

From the Commonwealth Serum Laboratories, Melbourne.

ALTERATION IN ACTIVITY OF GRASS POLLEN EXTRACT

By

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INTRODUCTION

For many years allergists have recognised that systemic reactions may occur unexpectedly during hyposensitization with pollen extracts (Gay 1929; Bruun, 1959). One of the causes of these reactions has been considered to be failure to reduce dosage when changing from an old to a fresh extract (Duke, 1930; Waldbott, 1931; Furstenberg & Gay 1937; Frankland, 1955; Van Arsdel & Sherman, 1937). Apparently, loss of activity in the older extract results in a higher dosage increment than intended. This loss of activity is considered to be due mainly to the adsorption of antigen on the surfaces of the glass containers or syringes (Hjorth, 1958; Malley *et al.*, 1962) and it is more apparent in weaker solutions of pollen extracts (Sheldon, 1953; Frankland, 1955; Augustin & Hayward, 1962). Hjorth (1957) has demonstrated that the activity of pollen solutions is related to the amount of filling of the container—the so-called “volume effect”. He observed that partial emptying of a glass vial containing a mixture of grass pollen extracts resulted in a considerable reduction in the activity of the remaining allergen solution. Subsequently, Hjorth (1958) found that a non-ionic detergent, “Tween 80”, acted as a stabilizer and prevented loss of potency.

PURPOSE

The purpose of this study was to consider certain factors which could alter the potency of pollen extracts, as currently

used in Australia, prepared in Coca's solution. The factors examined were dilution, type of container, volume in relation to the capacity of the container, time of storage and the addition of a surface-active agent, "Tween 80" (polyoxyethylene derivative of sorbitan mono-oleate, G 1725, Atlas Products).

MATERIALS USED

Rye grass pollen extract was selected because Rye grass (*Lolium perenne*) is the commonest cause of grass pollen hay fever in Victoria, and consequently Rye-grass-sensitive cases would be readily available for testing. 6 grams of dried ether-washed pollen was suspended in 100 ml of Coca's solution (0.5 % NaCl, 0.27 % NaHCO₃ and 0.4 % phenol in water) (Coca, 1922) and soaked at room temperature for 3 hours with occasional shaking. The supernatant liquid was filtered through paper and the sediment thoroughly washed twice with more Coca's solution. The whole extract was filtered through paper and then by suction through a Seitz bacterial filter pad into a sterile storage bottle. The total volume of Coca's solution used was 215 ml, and 165 ml of extract was eventually obtained. The total nitrogen content estimated by a micro-Kjeldahl method was 0.19 mg. per ml. One half of the extract was diluted to "D" strength (0.1 mg N per ml = 10,000 units/ml) with Coca's solution (CS), and the other half was diluted to "D" strength with Coca's solution to which had been added 0.01 % of "Tween 80" (CST). From each of these extracts "C" strength (0.01 mg N per ml. = 1,000 units/ml), "B" strength (0.001 mg N per ml = 100 units/ml) and "A" strength (0.0001 mg N per ml = 10 units/ml) dilutions were prepared in the respective extracting fluids (CS and CST).

The containers used were 13-ml. Pyrex glass bottles and 8 cm. capillary tubes of soda glass. The bottles contained either 10½ ml. (10½ "fill") or 1½ ml. (1½ "fill") and the capillary tubes were filled to a length of 5-6 cm. (CT "fill"). The filling of sufficient bottles and capillary tubes with each of the four strengths in both extracting fluids (CS and CST)

was completed within a few days of the preparation and dilution of the extract. The products were stored at 0-4° C till required. A number of 13-ml. bottles were filled with CS and CST for use as controls.

Samples of the preparations were tested for activity within a month of their production and again after approximately 6 months, 12 months, 18 months, 2 years and 3 years. As "A" strength gave such a high proportion of very small reactions at 12 months, no further tests were made with that strength.

METHOD

It was proposed to compare the potency of the various preparations by skin testing. As allergic patients show great variation in skin reactivity, preliminary scratch tests with dry pollen were made before deciding which of the volunteers would be suitable for testing the different strengths. Those giving very large skin reactions were chosen to test "C" strength by the prick method and "A" and "B" strengths by the intradermal method. Tests were made on the volar aspect of the forearm in two or three longitudinal rows according to the number of tests.

The prick test was used throughout for "D" strength and for most of "C" strength. The test material was dropped from the syringe on to the skin and a sharp prick made through it with a sterile surgical straight needle.

