the two sides of the back revealed a significant difference ($0{\cdot}01 > P > 0{\cdot}001$). This can probably be attributed to the difference in the nature of the diluent as an

analysis of variance (Table 9) of the results of testing on either side of the back failed to reveal any site bias.

TABLE 8

TWENTY MINUTE WEAL AREAS (MM^2) ELICITED BY SIMULTANEOUS TESTING OF ALBUMIN-DILUTED AND BUFFER-DILUTED SENSITIZING SERUM IN SAME RECIPIENT (R.D.)

Dates of testing: 8th–9th May 1961

Left side of back (diluent = 6 per cent HuSA in buffered saline pH 7·5)					*Right side of back (diluent = buffered saline pH 7·5)*				
A	B	C	D	E	E	D	C	B	A
76	51	41	6	1	6	39	98	98	73
D	E	A	B	C	C	B	A	E	D
2	0	61	93	47	92	89	134	8	60
B	C	D	E	A	A	E	D	C	B
72	37	4	0	107	132	0	52	87	85
E	A	B	C	D	D	C	B	A	E
2	110	79	60	0	0	31	110	105	0
C	D	E	A	B	B	A	E	D	C
27	4	1	91	103	114	182	0	50	60

Serum diln. tested	*Mean weal area (mm^2)*	*Coefficient of variation (%)*	*Serum diln. tested*	*Mean weal area (mm^2)*	*Coefficient of variation (%)*
A = Undiluted	89	21	A = Undiluted	125	30
B = 1/5	80	22	B = 1/5	99	11
C = 1/10	42	26	C = 1/10	74	34
D = 1/25	3	52	D = 1/25	40	36
E = 1/50	1		E = 1/50	3	

TABLE 9

ANALYSIS OF VARIANCE OF WEAL AREAS GIVEN IN TABLE 8

Source of variance	*Weals elicited by HuSA-diluted sensitizing serum*				*Weals elicited by buffer-diluted sensitizing serum*			
	Sum of squares	*Degrees of freedom*	*Mean squares*	*Significance*	*Sum of squares*	*Degrees of freedom*	*Mean squares*	*Significance*
Vertical site (rows)	1076	4	269	$P>0·20$	1410	4	353	$P>0·20$
Horizontal site (columns)	637	4	159	$P>0·20$	3246	4	812	$P>0·20$
Serum dilution (treatments)	38104	4	8526	Very great	46241	4	11560	Very great
Residual	2772	12	231		7880	12	659	
Total	38589	24			58777	24		

As is seen from Table 8, the weals elicited by the buffer-diluted sensitizing serum were appreciably larger than those produced by the corresponding dilution of albumin-diluted serum. This observation is in agreement with the previously described findings, where

recipient's serum was used as diluent. The quantitative responses of recipient R.D. to the albumin-diluted and buffer-diluted sensitizing serum are compared in Fig. 7. Characteristic

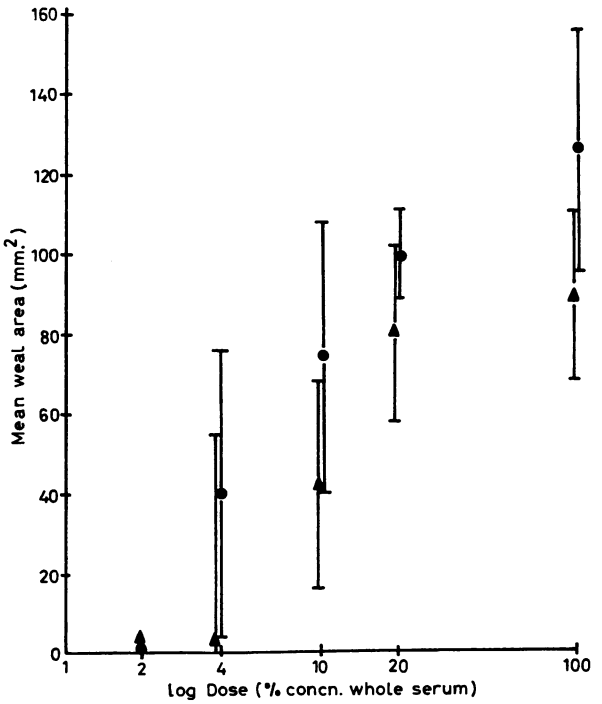

FIG. 7. Quantitative responses to P–K tests performed in the same recipient (R.D.) with sensitizing serum diluted with human serum albumin solution (▲) and with buffered saline (●). See Table 9 for site layout.

log(dose)–response curves were obtained, each of which showed the familiar tendency to flatten out above a dose level of about 1/5 diluted sensitizing serum. This effect appeared to be more marked in the curve referring to tests performed with the albumin-diluted sensitizing serum.

3. INFLUENCE OF TIME AND SITE OF ALLERGEN CHALLENGE ON THE P–K TEST

A quantitative comparison was made of the size of weal elicited by challenge with allergen at various times (ranging from 1 to 168 hours) after the initial passive sensitization to horse dandruff. At the same time, the influence of the siting of the challenge was also investigated. This is of particular importance, of course, where challenge involves the pricking in of allergen (as was the practice throughout the present studies) rather than its introduction by intradermal injection.

Twenty-four skin sites on the backs of four recipients (three of whom—G.W., I.P. and R.D.—had been used in previously described tests) were sensitized in the usual manner by intradermal injection of 0·1 ml. of horse dandruff allergic serum (diluted 1 : 1·5 for reasons of economy). The arrangement adopted is shown in Fig. 8(a), where an indication is given of the time of subsequent challenge of each sensitized site and of the position of the allergen prick.

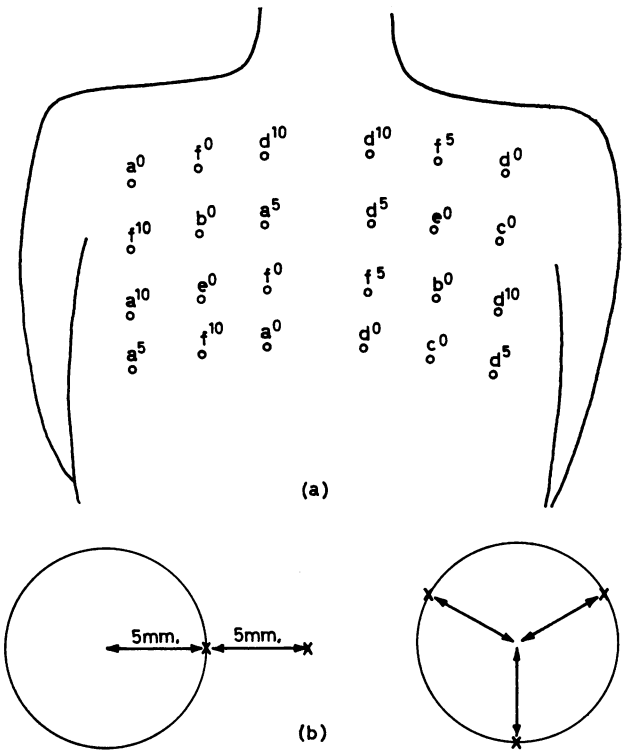

FIG. 8. Effect of time and site of allergen challenge on P–K activity to horse dandruff allergic serum diluted 1:1·5). (a) Arrangement of passive transfer sites (the suffixes refer to the site of challenge, expressed as distance in millimetres from centre of transfer injection bleb). (b) Siting of challenge prick in relation to outline of transfer injection bleb.