The intradermal test was used for "A" and "B" strengths in all the series and for "C" strength in some of them. A 26-gauge, 1/2 inch hypodermic needle was inserted as superficially as possible and sufficient liquid to form a bleb of 1-1.5 mm. diameter was introduced. A separate sterile needle was used for each patient, but the same syringes were used throughout a testing session.

New 1-ml. tuberculin syringes were procured for this work. After use they were at once rinsed well in cold tap water, separated and boiled up in water with sodium triphosphate. After being rinsed well in water, they were soaked in 3 % hydrochloric acid, rinsed well in water, soaked for at least half an hour in "Tween 80" (0.01 %) and again thoroughly rinsed in water and dried before being assembled and sterilized in dry air at 180° C.

Reactions were observed and recorded at their maximum size, usually about 10 minutes after the application of the test. Each wheal was measured directly, recording first the largest diameter and then the largest measure at right angles to this. The average of these two readings was taken as the average wheal diameter.

A series consisted of the variety of preparations used at a testing session, e.g. a series would consist of 10 1/2, 1 1/2 and CT "fills" in CS, and 10 1/2, 1 1/2 and CT "fills" in CST, all of the same strength, together with the two control fluids,

CS and CST. The same technique (either prick or intradermal) was used throughout a series. The code numbers 1 to 8 were given at random to these eight preparations. Just before testing began, a syringe was filled from each of the eight preparations and given the appropriate code number. These numbers were chosen in a random order and were written on the arm of the patient. At each numbered site the prick or the intradermal test was made from the syringe with the corresponding number. Depending on the proportion of large reactions expected, one to four series of tests were made on each patient. Except when comparing the reaction of "C" strength with that of "D" strength, only one strength of extract was used in a series. Throughout the work one operator performed the tests and another measured the reactions. The results were not decoded until the session was over. Fresh codes and randomizations were used at each session.

A sequential analysis of the comparison of pairs of factors was made after each session to decide whether a further session was needed to give sufficient tests. The number of series for each strength tested at each period varied from 8 to 38 and was sometimes limited by the number of suitable patients available. Only a few of the original patients were available for the whole period of three years.

All tests were carried out during February-March or July-August when the exciting pollen (Rye grass) was not present in the air.

RESULTS

The results are summarised in Tables 1, 2 and 3. In Tables 1 and 2 are given only the average results of all tests made with each preparation at the one time. It was considered unnecessary to list the reactions of individual tests in every series; but in Table 3 all the results were used in the comparisons examined statistically for significant differences.

In Table 1 are given the average skin reactions for all extracts tested, in all "fills" and strengths, as shown after each period of storage. It should be noted that only the reactions elicited by one strength at any one time are comparable.

It is apparent from an examination of the results furnished by the tests with preparations less than a month old that only with "D" strength material did reactions from all "fills" in CS approximate to those of "fills" in CST. With weaker dilutions the reactions produced by CST extracts were invariably larger than those produced by CS extracts, the differences being greater as the strength decreased. When the different "fills"

TABLE 1
Skin Reactions to Rye Grass Pollen Extract.

		1 month		6 months		12 months		18 months		2 years		3 years	
Strength	"fill"	CS	CST	CS	CST	CS	CST	CS	CST	CS	CST	CS	CST
A = 10 u/ml.	10½ ml.	3.5	7.2	3.1	6.8	1.4	7.1						
	1½ ml.	1.7	6.4	2.7	6.6	1.0	6.4						
	CT	5.7	7.9	4.9	6.5	2.5	6.2						
	Average	3.6	7.2	3.6	6.6	1.6	6.6						
B = 100 u/ml.	10½ ml.	7.0	8.8	6.2	8.3	4.8	7.7	4.8	10.0	4.4	9.6	5.3	10.6
	1½ ml.	3.9	9.6	2.8	8.7	2.3	9.0	1.2	9.4	0.6	9.6	2.6	10.2
	CT	6.1	9.6	5.1	6.9	5.0	7.9	4.6	8.9	4.8	9.9	6.2	9.2
	Average	5.7	9.3	4.7	8.0	4.4	8.2	3.5	9.4	3.3	9.7	5.7	10.0
C = 1000 u/ml.	10½ ml.	5.0	5.4	5.0	5.6	4.0	4.8	5.6	5.7	6.6	7.0	7.4	8.8
	1½ ml.	4.2	5.6	4.2	5.7	3.5	4.5	2.9	5.9	5.4	7.6	5.6	8.3
	CT	4.7	5.6	4.2	5.2	4.2	5.0	3.9	5.8	6.2	7.3	8.6	7.6
	Average	4.6	5.5	4.5	5.5	3.9	4.8	4.1	5.8	6.1	7.3	7.2	8.2
D = 10000 u/ml.	10½ ml.	7.0	6.5	6.0	6.4	6.6	5.6	6.8	8.8	5.8	6.5	6.0	5.8
	1½ ml.	6.9	6.4	6.1	6.4	6.2	6.7	6.7	7.1	6.0	5.8	5.3	6.1
	CT	6.1	6.4	6.7	6.5	6.0	6.3	7.2	8.5	5.8	6.4	6.4	—
	Average	6.7	6.4	6.3	6.4	6.3	6.2	6.9	8.1	5.9	6.2	5.9	6.0