(a) *Influence of the Time of Challenge on the Size of Weal Elicited (by Pricking in Allergen at the Centre of the Sensitized Site)*

It appears from the results given in Table 10 that in three of the recipients (G.W., I.P. and R.D.) the time interval between transfer and challenge has little effect on weal size. If the coefficient of variation of a single measurement in each of these recipients (obtained from the tests described earlier) is taken into account, the differences of the size of weal elicited in any one individual at the different times were not significant in most cases. In recipient G.W., however, challenge after 53 hours produced a significantly larger weal (mean area = 104 mm^2) than that obtained by challenging sensitized sites 2 hours after transfer (when the mean weal area = 79 mm^2). The differences in the size of weals evoked in recipients I.P. and R.D., by challenge at corresponding times (2 hours and 51 hours) were not as great. The tests in the fourth recipient (S.B.) were unsatisfactory, because of his slight sensitivity to horse dandruff, which could account for his poor reactivity. A general picture of the relationship between the response of the various recipients and time of challenge is provided by the curves plotted in Fig. 9. As will be seen maximal P–K activity in recipients G.W. and I.P. was demonstrable by challenge at about 50 hours after transfer. In contrast, the lower maximal response of recipient R.D. occurred following challenge at about 30 hours. It would obviously be necessary to

TABLE 10

EFFECT OF TIME OF CHALLENGE ON SIZE OF WEALS ELICITED BY DILUTED (1:1·5) HORSE DANDRUFF ALLERGIC SERUM IN FOUR RECIPIENTS

	Recipient G.W.						*Recipient I.P.*					
	Interval between transfer and challenge (hours)	10-*minute weal area* (mm^2)			20-*minute weal area* (mm^2)			*Interval between transfer and challenge (hours)*	10-*minute weal area* (mm^2)			20-*minute weal area* (mm^2)		
		Site 1	*Site* 2	*Mean*	*Site* 1	*Site* 2	*Mean*		*Site* 1	*Site* 2	*Mean*	*Site* 1	*Site* 2	*Mean*
(a)	2	34	60	47	60	98	79	2	34	62	48	70	95	83
(b)	$8\frac{2}{3}$	32	44	38	47	90	69	8	30	38	34	60	88	74
(c)	30				91	76	84	$30\frac{1}{6}$				95	85	90
(d)	$53\frac{1}{3}$				113	94	104	51				111	78	95
(e)	76				61			$76\frac{5}{12}$				64		
(f)														

	Recipient R.D.						*Recipient S.B.*					
	Interval between transfer and challenge (hours)	10-*minute weal area* (mm^2)			20-*minute weal area* (mm^2)			*Interval between transfer and challenge (hours)*	10-*minute weal area* (mm^2)			20-*minute weal area* (mm^2)		
		Site 1	*Site* 2	*Mean*	*Site* 1	*Site* 2	*Mean*		*Site* 1	*Site* 2	*Mean*	*Site* 1	*Site* 2	*Mean*
(a)	$1\frac{1}{4}$	45	37	41	65	67	66	1	66	57	62	65	85	75
(b)	7	39	31	35	85	64	75	7	36	12	24	21	12	17
(c)	28				89	89	89	29				22	35	19
(d)	$48\frac{3}{4}$				105	37	71	51				12	13	13
(e)	$77\frac{1}{2}$				68			72				3		
(f)	$168\frac{1}{2}$				66	69	68							

N.B. See Fig. 8(a) for site lay-outs (the letters a, b, etc., locate the positions of sites challenged at various times).

Controls: Direct prick tests with allergen solution at each time of challenge produced negative results in recipients G.W., I.P. and R.D. In contrast, recipient S.B. produced a small weal (20-minute mean area = 8 mm^2 at 1 hour challenge, 9 mm^2 at 7 hours and 2 mm^2 at 72 hours).

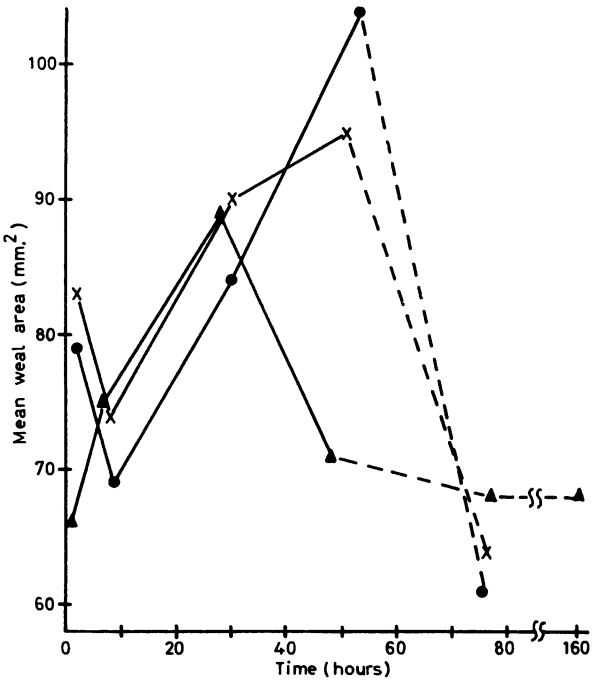

FIG. 9. Relationship between P–K response and time of challenge of sensitized skin site. G.W. (●), I.P. (×), R.D. (▲).

perform a greater number of repeat P–K tests (i.e. more than two), at more regular time intervals, in order to define the optimum time for maximal response more precisely.

The data presented in Table 10 also give an indication of the rate of increase in the size of the weal formed, after pricking allergen solution into sensitized skin sites. As will be seen, the weal size doubles approximately during the 10–20 minute period after challenge. It has become the custom, therefore, to measure the weals after 20 minutes, although statistical analysis showed that such a practice did not lead to greater accuracy than that achieved by determining 10-minute weal areas.

(b) *Influence of Siting of Allergen Challenge*

Studies of the effect of varying the position of the challenging prick (as shown in Fig. 8b) also provided interesting results (recorded in Table 11). On challenging sensitized sites in recipients G.W. and I.P. 2 hours after transfer, the mean area of weals elicited by challenge at the periphery of the initial transfer weal (i.e. at approximately 5 mm. from the centre) were almost 25 per cent less than the mean area of weals elicited by pricking in allergen at the centre of the transfer injection weal. On the other hand, a similar comparison of the size of weals elicited by pricking other sites in similar relative positions 51–53 hours after transfer revealed a greater 'site effect'. On this occasion, the weals elicited by challenge at the periphery of sensitized sites in recipients G.W. and I.P. were 75 per cent smaller than those elicited by a central challenging prick. In contrast, in recipients R.D. and S.B. the degree of difference in weal size as a result of variation in the position of challenging prick could be demonstrated in sensitized sites as early as 1 hour after transfer.

TABLE 11

EFFECT OF SITE OF CHALLENGE ON SIZE OF WEALS ELICITED BY DILUTED (1:1·5) HORSE DANDRUFF ALLERGIC SERUM IN FOUR RECIPIENTS

Recipient	Challenge: Site (Fig. 8a)	Challenge: Time after transfer (hours)	20-minute weal areas (mm^2) after pricking in allergen at varying distances from centre: 0 mm. 1	0 mm. 2	0 mm. Mean	5 mm. 1	5 mm. 2	5 mm. Mean	10 mm. 1	10 mm. 2	10 mm. Mean	
G.W.	a	2	60 (109)	98 (124)	79 (111)	46 (111)	71 (99)	59 (105)	0 (117)	0 (71)	0 (94)	
		8⅔	0	0	0	0	0	0	7	33	20	} Results of rechallenge of 2-hour sites
		30	12	8	10	11	5	8	0	0	0	
	d	53⅓	113 (113)	94 (120)	104 (117)	28 (103)	29 (92)	29 (98)	0	0	0	
						34	25	30	Remeasurement of 53⅓-hour weals			See Figs. 8(b), 10
		54				36	31 21	31	Results of second challenge at two further points on periphery			
I.P.	a	2	70 (109)	95 (132)	83 (121)	48 (110)	77 (96)	63 (103)	0 (95)	0 (68)	0 (82)	
		8	0	0	0	0	0	0	8	32	20	} Results of rechallenge of 2-hour sites
		30⅓	12	8	10	11	6	9	0	0	0	
	d	51	111 (111)	78 (118)	95 (115)	25 (105)	29 (91)	27 (98)	0	0	0	
						32	29	31	Remeasurement of 51-hour weals			See Figs. 8(b), 10
		52				36	35 22	31	Results of second challenge at two further points on periphery			
R.D.	a	1¼	65 (73)	67 (91)	66 (82)	9 (80)	21 (100)	15 (90)	0 (138)	0 (88)	0 (113)	
		7	6	4	5	0	0	0	0	4	2	} Results of rechallenge of 1¼-hour sites
		28	7	4	6	4	6	5	4	5	5	
	d	48½	105 (150)	37 (125)	71 (138)	18 (88)	23 (109)	21 (99)	1 (115)	0 (114)	1 (114)	
	f	168½	66 (136)	69 (109)	68 (123)	18 (124)	14 (148)	16 (136)	0	0	0	
S.B.	a	1	65 (131)	85 (114)	75 (123)	0 (127)	15 (86)	8 (107)	5 (136)	6 (90)	6 (113)	
		7	1	7	9	18	12	15	7	9	8	} Results of rechallenge of 1-hour sites
		29	17	4	11	6	10	8	11	9	10	
	d	51	12 (172)	13 (141)	13 (157)	8 (140)	10 (138)	9 (139)	6 (142)	8 (148)	7 (145)	