Average wheal diameter in mms.

In Coca's Solution (CS) and in Coca's Solution with 0.01 % "Tween 80" added (CST).

Storage at 0-4° C.

of each strength were compared for "A", "B" and "C" strengths, the CS extract in the 1½ "fill" consistently gave the smallest reaction, but in CST all "fills" gave closely similar results. Only for "D" strength did all "fills", both in CS and CST, give similar reactions.

After periods of storage, even up to three years, "D" strength extracts in all "fills" and in either CS or CST still gave similar reactions. Any differences were small and no one "fill" or extraction fluid consistently gave better results. (Unfortunately, the supply of CT ran out before the 3-year tests were completed). For weaker extracts in CS, however, there was a further gradual loss in activity when they were stored. For "C" strength there was little further change except possibly

in the $1\frac{1}{2}$ "fill". "B" strength showed further deterioration in 12 months, but thereafter little more except in the $1\frac{1}{2}$ "fill". "A" strength deteriorated little further in 6 months but so much in 12 months in all "fills" that further tests were not made. For "A", "B" and "C" strengths the CS extract in the partly filled bottles deteriorated both initially and with time, and more than in filled bottles or capillary tubes. All CST extracts in all "fills" remained stable over the 3-year period.

TABLE 2
Skin Reactions to Pollen Extract in Coca's Solution (CS).

Age of extract		1 mth.	6 mths.	12 mths.	18 mths.	2 years	3 years
Strength of extract	Size of "fill"						
A = 10 u/ml.	$10\frac{1}{2}$ ml.	49	47	21			
	$1\frac{1}{2}$ ml.	24	41	15			
	CT	79	74	38			
B = 100 u/ml.	$10\frac{1}{2}$ ml.	75	78	59	51	45	53
	$1\frac{1}{2}$ ml.	42	35	28	13	6	26
	CT	66	64	61	49	50	62
C = 1000 u/ml.	$10\frac{1}{2}$ ml.	91	91	83	97	90	90
	$1\frac{1}{2}$ ml.	76	76	73	50	74	68
	CT	86	76	88	67	85	105
D = 10000 u/ml.	$10\frac{1}{2}$ ml.	109	94	106	84	94	100
	$1\frac{1}{2}$ ml.	108	95	100	83	97	88
	CT	95	105	97	89	94	107

Expressed as percentage of mean reactions of extracts in Coca's Solution with "Tween 80" (CST). All extracts stored at $0-4^{\circ}$ C.

As already shown, for each strength, the CST extracts gave closely similar wheals. To make results comparable over the whole period, the average reaction of each "fill" of CS extract was reduced to the percentage of the average of the reactions of all CST "fills" of the same strength at that time. These percentages are listed in Table 2. Figure 1 shows Table 2 graphically. It is obvious from Table 2 and Figure 1 that for "D" strength there was no apparent change in potency due either

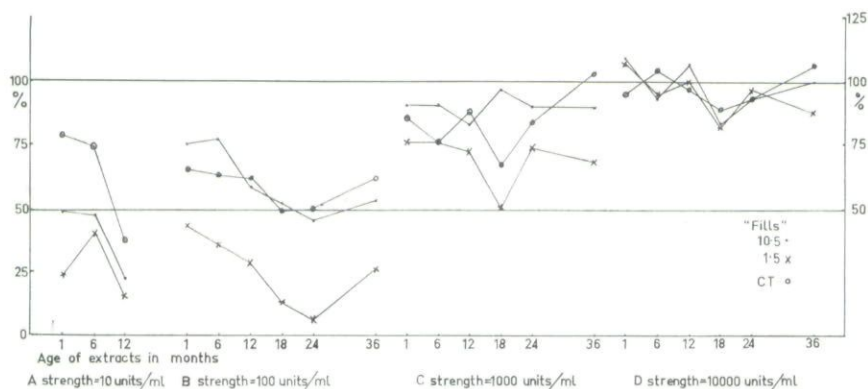


Fig. 1.