The values in parentheses refer to the areas of the sensitizing injection blebs (outlined in Indian ink immediately after transfer).

In recipients G.W. and I.P. the sites (a^5 in Fig. 8a) which were challenged initially after 53 hours and 51 hours respectively by pricking in allergen at a single point near to the periphery of the transfer injection weal were rechallenged after a further 40 minutes by pricks at two other points on the periphery as indicated in Fig. 8(b). This resulted in the production of one extra weal in one of the pair of sites rechallenged and *two* extra weals in the other site, in each of the recipients (as illustrated by the weal tracings reproduced in Fig. 10). The areas of the first weal and of the new weals, measured at the

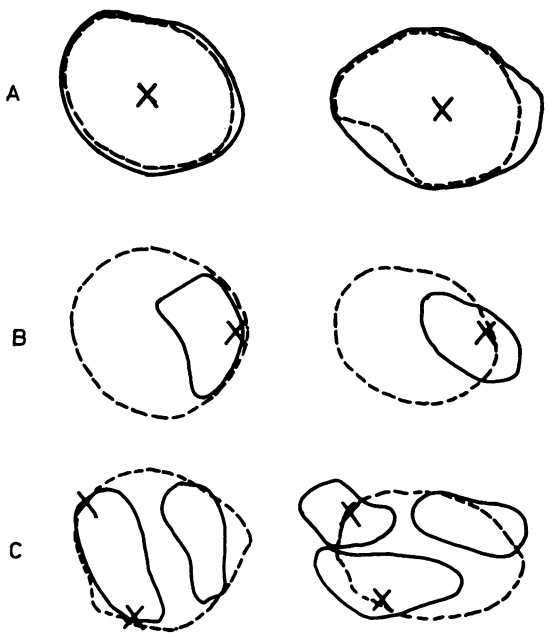

FIG. 10. Tracings of the outlines of weals evoked by multiple peripheral challenge of sensitized skin sites in recipient G.W. A. Central challenge 53 hours after sensitization. B. Peripheral challenge 53 hours after sensitization. C. Challenge at two other points on the periphery 53 hours 40 minutes after sensitization. The dotted lines outline the sensitizing injection blebs and the full lines outline the weals elicited by pricking in allergen at the points marked with a cross.

same time, are included in Table 11. It is interesting to note that, where a total of three peripheral weals were elicited at a sensitized site, the combined areas are of comparable magnitude to the area of the single weal elicited in other sensitized sites by a *central* challenge prick. This provides a quantitative demonstration of the additive discharge of a passively sensitized skin site.

Other sensitized sites in the four recipients were challenged by pricking in allergen at a point outside the initial transfer weal area, arbitrarily selected at 10 mm. from its centre (as illustrated in Fig. 8b), in a further attempt to measure the outward diffusion of unbound skin sensitizing antibody. As will be seen (Table 11), no activity could be detected as a result of first challenge at this position in 1–2 hours after transfer in recipients G.W., I.P. and R.D. When, however, the *same* sites were retested at 7–8 hours by pricking in allergen solution at the *same position* weals of appreciable size (about 25 per cent of the area of weals elicited by central challenge) were produced in recipients G.W. and I.P.