Reactions of CS extracts as percentage of CST reactions.

to "fill" or to the presence of "Tween 80". Variation occurred either way but never exceeded 20 %. For "C" strength CS extracts were usually weaker than CST extracts, and CS extracts were always decidedly weaker for "A" and "B" strengths. The 1½ ml. "fill" was always the weakest of the CS extracts for "A", "B" and "C" strengths. For "B", "C" and "D" strengths 10½ ml. and CT "fills" gave very similar reactions, while for "A" strength the CT "fill" consistently gave the best reaction.

The six preparations of pollen extract, namely, 10½, 1½ and CT "fills" in CS and 10½, 1½ and CT "fills" in CST for each of four strengths ("A", "B", "C" and "D"), were compared in pairs for skin reactivity within a month of production and after various periods of storage at 0-4° C.

In Table 3 the reactions to the "fills" being compared are given as = when the difference between their reactions is not significant at the 5 % level on the basis of the chi square test. Otherwise the "fill" in each pair giving the significantly larger reaction is entered.

Firstly, each "fill" in CS was compared with the corresponding "fill" in CST to find in which extracting fluid activity was better retained. In all tests with "A" and "B" strengths (for all "fills") the CST extracts were significantly better than the CS extracts. With "C" strength the CST extracts

TABLE
Comparison in size of Skin Reactions

Preparations compared	A Strength = 10 units/ml.			B Strength = 100 units/ml.					
	1 mth.	6 mths.	12 mths.	1 mth.	6 mths.	12 mths.	18 mths.	2 years	3 years
10½ 10½ (T)	T	T	T	T	T	T	T	T	T
1½ 1½ (T)	T	T	T	T	T	T	T	T	T
CT CT (T)	T	T	T	T	T	T	T	T	T
10½ 1½	10½	10½	10½	10½	10½	10½	10½	10½	10½
10½ (T) 1½ (T)	=	=	=	=	=	=	=	=	=
10½ CT	CT	CT	CT	=	=	=	=	=	=
10½ (T) CT (T)	=	=	=	=	=	=	=	=	10½ (T)

Comparison of pairs of 6 different preparations, viz. 3 "fills" in CS-10½, 1½ and CT, and 3 For 4 strengths A, B, C and D after different periods of storage at 0-4° C. Significantly larger

were in general significantly better than the CS extracts. With "D" strength there was usually no significant difference between the CS and the CST extracts. On the few occasions when a difference was shown, there was no consistent trend towards the superiority of either extracting fluid.

Secondly, the 10½ "fills" were compared with the 1½ "fills". In CS the 10½ "fills" were significantly better throughout the 3-year period than the 1½ "fills" with "A" and "B" strengths; and also this was so for "C" strength up to 18 months storage. In contrast, no significant difference was shown between 10½ and 1½ "fills" in CST for "A", "B" and "C" strengths. No consistent variation in activity was shown for "D" strength in either of the extracting fluids.

Thirdly, the 10½ "fills" were compared with the CT "fills". Only for "A" strength in CS was a persistent difference shown, the CT "fills" being significantly better than the 10½ "fills". In some series for "B" and "C" strengths the 10½ "fill" was found to be better than the CT "fill" in one or other of the extracting fluids, but in most series no significant difference was shown. For "D" strength no difference was found.

C Strength = 1000 units/ml.						D Strength = 10.000 units/ml.					
1 mth.	6 mths.	12 mths.	18 mths.	2 years	3 years	1 mth.	6 mths.	12 mths.	18 mths.	2 years	3 years
=	T	T	T	T	T	=	=	10½	T	=	=
T	T	T	T	T	T	=	=	=	=	=	T
T	T	T	T	T	=	=	=	=	=	=	\
10½	10½	10½	10½	=	=	=	=	=	=	=	10½
=	=	=	=	=	=	=	=	1½T	=	=	=
=	10½	=	10½	=	=	=	=	=	=	=	=
=	10½(T)	=	=	=	=	=	=	=	=	=	\

"fills" in CST-10½(T), 1½(T) and CT(T).

reactions are indicated. No significant difference =.

DISCUSSION

Value of the Skin Test for Comparison of Strength of Extracts.

Every effort was made to reduce factors known to cause variation in skin reactions. Persons show wide diversity in their response to the same test material; so several patients were used for each comparison. Various sized reactions are given at different sites on the same individual; so all tests were made on the volar aspect of the forearm. Care was taken to ensure that comparative tests were as uniform as possible in depth and area of penetration of the skin and also in the amount of test dose. The question may be asked whether under such uniform conditions does the size of the wheal produced vary with the strength of the test solution? There is a difference of opinion as to what extent the size of reaction may be relied on to distinguish differences in potency. According to *Bowman* (1935) 25 % or greater differences in the strength of an allergen solution could be detected, but a difference of 15 % could not be revealed by skin response. *Arbesman* (1939) claimed that a tenfold difference in dilution could not always be shown. *Dorfman* (1938) found that a ten to one-

TABLE 4

Strength Diluent		Average wheel diameter in mms.																		
C	CS	4	3	4	3	3	3	2	3	2	2	2	3	2	3	3	6	5	6	3.3
D	CS	6	4½	7	5½	4½	6	5	5½	3½	5	5	5	5½	5	5½	5½	10	10	5.8
C	CST	4	3	7	3½	4	5	3	4	2	2	3	3	2½	4	4	5½	7½	12½	4.4
D	CST	6	6	7	6	6½	7	4½	5	3½	5½	5	5½	4½	6½	6	7	8½	17½	6.5
Series No.		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	Av.

Reactions with "C" strength and "D" strength Rye grass pollen extracts. (CS) Coca's solution.
 (CST) Coca's solution with "Tween 80" 0.01 %.

hundredfold variation in concentration produced little difference in wheal size. *Augustin* (1959) found the prick test a very sensitive one, but that it could not be used to detect a 25 %–50 % difference in concentration. *Effron* (1939), with house dust extract and *Rinkel* (1949), with pollen extract, produced dose response curves, but found these curves to be discontinuous or with plateaus when increase in concentration caused little or no change in wheal size. *Collins* (1960) obtained reproducible response curves with skin test doses of histamine.

To ascertain whether a tenfold difference in dilution could be distinguished under the conditions of this study the following experiment was carried out. Eighteen series of skin tests were made with "D" strength and "C" strength (tenfold dilution) by the prick method. Each series consisted of six preparations, viz. $10\frac{1}{2}$ "fill" of "C" strength in CS, $10\frac{1}{2}$ "fill" of "D" strength in CS, $10\frac{1}{2}$ "fill" of "C" strength in CST, $10\frac{1}{2}$ "fill" of "D" strength in CST and the two controls, CS and CST. All tests with the control fluids were negative. When the 18 series of tests were compared, the reactions to "D" in CS were greater than those of "C" in CS in 17 of the 18 cases, i.e. they were significantly greater to the 0.01 % level, and the reactions of "D" in CST were all greater than those of "C" in CST. The actual readings are given in Table 4.

"D" strength, which is ten times the strength of "C", was readily demonstrated by skin reactions to be the stronger. At that range of strength, 1,000–10,000 units/ml. at least a tenfold difference could be detected.

After all the evidence on the relation of the size of skin reactions to varying concentrations of pollen allergen was considered, it was decided that skin testing is a reliable way of detecting differences in activity between weaker solutions. However, with strong extracts, consistent variation in wheal size can be expected only with relatively large differences in concentration. Wheal size is a comparative rather than a quantitative measure of activity.

Cause of Alteration in Potency.

It is obvious from the results that there was a definite difference in potency between some preparations in CS and corresponding ones in CST. Where the results were consistent the more potent extract was always the one diluted in CST. This difference in potency was noticed only in the weaker dilutions and was greatest in the small ($1\frac{1}{2}$) "fill", and moreover was immediate. In some series there was a further change in potency after a period of storage, again in the CS extracts but not in the CST extracts which gave consistently similar reactions throughout the test period. How can this difference in skin reactivity between extracts in CS and the corresponding ones in CST be explained? It could be due to either a lowering of potency in the CS extracts or to an enhancing effect of the "Tween 80" in the CST extracts. That "Tween 80" did not itself irritate the skin was shown by the completely negative result of all control tests with both CS and CST. In all series of "D" strength those extracts containing "Tween 80" did not give significantly greater reactions than those without "Tween 80". If "Tween 80" enhances skin reactions, one would have expected the average wheal size of all extracts in CST to have been larger than the corresponding ones in CS. It appears, therefore, that activity was lost from some extracts in CS but was retained in the corresponding CST extracts.