In comparison, repeat challenge (at 7–8 hours) of sites in recipients G.W. and I.P., initially challenged at 1–2 hours by pricking in allergen *centrally* or at *5 mm.* from the

centre of the transfer weal, failed to elicit further response (as seen in Table 11). A further repeat challenge of these same sites in the same positions at 30 hours, produced a slight response at 0 and 5 mm. distance from the centre, in contrast to the failure now to evoke a reaction at the 10 mm. position. Although the responses resulting from these rechallenge tests were relatively weak, involving the production of only small weals, there was a remarkably close parallelism between the results obtained in the two recipients (G.W. and I.P.). Recipient R.D. behaved somewhat differently in such tests in that measurable activity was not detected in the 10 mm. position until the third challenge at 28 hours after the initial transfer. This recipient also showed a slight residual activity on both the first (at 7 hours) and second (at 28 hours) repeat challenge of the sites initially challenged by central pricking in of allergen. As mentioned earlier, the slight immediate-sensitivity of S.B. complicated the results of tests performed in this recipient. For this reason relatively little attention has been given to these findings, although they do provide an indication of the different behaviour of the skin of a hypersensitive recipient.

DISCUSSION

The data presented provide an indication of the usefulness of the P–K test as a quantitative method of measuring skin sensitizing activity. As far as is known, a critical examination of the accuracy of the passive sensitization procedure has never before been attempted. Becker and his associates (Becker, 1948; Rappaport and Becker, 1949; Swain and Becker, 1952) have, however, carried out extensive quantitative studies of the *direct* skin test (with allergen or histamine) utilizing both 'all or none' and quantitative responses. Squire (1950) too, has made a quantitative evaluation of the accuracy of direct skin testing, where varying dilutions of histamine were introduced into the skin by pricking rather than by the scratching or intradermal injection methods employed by the Becker group. It is interesting to compare the accuracy of the P–K test results reported here with that achieved in the direct testing procedures (all of which employed sites in the recipients' arms rather than backs).

The demonstration of a lack of site variance amongst the six suitable P–K test recipients contrasts with previous investigators' findings from quantitative studies of the direct skin test. These indicated that the responsiveness of hypersensitive individuals to injected ragweed allergen (Becker and Rappaport, 1948) and to histamine (Swain and Becker, 1952) decreases as the forearm is descended and on passing from the ulnar to the radial side. It is concluded, therefore, that if site variance is to be avoided in skin testing, the back of the recipient should be used in preference to the forearms. This also has the advantage, of course, of providing many more test sites in any one recipient.

The reactivities of various recipients employed in the present series of P–K tests are compared in Table 12, which records their response to testing with 1/5 diluted sensitizing serum. Apart from facilitating the comparison of the performances of different recipients, this table permits a comparison of the same recipient's reactivity (to the same dose of sensitizing serum) on different occasions. The results of tests with allergic serum diluted with human serum albumin solution or whole serum are also included. Excluding the recipients whose skin showed some undesirable feature (recorded in last column of Table 12), those tested at the same time (such as H.S. and G.W. on 26th July or G.W. and M.D. on 6th September) showed remarkably similar responses. The differences in response (of the order of 20 per cent) shown by the same recipient (e.g. G.W. or R.D.) on different

TABLE 12

SUMMARY OF RESPONSES OF VARIOUS RECIPIENTS TO P–K TESTING WITH DILUTED (1/5) HORSE DANDRUFF ALLERGIC SERUM ON DIFFERENT OCCASIONS

Date of challenge (1961)	*Recipient*	*No. of tests with 1/5 diluted serum*	*Total no. of tests in series (excluding controls)*	*Mean weal area* (mm^2)	*Coefficient of variation* (%)	*Noticeable features of recipient's skin*
7th April	S.K.	4	48	55	35	Very hairy (required shaving)
	R.D.	4	48	71	11	
9th May	R.D.	5	25	99	11	
		5*	25*	80	22	
26th July	J.F.	6	36	71	19	Sun-tanned
	H.S.	6	36	98	17	
	G.W.	6	36	91	20	
6th September	G.W.	36	36	73	25	
	M.D.	36	36	66	32	
	S.K.	36	36	22	85	(See above)
9th November	G.W.	6†	36†	73	22	
	I.P.	6†	36†	51	17	Readily showed flaring