Any immediate loss of potency of extracts in CS could be explained by adsorption on the surface of containers. This adsorption would be prevented by "Tween 80", a surface-active agent. Loss due to adsorption would be more evident in weak dilutions and in small "fills" than in strong extracts where the proportion of activity adsorbed would be very small in relation to the total activity.

Hjorth (1957) showed that for mixed grass pollen extract there was a difference in potency between extract from full and partly filled bottles, the potency being less in the partly filled bottles. Subsequently *Hjorth* (1958) showed that this loss of potency, which also was more pronounced in weak extracts,

could be prevented by the addition of "Tween 80" to the extracting fluid. He explained the loss as probably due to adsorption on the glass of the container or of the vessels used during preparation.

Although our work differed in some respects from that of *Hjorth*, our results support his findings. He showed an immediate loss, attributed to adsorption, from pollen extract over a range of strength of 0.01 % to 0.00001 % w/v. We were able to demonstrate it over a range of 0.15 % w/v ("C" strength) to 0.0015 % w/v ("A" strength) but could not detect any loss at the highest strength used, namely, 1.47 % w/v ("D" strength). Our extracts in Coca's solution showed this adsorption loss although such extracts have been considered more stable than those in the phosphate-buffered saline used by *Hjorth* (*Grove & Coca*, 1925; *Coca et al.*, 1931; *Vaughan*, 1939). In addition to preventing immediate adsorption loss from extracts in Coca's solution, "Tween 80" appeared to prevent deterioration of activity during their storage over a period of three years. While preventing the loss from weak dilutions, it did not apparently alter the activity of strong extracts.

Other means of preventing adsorption, such as siliconing of containers, were not tested. It has been claimed by *Malley* (1962) that siliconing does prevent the adsorption of pollen allergen. *Magnus* (1956), however, found that the loss of activity of old tuberculin was not prevented in siliconed bottles.

Danger of Addition of "Tween 80" to Pollen Extracts.

It would appear from the foregoing that "Tween 80" would be a useful additive to pollen extracts, ensuring their stability over a long period. This would be so if all syringes and vessels used for pollen extracts were chemically clean. In general medical practice the same syringes are repeatedly used, and even careful cleaning may not remove adsorbed protein from the glass. Such adsorbed material is removed by "Tween 80" solutions. This was confirmed by us accidentally. On one occasion, owing to a shortage of our carefully prepared syringes,

a borrowed one (understood to have been well washed and sterilized) was used for the control test with CST on two patients. Instead of the usual negative results these patients both gave positive reactions to the CST. This syringe had previously been used during hyposensitization treatment with pollen extract. After this episode, several syringes (routinely washed and sterilized) were borrowed from a large allergy clinic. Some filled with CS and others with CST were used to make intradermal tests on a number of pollen-sensitive patients. All tests with CST gave positive results. Our experience confirms what one would expect, namely, that there is a danger in adding a surface-active agent such as "Tween 80" to pollen extracts. Unknown amounts of foreign protein could be eluted by "Tween 80" from syringes considered clean.

EFFECT OF LOSS OF POTENCY ON THE CLINICAL USE OF POLLEN EXTRACTS

Skin Testing.

Pollen extracts for skin testing are usually supplied in "D" strength, either in capillary tubes for individual tests or in full or partly filled bottles to allergists and hospital clinics. As "D" strength extract showed no deterioration in any preparation tested, even after three years' storage, these extracts in Coca's solution, as at present used in Australia, are reliable for skin testing. If "Tween 80" were added to the extracting fluid, there would be no advantage either in respect of initial potency or stability over three years, but there would be a risk that some foreign protein might be eluted from a syringe used to withdraw extract for a skin test.

Treatment.

Hyposensitization treatment with pollen extract is usually commenced with "A" strength and continued through "B", "C" and "D" strengths with increasing dosage until an opti-

TABLE 5
Effect of Adsorption Loss on Schedule of Dosage.

1	2	3	4	5	6	7	8
Strength of extract	Volume of dose	Unitage of dose			Increase of dose		
		Theoretical	Probable if maximum loss	Probable if from full bottle	Theoretical	Probable if maximum loss	Probable if from full bottle
A.	0.1 ml.	1	25%	50%			
			1/4	1/2			
	0.2 ml.	2	1/2	1	1	1/4	1/2
					2	1/2	1
	0.4 ml.	4	1	2	4	1	2
B.	0.1 ml.	10	50%	75%			
			5	7 1/2			
	0.2 ml.	20	10	15	10	5	7 1/2
					20	10	15
	0.4 ml.	40	20	30	40	20	30
C.	0.1 ml.	100	75%	100%			
			75	100			
	0.2 ml.	200	150	200	100	75	100
					200	150	200
	0.4 ml.	400	300	400	400	300	400
D.	0.1 ml.	1000	100%	1000			
			1000	1000			
	0.2 ml.	2000	2000	2000	1000	1000	1000
					1000	1000	1000
	0.4 ml.	4000	4000	4000	1000	1000	1000

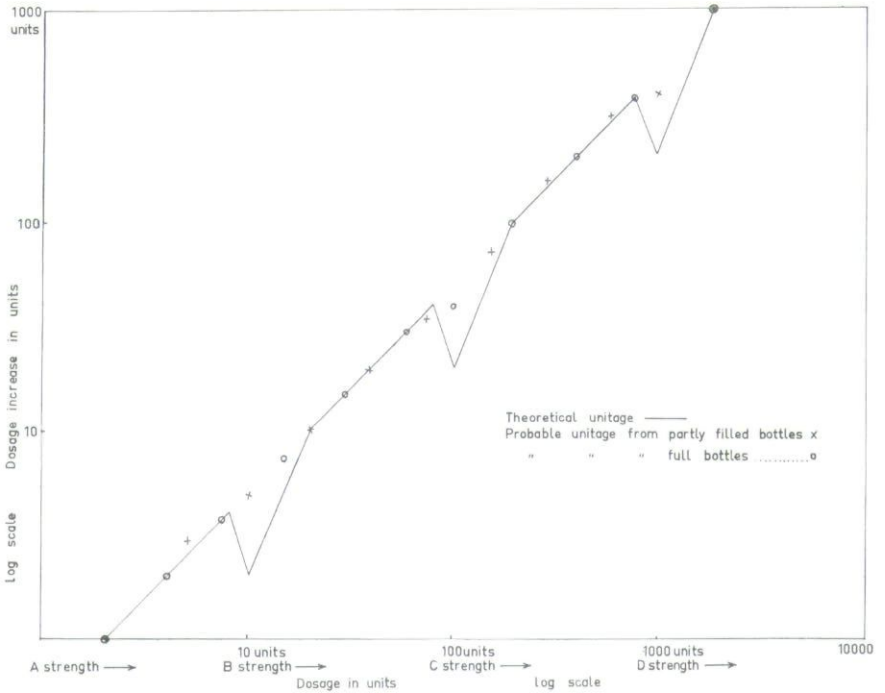


Fig. 2.
Effect of adsorption on schedule of dosage.

num dose between 0.1 ml. of "C" strength and 1.0 ml. of "D" strength is reached. This optimum dose may be continued over a prolonged period. Unexpected, severe reactions have been encountered during treatment even when there has been an apparently safe increase or no increase in dose and when pollen is not present in the air. Such reactions do not occur with "A" and "B" strengths, and only in very sensitive cases with "C" strength. Most frequently they are encountered when the patient is being given "D" strength.

Table 5 and Figure 2 show a typical schedule of dosage advised for the average patient. (The figures given in columns 4, 5, 7 and 8 of Table 5 are based on Table 2). For this reason they must be regarded as approximate and probably less accurate with higher concentrations of extract than with the

lower ones. In Table 5, beside the theoretical number of units in each dose (column 3) are given the probable number of units if there is maximum loss due to partly filled bottles (column 4) and also the possible number of units in the same doses from full bottles (column 5). The increase (in units) between consecutive doses is also given—theoretical increase (column 6), probable increase if there is maximum loss due to partly filled bottles (column 7) and probable increase if full bottles are always used (column 8). It will be obvious that with this schedule the increase is more uniform for either probable dosage than for the theoretical dosage. This schedule, which diminishes the size of the first dose of each new strength was originally so designed because the change to a new bottle was known to be a danger point. Our work confirms the suitability of this schedule and at least partly explains the reason for caution at these steps in treatment.