* Sensitizing serum diluted with 6 per cent human serum albumin solution
† Sensitizing serum diluted with recipient's serum.

occasions can be attributed to a day-to-day variation similar to that observed in direct prick testing with histamine (Squire, 1950).

The lower response shown by recipient R.D. to sensitizing serum diluted with human serum albumin solution rather than buffer, in simultaneous P–K testing, is not readily explained. Possibly serum albumin molecules present in the transfer site impede the free passage of reagin molecules to their tissue binding sites. From a practical stand-point this finding, and also the results of tests (in recipients G.W. and I.P.) with sensitizing serum diluted with recipient's serum, indicate that no advantage was gained by using a protein solution as diluent. Nevertheless, this practice is known to enhance the stability of isolated reagin fractions.

The coefficient of variation of a single P–K test with 1/5 diluted allergic serum was unexpectedly low in most recipients (as is seen in Table 12), considering the many possible sources of error of passive skin testing. Even after excluding site and day-to-day variation, Squire (1950) found that the coefficient of variation, of a single *direct* prick test with histamine solution (in the forearms of normal individuals) was about 16 per cent. The difficulty of injecting into the skin a constant volume of sensitizing serum at a constant depth, even when using accurate syringes with tightly fitting barrels, constitutes an additional potential source of error in passive transfer tests as compared to direct skin testing. In fact, it is probable that the variability of the P–K measurements recorded in this study could have been further reduced if only those test sites showing a transfer injection bleb of constant size had been selected for subsequent challenge with allergen. This point is emphasized by the findings of Rappaport and Becker (1949), who demonstrated the difficulty of injecting solutions to a constant depth into the dermis, by comparing the injection volumes (delivered in direct skin tests) with the sizes of the initial blebs produced and with the sizes of the weals subsequently evoked.

Measurement of an individual's quantitative response to varying dilutions of sensitizing

serum provides a better indication of his suitability as a P–K test recipient than that obtained by merely considering his response to a single, standard, serum dilution (e.g. 1 in 5). As was shown for the various recipients tested, a linear log(dose)–response relationship held (within certain limits). Moreover, the variability of single P–K measurements was not concentration-dependent for weals of about 50 mm^2 or greater. Below this weal size, however, the variability increases and becomes excessive for weals of minimal area (e.g. 10 mm^2 or less). Admittedly the shallowness of the slope of the log(dose)–response curve (cf. Fig. 5)—a ten-fold increase in dose leads to only about a four-fold increase in weal area over the linear range—constitutes a limitation to the use of the P–K test as a quantitative procedure. Nevertheless, many other biological assays depend upon a similar relationship. The results presented here provide ample evidence that the accuracy of the P–K tests could be improved by making use of this quantitative relationship in all assays of skin-sensitizing antibody activity. For instance, in measuring the reaginic antibody activity of isolated allergic serum fractions, it is suggested that as a routine procedure tests should be made in quadruplicate with three or four dilutions of the parent allergic serum in order to provide a standard log(dose)–response curve. This would leave twenty to twenty-four sites (at least 5 cm. apart in each direction) available on the average size back for simultaneous tests with reagin fractions.

The results presented in section 3 provide a quantitative assessment of the influence of the timing and siting of the allergen challenge prick on the accuracy of P–K measurements. An early observation (Coca and Grove, 1925) that transferred reagins become attached firmly and quickly to the fixed tissue elements was convincingly substantiated. Evidence was also obtained in support of the observation of Vaughan and Black (1954) that the point of inoculation of allergic serum is surrounded by a passively sensitized zone of skin (1 in. or more in diameter) with highest sensitization at its centre. Further quantitative tests will be required, however, to define this zone more precisely.