Although any loss of activity by adsorption was not apparent for "D" strength and full bottles of "C" strength, it is possible that some loss, too small in proportion to be detected, may have occurred. Precautions should, therefore, be taken when commencing to use a new bottle of "C" strength or of "D" strength. It is obvious that any increase in volume of dose with "C" and "D" strengths involves a much greater additional unitage than with "A" and "B" strengths. Very sensitive individuals may not be able to assimilate this more rapid increase readily, and so must be given a more conservative schedule of dosage. When treatment with "C" strength is prolonged and a new bottle of "C" strength is commenced, it would be wise to reduce the dose from the new bottle to one half that of the last dose from the old bottle, or to discard the old bottle while still half full. Again, when a change is made from a partly filled bottle of "C" strength to "D" strength, it would be wise to give only one-half the theoretically safe unitage. Such precautions should minimise the risk of untoward reactions while using "C" and "D" strength extracts. *Duke* (1930) suggested that reactions encountered at the change to a new bottle could be avoided by diluting the early

small-volume doses to a constant volume with physiological saline in the syringe. This idea for diminishing the risk of unexpected reactions when beginning a bottle of "C" or "D" strength may be worth further consideration.

CONCLUSIONS

1. When fresh dilutions of pollen extract in Coca's solution were compared for potency with similar dilutions in Coca's solution to which "Tween 80" (0.01 %) had been added, it was found that for the strongest solution ("D" strength) there was no apparent difference. However, as the solutions were diluted there was a progressive decrease in potency of the extracts in CS beyond that of extracts in CST. This loss of activity was most evident for "A" strength, the weakest extract.

2. The initial loss of potency of these extracts in CS was also related to the fullness of the container. Extracts from partly filled bottles ($1\frac{1}{2}$ "fill") of "A", "B" and "C" strengths showed greater loss than extracts from either full bottles ($10\frac{1}{2}$ "fill") or capillary tubes (CT "fill"). This effect of "fill" on potency was not evident for "D" strength.

3. For "B", "C" and "D" strengths there was no apparent difference in potency between the extracts from a full bottle ($10\frac{1}{2}$ "fill") and that from a full capillary tube (CT "fill"), but, for "A" strength the extract from the CT "fill" was more potent than that from the full bottle ($10\frac{1}{2}$ "fill").

4. The presence of "Tween 80" in the extracting fluid prevented the initial loss of potency shown by the weaker dilutions in Coca's solution, but it did not enhance the activity of the stronger extracts or have any effect when used in control skin tests.

5. The initial loss of potency, both in weak solutions and in partly filled bottles, could be explained by adsorption on the surface of the container. A surface-active agent such as "Tween 80" would tend to prevent this.

6. Any loss of activity due to adsorption would be too small

to affect extracts commonly used in skin testing. However, during hyposensitization treatment the loss could be sufficient to alter materially the unitage of the last dose from one bottle in relation to the first dose from a new bottle of extract.

7. When pollen extracts in Coca's solution were stored in the refrigerator ($0-4^{\circ}\text{C.}$) the stronger extracts ("C" and "D" strengths) apparently remained stable for at least three years. The activity of weaker extracts declined in this time, and most rapidly in partly filled containers. All dilutions containing "Tween 80" remained stable for three years.

In spite of the value of "Tween 80" as a stabilizer of the potency of pollen extracts, its use for this purpose is considered inadvisable. Unless all vessels, including syringes, are chemically clean, "Tween 80" could, by elution, add an unknown amount of foreign protein or allergen to the extracts used.

Our thanks are due to the patients who cheerfully accepted the discomfort of skin reactions.

SUMMARY

Rye grass pollen extract in Coca's solution in four dilutions was examined for stability when stored at $0-4^{\circ}\text{C}$ in filled and partly filled Pyrex glass bottles and also in soda glass capillary tubes. These extracts were compared by skin testing, immediately after preparation and at intervals over three years, with similar bottled extracts to which "Tween 80" (0.01 %) had been added.

When the fresh extracts in Coca's solution were examined, no deterioration in potency was detected in the strongest pollen extracts, namely, 1.47 % w/v (10,000 units/ml.), but marked loss was shown in the weakest extract (10 units per ml.). As this loss was immediate and most obvious in the partly filled bottles, it was presumed to be due to adsorption on the glass. In extracts up to 1,000 units per ml. the extract from partly filled bottles was weaker than that from full bottles. Time of storage to three years had little effect except on the weakest dilutions.

This adsorption loss would not materially affect extracts of strengths used in skin testing but could cause unexpected, severe reactions during a prolonged course of hyposensitization treatment at the change to a new bottle of extract of the same or the next strength.

In all containers the extracts of all four dilutions containing "Tween 80" remained stable. However, the addition of "Tween 80" as a stabilizer in pollen extracts would introduce the danger of elution of foreign protein from the syringes and the other vessels used.

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