The experiments demonstrating the quantitative discharge of a sensitized site by multiple (i.e. three) challenges at its periphery emphasize the need for accurate positioning of the challenging allergen in routine prick tests. For this reason, the practice of pricking in allergen at the needle mark left by the initial transfer injection is to be recommended.

ACKNOWLEDGMENTS

The authors thank Professor J. R. Squire for his interest in this work. They are also grateful to the donor of the allergic serum (obtained with the kind assistance of Dr. W. Weiner and his Staff at the Birmingham Blood Transfusion Service) and to the recipients of the P–K tests for their patient co-operation.

The investigations were undertaken whilst one of us (D.R.S.) was holding a Travelling Research Fellowship at the Department of Medicine, New York University, through the generosity of the Wellcome Foundation (London).

REFERENCES

BECKER, E. L. (1948). 'Quantitative studies in skin testing. I (A). The assay of ragweed extracts by means of scratch test utilising an "All or None" response. I (B). The graphic solution of the assay of ragweed extracts by means of scratch test utilising an "All or None" response.' *J. Allergy*, **19**, 108 and 118.

BECKER, E. L. and RAPPAPORT, B. Z. (1948). 'Quantitative studies in skin testing. II. The form of the dose–response curve utilising a quantitative response.' *J. Allergy*, **19**, 317.

BOWMAN, K. and WALZER, M. (1932). 'A study of the refractiveness to whealing in normal (nonatopic) skin.' *J. Allergy*, **3**, 503.

BOWMAN, K. L. and WALZER, M. (1953). 'Studies in reaginic and histaminic wheals. 1. The effects of reaginic and histaminic wheals upon the subsequent responsiveness of passively sensitised cutaneous sites.' *J. Allergy*, **24,** 126.

BROWNLEE, K. A. (1948). *Industrial Experimentation*, 3rd edn., chap. XII. H.M. Stationery Office, London.

COCA, A. F. and GROVE, E. F. (1925). 'Studies in hypersensitiveness. A study of the atopic reagins.' *J. Immunol.*, **10,** 445.

COOKE, R. A. (1947). *Allergy in Theory and Practice*. Saunders, Philadelphia.

FISHER, R. A. and YATES, F. (1948). *Statistical Tables for Biological, Agricultural, and Medical Research*, 3rd edn. Oliver & Boyd, Edinburgh.

GORDON, J., ROSE, B. and GEHON, A. H. (1958). 'Detection of "non-precipitating" antibodies in sera of individuals allergic to ragweed pollen by an "in vitro" method.' *J. exp. Med.*, **108,** 37.

HARLEY, D. (1953). 'The prick method of skin testing.' *Int. Arch. Allergy*, **4,** 455.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J. (1951). 'Protein measurement with the folin-phenol reagent.' *J. biol. Chem.*, **193,** 265.

PRAUSNITZ, C. and KÜSTNER, H. (1921). 'Stuchenuber die Uebercrip findlichkeit.' *Zbl. Bakt., I. Abt., Orig.* **86,** 160.

RAPPAPORT, B. Z. and BECKER, E. L. (1949). 'Quantitative studies in skin testing. IV. The volume–response relationship.' *J. Allergy*, **20,** 358.

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

STANWORTH, D. R. (1959). 'Studies on the physicochemical properties of reagin to horse dandruff.' *Immunology*, **2,** 384.

SWAIN, H. H. and BECKER, E. L. (1952). 'Quantitative studies in skin testing. V. The whealing reactions of histamine and ragweed pollen extract.' *J. Allergy*, **23,** 441.

VAN ARSDEL, P. P. and SELLS, C. J. (1963). 'Antigenic histamine release from passively sensitised human leucocytes.' *Science*, **141,** 1190.

VAUGHAN, W. T. and BLACK, J. H. (1954). *Practice of Allergy*. 3rd edn., chap. XVIII. Henry Kimpton, London.

WRIGHT, G. P. and HOSKINS, S. J. (1941). 'Reversed passive skin sensitisation to horse serum in human beings.' *J. Path. Bact.*, **53,** 243.